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Ethics Declaration

Family 1 was enrolled in a research study approved by the IRB of Duke University Health System (Pro00031066) and transferred to a research study approved by the Lurie Children's Hospital of Chicago IRB (IRB2019–3084). Family 2 was enrolled in a research protocol that was reviewed by the IRB at the Children's Hospital of Philadelphia. Family 3 was enrolled in a research study approved by the Conjoint Research Ethics Board (CHREB) of the University of Calgary (REB17–1168). Family 4 was enrolled in a research study approved by the central IRB at the National Human Genome Research Institute (NHGRI, 15-HG-0130) and locally by the Duke University IRB (Pro00056651). All families provided informed consent before participation.

Conflict of Interest

Rebecca Ganetzky is a paid consultant for Minovia Therapeutics and Nurture Genomics.

Neal Sondheimer is employed by Synlogic, Inc. All other authors declare no conflicts of interest.

Additional Information

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Biallelic variants in ribonuclease inhibitor (*RNH1*), an inflammasome modulator, are associated with a distinctive subtype of acute, necrotizing encephalopathy

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Abstract

Purpose: Mendelian etiologies for acute encephalopathies in previously healthy children are poorly understood, with the exception of RAN binding protein 2 (*RANBP2*)–associated acute necrotizing encephalopathy subtype 1 (ANE1). We provide clinical, genetic, and neuroradiological evidence that biallelic variants in ribonuclease inhibitor (*RNH1)* confer susceptibility to a distinctive ANE subtype.

Methods: This study aimed to evaluate clinical data, neuroradiological studies, genomic sequencing, and protein immunoblotting results in 8 children from 4 families who experienced acute febrile encephalopathy.

Results: All 8 healthy children became acutely encephalopathic during a viral/febrile illness and received a variety of immune modulation treatments. Long-term outcomes varied from death to severe neurologic deficits to normal outcomes. The neuroradiological findings overlapped with ANE but had distinguishing features. All affected children had biallelic predicted damaging variants in *RNH1*: a subset that was studied had undetectable RNH1 protein. Incomplete penetrance of the *RNH1* variants was evident in 1 family.

Conclusion: Biallelic variants in *RNH1* confer susceptibility to a subtype of ANE (ANE2) in previously healthy children. Intensive immunological treatments may alter outcomes. Genomic sequencing in children with unexplained acute febrile encephalopathy can detect underlying genetic etiologies, such as *RNH1*, and improve outcomes in the probands and at-risk siblings.

Keywords

Acute demyelinating encephalopathy; Acute necrotizing encephalopathy; Inflammasome; RANBP2; RNH1

Introduction

Acute encephalopathy syndromes are rare disorders of rapid and serious central nervous system dysfunction, resulting in altered sensorium/loss of consciousness, motor and sensory impairments, and, sometimes, seizures.[1] In childhood, acute encephalopathy can occur either with known neurologic/genetic disorders (eg, severe epilepsies, urea cycle defects) or unexpectedly in previously typical children. Subtypes of acute encephalopathy that can affect previously healthy children include acute necrotizing encephalopathy (ANE) and acute demyelinating encephalomyelitis (ADEM), which have overlapping clinical features but distinctive neuro-imaging findings and serum biomarkers.[1] Both disorders are inflammatory/autoimmune in nature, and recent evidence indicates that genetic factors may confer susceptibility to ANE and perhaps ADEM.[2,3] We expand upon this genetic

architecture by describing biallelic variants in the ribonuclease inhibitor (*RNH1)* gene, in previously healthy children who developed a distinctive subtype of ANE. Our report of *RNH1*-associated encephalopathy in healthy children expands upon a very recent report by Hedberg-Oldfors et al,[4] in which 2 siblings with a homozygous damaging variant in *RNH1* developed acute encephalopathy (akin to ANE) but superimposed upon pre-existing global developmental delays, myopathy, and congenital cataracts.

The exact incidence of ANE is unknown; it often affects children younger than 4 years and appears catastrophically within 1 to 3 days of a viral infection, such as influenza/parainfluenza.[5] On brain magnetic resonance imaging (MRI), the hallmark is symmetrical lesions of the thalami, and variably, the periventricular white matter, internal capsule, putamen, and brain stem.[6] A systemic inflammatory response to infections, leading to a cytokine storm, results in brain edema rather than a direct invasion of the brain by the pathogen.[7] A subtype of ANE, referred to as ANE1, is associated with variably penetrant (40%) heterozygous variants in *RANBP2* (RAN binding protein 2). ANE1 is clinically distinguished from sporadic ANE by recurrent episodes, a family history, and additional bilateral involvement of the amygdala, insular cortex, and hippo-campi. The mechanism for ANE1, however, remains elusive, with several nonmutually exclusive hypotheses related to the cellular functions of *RANBP2*.[3,8] Beyond *RANBP2*, healthy individuals with heterozygous polymorphisms in carnitine palmitoyl transferase 2 (*CPT2*), which is associated with an autosomal recessive disorder, have also experienced ANE with viral infections, thought to be due to impairment of mitochondrial metabolism during fever.[9] Irrespective of etiology, ANE can be devastating, with high mortality and residual neurologic deficits, with only 10% recovering fully.[7]

ADEM has an incidence of about 1:125,000 individuals annually,[10] predominantly affects children <10 years of age, and occurs 1 to 2 weeks after a febrile infectious illness. On brain MRI, its distinguishing features are asymmetric, large, multiple lesions, typically involving subcortical and central white matter, consistent with demyelination,[11] and rarely the thalami and basal ganglia.[10] The pathogenesis of ADEM is still unclear, but it is thought to be autoimmune, partly because of formation of autoantibodies to myelin.[2,4,12–14] The genetic loci that may be associated remain largely unknown, with 1 report of ADEM in an individual with a heterozygous variant in *TLR3*, which is associated with susceptibility to viral infections.[2] Over 2 of 3 children with ADEM recover completely, with 7% to 10% dying and the rest left with neurologic deficits.[15]

It is likely that with the current exponential increase in genome-wide sequencing that further genetic loci will be identified for acute encephalopathies in previously healthy individuals. In this study, we report 8 previously healthy children from 4 families, who presented with an acute encephalopathy, variably diagnosed as ANE or ADEM, and had biallelic variants in a gene not yet associated with human disease at that time, *RNH1.* The neuroradiological findings are distinctive and in combination with the genetic findings, likely indicate a subtype of ANE (proposed as ANE2) of variable penetrance, similar to *RANBP2*-associated ANE1.

Materials and Methods

Ethics statement, recruitment, and participant evaluation

Participating research centers were connected through GeneMatcher.[16] All participants were enrolled because they were probands with an acute encephalopathy, or affected or unaffected siblings or parents. Clinical assessment, exome sequencing (ES) or genome sequencing (GS), bioinformatic analyses, and skin punch biopsies were approved by local institutional review boards (IRBs). A legal guardian provided written informed consent. All affected individuals' clinical information, including medical history, physical examination, and neuroradiology findings, were obtained from their medical records and self-report updates and reviewed independently.

ES, GS, and variant analyses

Trio or quartet-based ES was performed for families 1, 2, and 3, with varying exome library capture kits and Illumina sequencing platforms as published[17–19]; quartet GS was performed for family 4 as published (sequencing capture details in Supplemental Tables 1–4).[20] Detailed bio-informatic analyses are described in the Supplementary Methods.[21,22] All candidate variants identified by ES or GS in each family are in Supplemental Table 5.

Sanger sequencing

To perform segregation analysis and secondary validation of candidate variants identified by ES or GS, bidirectional Sanger sequencing was performed, with standard procedures. Primer sequences are available upon request.

Primary cell culture and RNH1 immunoblotting

We established primary dermal fibroblast cell cultures from epidermal skin punch biopsies from affected individuals and unaffected parents from family 1 and family 2, and RNH1 immunoblotting was performed. We used the following primary antibodies against RNH1 (Novus Biologicals, H0006050-M07; 1:500) and B-actin (ThermoFisher Scientific PA1–183; 1:2000) (Supplemental Data).

Results

Clinical reports

Family 1—Individuals 1-II-1 and 1-II-3 (Figure 1, Table 1, Supplemental Data) are male and female siblings, respectively, of nonconsanguineous parents. The male sibling presented at 32 months, with acute encephalopathy during a rhinovirus infection. On brain MRI, symmetrical lesions of the periventricular and deep white matter were evident (Table 1), and he was given a diagnosis of ADEM. He was treated with levetiracetam and high-dose methylprednisolone. Anti–myelin oligodendrocyte glycoprotein (anti-MOG) antibodies, autoimmune testing, and cytokine levels were not obtained. After steroid taper, he developed hemolytic uremic syndrome. He was subsequently hospitalized 5 times, over a 13-month period. Additional treatments included, intravenous immune globulin (IVIG) and eculizumab. He continued to have respiratory, cardiovascular, renal, gastrointestinal, hematological, immune, and neurologic symptoms. Brain MRIs at 36 and 42 months showed

progressive cavitation of the affected areas and interval parenchymal volume loss (Figure 2A) with no further change on a brain MRI at 44 months. He died at 45 months of age when supportive care was withdrawn because of worsening respiratory status and overall poor prognosis.

Subject 1-II-3 presented at 6 months of age with seizure-like symptoms, but no altered sensorium, during a febrile illness from parainfluenza and rhinovirus. She had neutropenia, low complement-3 level, mildly elevated transaminases, and hypogammaglobulinemia. The C-reactive protein level was elevated, cytokine panel testing was notable for elevated IL2 receptor and IL13 levels, but anti-MOG antibodies were not obtained. A brain MRI showed T2 signal abnormality in the thalami, basal ganglia, internal capsule, brainstem, and dentate nuclei (Table 1, Figure 2B–D). Other infectious and metabolic studies were non-diagnostic. She was given a diagnosis of ADEM and was treated with high-dose methylprednisolone and IVIG. Notably, repeat brain MRI performed 11 days after the initial study showed resolution of previously identified abnormalities. She continues to receive IVIG periodically. At her last evaluation at 10 years of age, she was developing typically, with no seizures.

Family 2—Individuals 2-II-3 and 2-II-4 are male siblings of non-consanguineous parents (Figure 1, Table 1, Supplemental Data). Individual 2-II-3 at 4 months of age developed acute motor regression in the setting of a febrile illness. Spinal MRI showed demyelination in the lower thoracic spinal cord and conus medullaris (Figure 2E), and he was diagnosed with myelitis. Two months later, he developed acute encephalopathy, with status epilepticus and global neurologic regression. Brain MRI showed symmetrical T2 abnormalities in the periventricular and deep white matter of the cerebral hemispheres (Table 1). Cerebrospinal fluid (CSF) studies were notable for multiple elevated cytokines, including IL-2, 4, 6, and 8, IFN-γ, and TNF-α, and he was given a working diagnosis of ANE. He had nondiagnostic infectious and metabolic studies. He was treated with IVIG and pulse methylprednisolone. Subsequent brain MRIs showed extensive cavitation with internal hemorrhages, of previously affected areas (ages 17 mo, 2 y, and 3 y). At last follow-up at 5 years of age, he had static encephalopathy with frequent breakthrough seizures, minimal voluntary movement, and a 2-word vocabulary.

Individual 2-II-4, the younger brother of individual 2-II-3, presented in infancy with an episode of acute cerebral edema and status epilepticus and died. He had anemia and thrombocytopenia, but inflammatory markers were not tested. In addition to diffuse edema, he had ventriculomegaly with hypoattenuation in the thalamus, basal ganglia, mid-brain, and deep white matter on CT. He was treated with dexamethasone.

Family 3—Individuals 3-II-3 and 3-II-5 are female and male siblings, respectively, of nonconsanguineous parents (Figure 1, Table 1, Supplemental Data). Individual 3-II-3 presented with acute encephalopathy and seizure-like episodes at 2 years, during influenza A infection. Lymphopenia was evident on a blood count. Inflammatory markers were not measured. Metabolic studies were nondiagnostic. A head computed tomography showed cerebral and cerebellar edema. Individual 3-II-3 passed away during her acute presentation, previously published.[23] On a postmortem, necrosis of the thalami, pontine tegmentum, and dentate nuclei were seen, consistent with ANE.

Individual 3-II-5 presented with acute encephalopathy, during influenza A infection, at 6 years of age but without seizures. Hematologic findings included lymphopenia and anemia. Anti-MOG antibodies, C-reactive protein, and most plasma cytokines were within normal limits; in the CSF, IL-1RA and MCP-1 were elevated. Metabolic studies were nondiagnostic. Because of the clinical presentation, serial MRIs being consistent with ANE (Table 1) and the family history, he was admitted to the pediatric intensive care unit, under continuous electroencephalography monitoring, mechanical ventilation, and external cooling; he was treated with IVIG, high-dose pulse corticosteroids, plasmapheresis, acyclovir, broad-spectrum antibiotics, anakinra, and colchicine. He made a near-complete recovery over the course of a 5-week hospitalization. At last follow-up at 9 years of age, he was neurologically normal and had mild attention-deficit/hyperactivity disorder and behavioral problems.

Family 4—Individuals 4-II-1 and 4-II-2 are female and male siblings of nonconsanguineous parents (Figure 1, Table 1, Supplemental Data). Individual 4-II-1 presented with an acute encephalopathy during a febrile illness at 14 months, with no seizures. Infectious and metabolic studies were nondiagnostic; CSF showed normal white blood cell count (WBC) and protein levels. Cytokines and anti-MOG antibodies were not measured. Brain MRI showed restriction on diffusion-weighted imaging (DWI) and symmetrical T2 signal abnormality in periventricular and deep white matter (Figure 2F); she was diagnosed with ADEM. At age 3.5 years, she had a recurrence of acute encephalopathy and on brain MRI had periventricular and deep white matter abnormalities. She was treated with pulse steroids with each acute presentation. Chronic MRI changes included extensive cavitation of previously affected areas (17 m, 3 y, 4 y, and 17 y).

Individual 4-II-2 also presented with an acute encephalopathy at age 8 months during a febrile illness. Infectious and metabolic studies and CSF protein and WBC were normal. Cytokines and anti-MOG antibodies were not measured. Brain MRI showed symmetrical white matter disease, involving the periventricular white matter, as well as the bilateral cerebral hemispheres and dentate nuclei region (Figure 2G–I). He was diagnosed with ADEM. He developed acute encephalopathy again at 4.5 years of age and had similar MRI findings. The episodes were treated with pulse steroids. He developed seizures at 16 years of age; chronic MRI changes included progressive periventricular white matter disease with cavitation, thinning of the corpus callosum, and focal lesions in the dentate nuclei (Figure 2J–O).

Both siblings underwent a diagnostic evaluation at the Undiagnosed Diseases Network at 22 years of age and 16 years of age, respectively. Both had poor head control, feeding difficulties, no speech, spasticity with little movement of the extremities, and relatively preserved cognition. Interestingly, both had retinal bone spicules in the retinal periphery but no optic atrophy. On optical coherence tomography, they had rod pigmentary retinopathy.

Genomic studies identify rare biallelic variants in RNH1

Because of recurrences of the acute encephalopathy in siblings, genomic sequencing was pursued in all families. In family 1, we performed research quartet ES (Figure

1, Supplemental Table 1).[24] Following our agnostic bionformatic filtering criteria, we identified 2 rare heterozygous missense variants in trans (NM_203387.3: c.279G>T; p.Gln93His and c.626G>A; p.Cys209Tyr; Figures 1 and 3) in *RNH1*. Neither variant is reported in homozygosity in gnomAD; both are localized to leucine-rich repeat (LRR) domains and are conserved across vertebrate species (Figure 3C). Bidirectional Sanger sequencing confirmed appropriate segregation of both changes with disease in the pedigree (Figure 1, Supplemental Table 5). The healthy sibling (individual 1-II-2) was heterozygous for the (p.Gln93His) *RNH1* variant.

In family 2, singleton ES was performed clinically for individual 2-II-3 (Supplemental Table 2), and a homozygous nonsynonymous variant in *RNH1* was identified (NM_203387.3: c.887T>C; p.Leu296Pro). Sanger sequencing showed that the change was heterozygous in both parents, homozygous in the affected sibling 2-II-4, and heterozygous in all 3 unaffected siblings tested (Figure 1, Supplemental Table 5). The p.Leu296Pro variant is absent from gnomAD, localized to an LRR repeat domain and conserved among vertebrate species to chicken (Figure 3).

For family 3, rapid research-based and clinical trio-ES (Supplemental Table 3) were performed concurrently and identified individual 3-II-5 to have biallelic rare variants in *RNH1*: a presumed truncating change (NM_203387.3: c.40G>T; p.Glu14Ter) and a nonsynonymous variant (c.1117C>T; p.Arg373Trp) (Figure 1, Supplemental Table 5). The p.Glu14Ter change is present in a single allele in gnomAD and is in the N-terminal region preceding the LRRs; the p.Arg373Trp variant is ultrarare in gnomAD, localizes to the antepenultimate LRR domain, and is conserved among amino acids with strongly similar properties at p.Arg373 (Figure 3). Bidirectional Sanger sequencing confirmed that both variants are present in the deceased affected sibling (II-3). Notably, 2 unaffected siblings harbor both *RNH1* variants (3-II-2 and 3-II-6), and the other 3 healthy siblings are either heterozygous or wild type at the *RNH1* locus (Figure 1).

Subsequent to clinical evaluation as part of the Undiagnosed Diseases Network, family 4 quartet underwent GS (Supplemental Table 4). Bioinformatic filtering for candidate variants identified biallelic rare variants in *RNH1*: NM_203387.3: c.682_685delins CTGGGCCTTGGGCA; p.Ser228LeufsTer17 and c.1117C>T; p.Arg373Trp (Supplemental Table 5). The truncating variant is absent from gnomAD and localizes to LRR domain 8 of 15 (Figure 3). Bidirectional Sanger sequencing confirmed that both *RNH1* variants are present in both affected siblings.

Notably, the p.Arg373Trp variant is shared between 4 affected individuals in families 3 and 4, and it is trans with a presumed loss-of-function variant, lending support to the association of *RNH1* with the acute encephalopathy. On the immunoblot, the missense variants in families 1 and 2 were associated with nearly undetectable RNH1 protein levels in fibroblasts from all 4 affected individuals (Figure 4). Although these data are not of direct physiological relevance, it is evident that p.Gln93His, p.Cys209Tyr, and p.Leu296Pro result in loss of protein function.

Discussion

Although most acute encephalopathies in previously healthy individuals are thought to be sporadic, it is becoming evident that Mendelian etiologies can confer susceptibility, with *RANBP2* as the prototype.[25] We provide clinical, neuroradiological, and genetic data that associate biallelic variants in a novel gene, *RNH1*, with a distinctive acute febrile encephalopathy, which we propose designating as ANE2. Because there are no biallelic loss of function and/or moderate to strong missense variants in *RNH1* in gnomAD, ANE2 is likely to be ultrarare. However, knowledge of this entity and the underlying genetic susceptibility can enable prompt aggressive immune modulation to improve outcomes, prevention/preemptive treatment of recurrences, and genetic screening of at-risk family members to prevent devastating neurologic consequences.

Before the association with *RNH1* was known, the children in this study had been variably diagnosed with ANE ($n = 3$) or ADEM ($n = 4$). The early presentation of the encephalopathy (<48 hours during a viral or febrile illness) is consistent with ANE. However, laboratory findings that would support ANE were not seen consistently or were not available: these included normal CSF WBC count ($n = 2$ of 8) and elevated cytokines ($n = 3$, but not in a consistent pattern). The lack of comprehensive data on cytokines especially limits the comparison to ANE because cytokines (most often TNFα, IL-1, and IL-6) mediate the disease in ANE.[3] Notably, the acute hemolytic uremic syndrome in individual 1-II-1 could have occurred because of thrombotic microangiopathy, a complication described in ANE.[26,27] Another interesting observation is that the 2 affected individuals from family 4, did not have optic atrophy, as expected from a demyelinating process such as ADEM but had pigmentary retinopathy instead. Acute retinal necrosis with subsequent pigmentation has been observed in herpes encephalitis, but the pathophysiology may be different because of direct viral invasion of the retinae.[28] Therefore, further case reports are needed to clarify if retinal findings are associated with ANE2 as well as to study its pathophysiology.

The neuroradiological findings in our cohort overlapped with ANE considerably, including the DWI restriction in affected areas ($n = 5$ of 6 individuals with DWI images), the symmetrical involvement of the dentate nuclei ($n = 3$ of 8), thalami ($n = 4$ of 8), and chronic cavitation ($n = 4$ of 8). The postmortem necrosis of affected brain regions ($n = 1$) was also consistent with prior reports of brain autopsy changes in ANE.[29] However, the thalami would be expected to be affected in almost 100% of children with ANE, and hemorrhages are much more common in ANE than it is evident in our cohort. Furthermore, the frequent involvement of the periventricular white matter as seen in our cohort (5 of 8) is not common in typical ANE. In contrast to ANE, the neuro-radiological findings are mostly inconsistent with ADEM, in which asymmetrical large lesions often spare the periventricular white matter, and DWI changes tend to be more diffuse. We do not have information on anti-MOG antibodies (normal in 1, unavailable in the rest), that would support the possibility of ADEM. Thus, overall the findings overlap with ANE but with distinctive radiological features that are suggestive of a subtype, and we propose it to be ANE2.

The treatment of ANE/ADEM overlap, and in our cohort, steroids and IVIG were commonly used. The early use of IVIG and other immune modulating treatments in individuals 1-II-3

and 3-II-5 could have decreased brain swelling and suppressed the systemic inflammatory response and may have resulted in their excellent outcomes (individual 1-II-3 may also have had milder disease, as suggested by her normal sensorium and milder brain MRI changes).[30] Overall, the long-term neurologic outcome varied from death during an acute episode ($n = 3$) to severe neurologic deficits and intellectual disabilities ($n = 3$) and typical neurodevelopment with no neurologic deficits ($n = 2$). Thus, early recognition and aggressive immunological treatment to suppress inflammation and cytokine storm may improve outcomes in the *RNH1*-associated ANE2, similar to ANE1.[13]

We compared ANE1 that is associated with *RANBP2* with the *RNH1*-associated ANE2: brain areas specifically affected in ANE1, such as the amygdala, hippocampi, and medial temporal lobes,[13] were not involved in our cohort. We did see evidence of incomplete penetrance with *RNH1*, similar to *RANBP2*—2 healthy individuals in family 3 with the same biallelic damaging *RNH1* variants have not experienced an acute encephalopathy at ages 7 and 24 years, but they may continue to be at risk because ANE/ANE1 can occur at any age.[3,25] Similar to ANE1, we saw recurrence of the acute encephalopathy in 2 of our individuals, underscoring the increased vulnerability conferred by the genetic variants in *RNH1*. Unlike the autosomal dominant inheritance of *RANBP2* associated ANE1, ANE2 is autosomal recessive. Nonetheless, similar to *RANBP2*-associated ANE1,[31] loss of function is likely to be the mechanism with *RNH1*, as evidenced by the occurrence of truncating variants and the missense variants in families 1 and 2 resulting in a lack of RNH1 protein production. Further functional assays, such as animal modeling, are necessary to confirm this mechanism. Similar to *RANBP2*, most of the *RNH1* variants in our cohort are also localized to the N-terminal LRR domains of the protein; the LRRs are important for protein-ligand interactions.[32] Notably, another gene *RANGAP1,* whose protein becomes mislocalized with RANBP2 depletion (and being actively studied in ANE1), is also a member of the RNH1 subfamily, thus containing LRRs.[33] Therefore, our report that links *RNH1* to an acute encephalopathy may stimulate further insights into a molecular mechanism for ANE1.

Despite overlap in the location of variants in the LRRs for *RANBP2* and *RNH1*, the pathogenesis of the *RNH1*-associated acute febrile encephalopathy is unknown. However, recent observations independent of our report provide some potential mechanistic insight. RNH1 protein is ubiquitously expressed and was the first cytosolic protein identified to contain LRRs.[33] The LRRs in RNH1 (Figure 3D) are similar to the LRRs in NLRP proteins that form inflammasome complexes.[33] Inflammasome complexes, composed of sensors such as NLRP3, are critical components of the innate immune response, mediating caspase-1 inactivation and the secretion of proinflammatory cytokines in response to microbial infection; they have been linked to varied disorders, such as the periodic fever syndromes and Alzheimer disease.[34] Recently, Bombaci et al,[33] in elegant experiments in the context of SARS-CoV-2 infection, demonstrated that RNH1 deficiency in macrophages results in increased NLRP3 inflammasome activity, with increased interleukin-1β production and pyroptosis, a form of cell death. Next, they demonstrated that conditional knockout of *Rnh1* in mice promoted excessive inflammasome activity and lethality. Finally, in human patients with SARS-CoV-2, they demonstrated that RNH1 levels were negatively associated with SARS-CoV-2 severity—patients in intensive care had lower levels of RNH1

protein relative to those in regular wards; RNH1 expression levels in lung tissue were also dramatically decreased (indeed, largely absent) in patients who died of SARS-CoV-2–related respiratory illness compared with those who died because of nonviral causes.[33] Similarly, another recent publication reported that increased activation of the NLRP3 inflammasome was associated with increased cytokine release and pyroptosis, in influenza A and SARS-CoV-2 infections.[35] Thus, the collective evidence from these studies raises the possibility that germline damaging variants in *RNH1* may result in excessive and maladaptive inflammasome activation in SARS-CoV-2 and other viral infections, potentially increasing the risk of acute encephalopathy. Of related interest, both ANE and ADEM-like encephalopathy have been described in humans with SARS-CoV-2 infection,[3,36] and a pathogenic *RANBP2* variant was found in 1 individual with SARS-CoV-2–related ANE.[37]

It is plausible that screening with genomic sequencing may identify biallelic germline *RNH1* variants in healthy individuals, identifying them to be at risk for ANE2. Interestingly, *RNH1*-related acute encephalopathy with transient anemia was reported recently in 2 siblings with prior neurologic manifestations by Hedberg-Olfors et al[11]; and similar to our cohort, 1 sibling had thalamic findings, the homozygous variant was in an LRR domain, and functional studies demonstrated loss of function. However, distinctive differences are evident: there was no periventricular or dentate nuclei involvement in the 1 sibling who had a brain MRI in the Hedberg-Oldfors paper, and our cohort was previously healthy, compared with the pre-existing/additional features of myopathy, cataract, and developmental delays reported by Hedberg-Oldfors. The reason(s) for these discrepancies is/are unclear. It is possible that RNH1 has variable broader/direct effects on the brain (the supposition of a direct effect on the brain is supported by an in vitro study showing that Rnh1 may contribute to oligodendrocyte differentiation/myelination).[38] Alternatively, because the parents in the report by Hedberg-Oldfors are consanguineous, and there was a region of homozygosity on chromosome 11 (including *RNH1*), there could be a second, potentially linked etiology to explain the pre-existing features in the 2 siblings. Ultimately, more clinical reports on the *RNH1*-associated acute encephalopathy will provide more information on its relevance to healthy individuals and to those with prior manifestations.

The limitations of our study are that our individuals were studied retrospectively, with the acute encephalopathies having occurred over 2 decades ago in some and across multiple institutions; thus, we do not have comprehensive data on inflammatory markers to compare our findings with those in ANE. Our functional data are limited, with the immunoblot only demonstrating lack of protein. All the individuals we ascertained were affected as children, but it is likely that adults with biallelic damaging *RNH1* variants may also be at risk, but we do not know the clinical and neuroradiological findings in older individuals. However, awareness of *RNH1* as a genetic factor in acute encephalopathy can enable identification of at-risk individuals and stimulate further research into pathogenesis; hence, we strongly feel that these data are of immediate relevance.

In summary, the clinical, genetic, and neuroradiological data in this study provide evidence that biallelic variants in *RNH1* confer a variably penetrant predilection to an acute viral/febrile encephalopathy that overlaps ANE and ANE1; because of salient distinguishing features, it likely represents a recognizable subtype, and we propose it be referred to

as ANE2. Reports of further similar patients will confirm the veracity of ANE2 as an entity, its clinical features, and optimal treatment, and functional mechanistic studies will help unravel the underlying disease pathogenesis. We recommend genomic sequencing for underlying predisposition genes, such as *RNH1* and *RANBP2*, be undertaken in all previously healthy patients with an acute infectious encephalopathy to improve outcomes for affected individuals and for at-risk family members.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

Additional data are available from the corresponding authors upon request.

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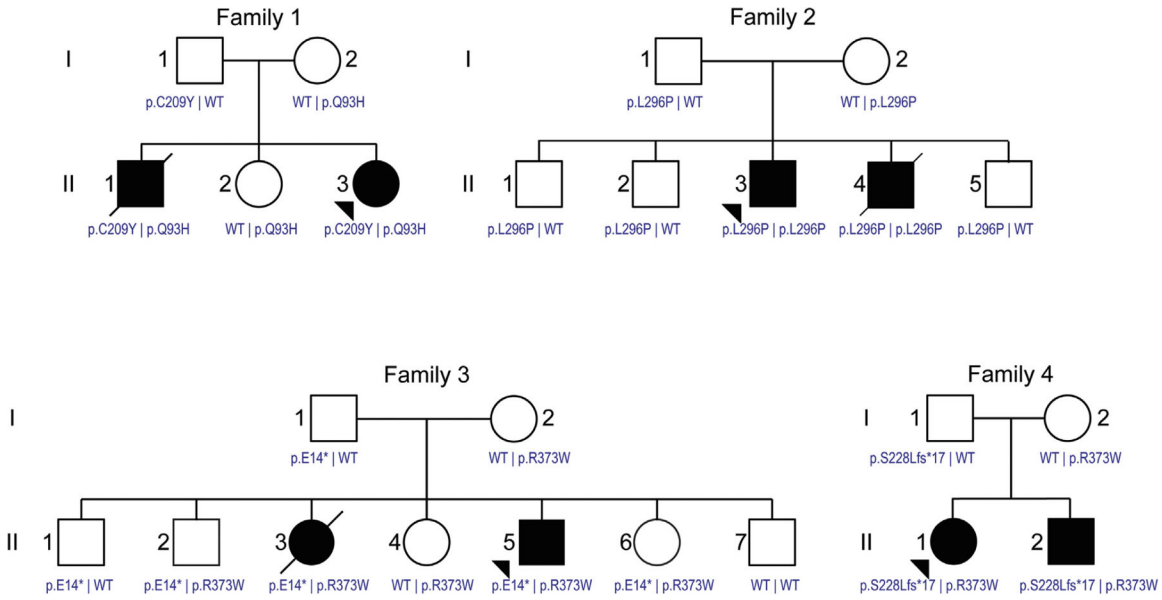

Figure 1. Pedigrees of families with biallelic rare variants in *RNH1*.

Filled shapes, affected individuals; unfilled shapes, healthy individuals; square, male; circle, female. Segregation of RNH1 variants as confirmed by bidirectional Sanger sequencing are indicated in blue. WT, wild type.

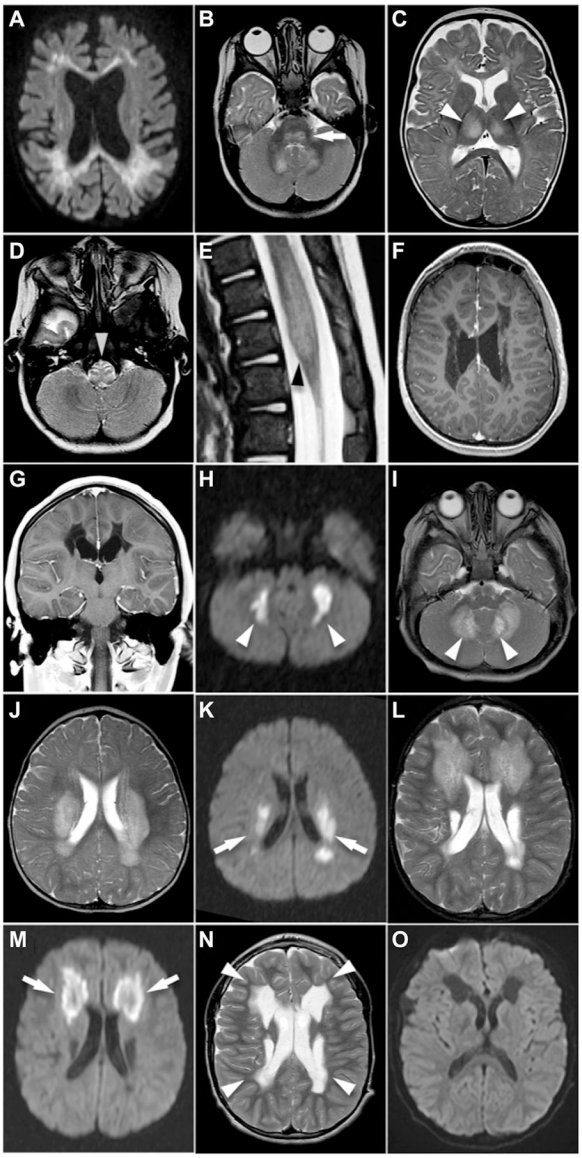

Figure 2. Representative magnetic resonance imaging from affected individuals harboring biallelic *RNH1* variants

Images (A-I) illustrate the location of lesions in 5 individuals. Axial DWI of individual 1-II-1 at age 3 years 6 months (A), axial T2-weighted images from individual 1-II-3 at age 6 months (B-D), sagittal T2-weighted image of the spinal cord in individual 2-II-3 at 4 months of age (E), axial postcontrast T1 SPGR of individual 4-II-1 at age 17 years (F), coronal postcontrast T1 SPGR of individual 4-II-2 at age 12 years (G), and axial DWI (H) and T2-weighted (I) images from individual 4-II-2 at 8 months are shown. Involvement of deep and periventricular white matter was common (A, F, and G). Involvement of the dentate nuclei of the cerebellum (B, H, and I) was also frequently observed. Less commonly observed areas included the dorsal pons (B), thalami (C), medulla (D), and spinal cord (E). Images (J-O) illustrate the temporal evolution of lesions in individual 4-II-2. Axial T2 (J) and DWI (K) images at 8 months of age show areas of T2 hyperintensity and swelling in the periventricular white matter associated with restricted diffusion (arrows in K). Repeat

T2 (L) and DWI (M) imaging performed 4 years later shows new lesions in the frontal periventricular and deep white matter (arrows in M). Cavitation is seen at the site of the prior lesions. Follow-up T2 (N) and DWI (O) imaging performed 15 years after the initial scan shows no new lesions, and cavitation of all prior lesions (arrowheads in N). DWI, diffusion-weighted imaging; SPGR, spoiled gradient.

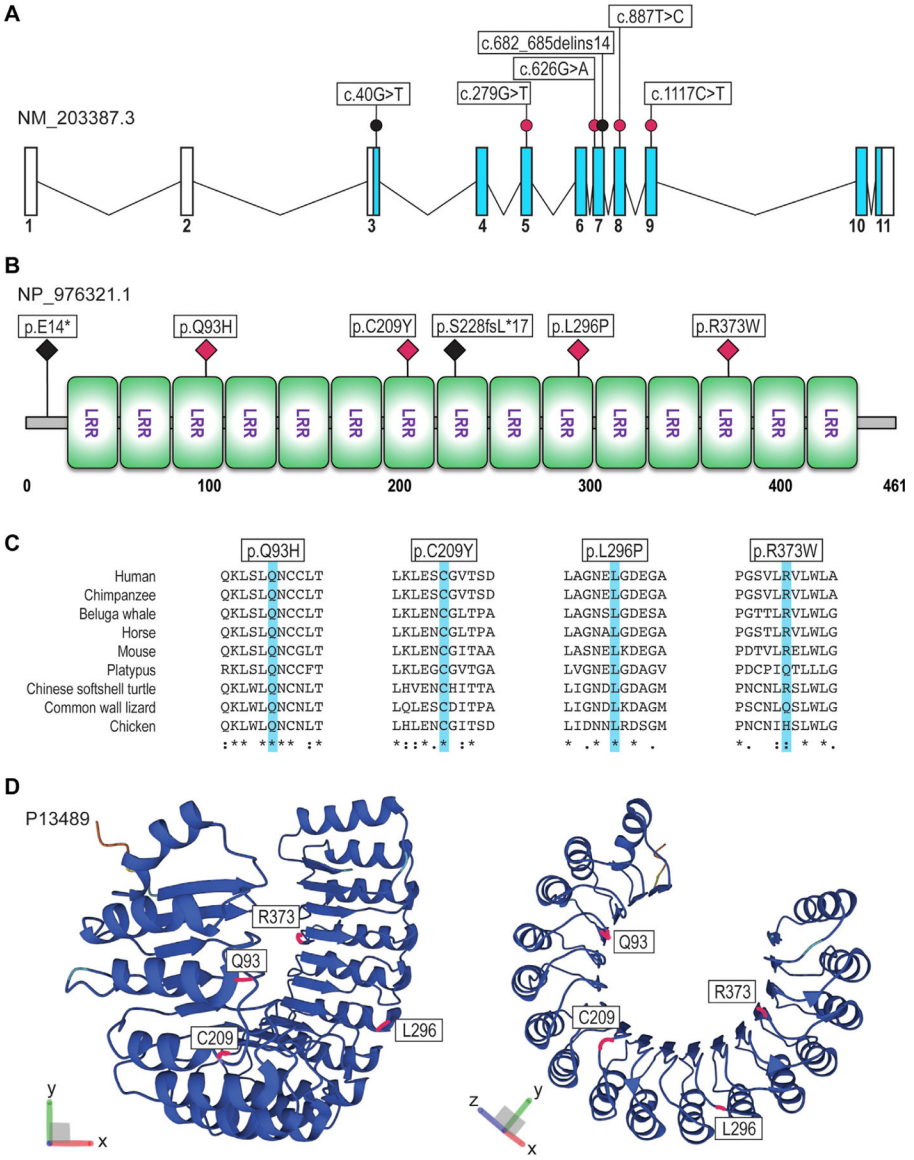

Figure 3. Gene and protein schematics showing localization of *RNH1* variants

A. Schematic representation of *RNH1* on chromosome 11. Blue boxes, coding exons; unfilled boxes, untranslated regions; lines, introns. Exon number is listed below each box. Variants named according to GenBank ID: NM_203387.3 transcript are indicated with black (presumed truncating) and red (missense) lollipops. B. Schematic representation of RNH1 amino acid sequence. Green boxes, leucine-rich repeat domains. Amino acid number is listed below the schematic; variants named according to NP_976321.1 are indicated with black (presumed truncating) and red (missense) lollipops. C. Conservation of human RNH1 missense variants as compared with 8 vertebrate species (blue box). Clustal Omega (v1.2.4) was used to generate a multiple sequence alignment using human (NP_976321.1), chimpanzee (NP_001009060.1), beluga whale (XP_022421109.1), horse (XP_001488525.1), mouse (NP_660117.2), platypus (XP_028917039.1), Chinese softshell turtle (XP_006124919.1), common wall lizard (XP_028591537.1), and chicken

(XP_040556805.1) amino acid sequences. D. Three-dimensional structure of human RNH1. AlphaFold (version 2022-11-01) was used to generate a predicted model; 2 different orientations are shown with localization of missense variants indicated. LRR, leucine-rich repeat.

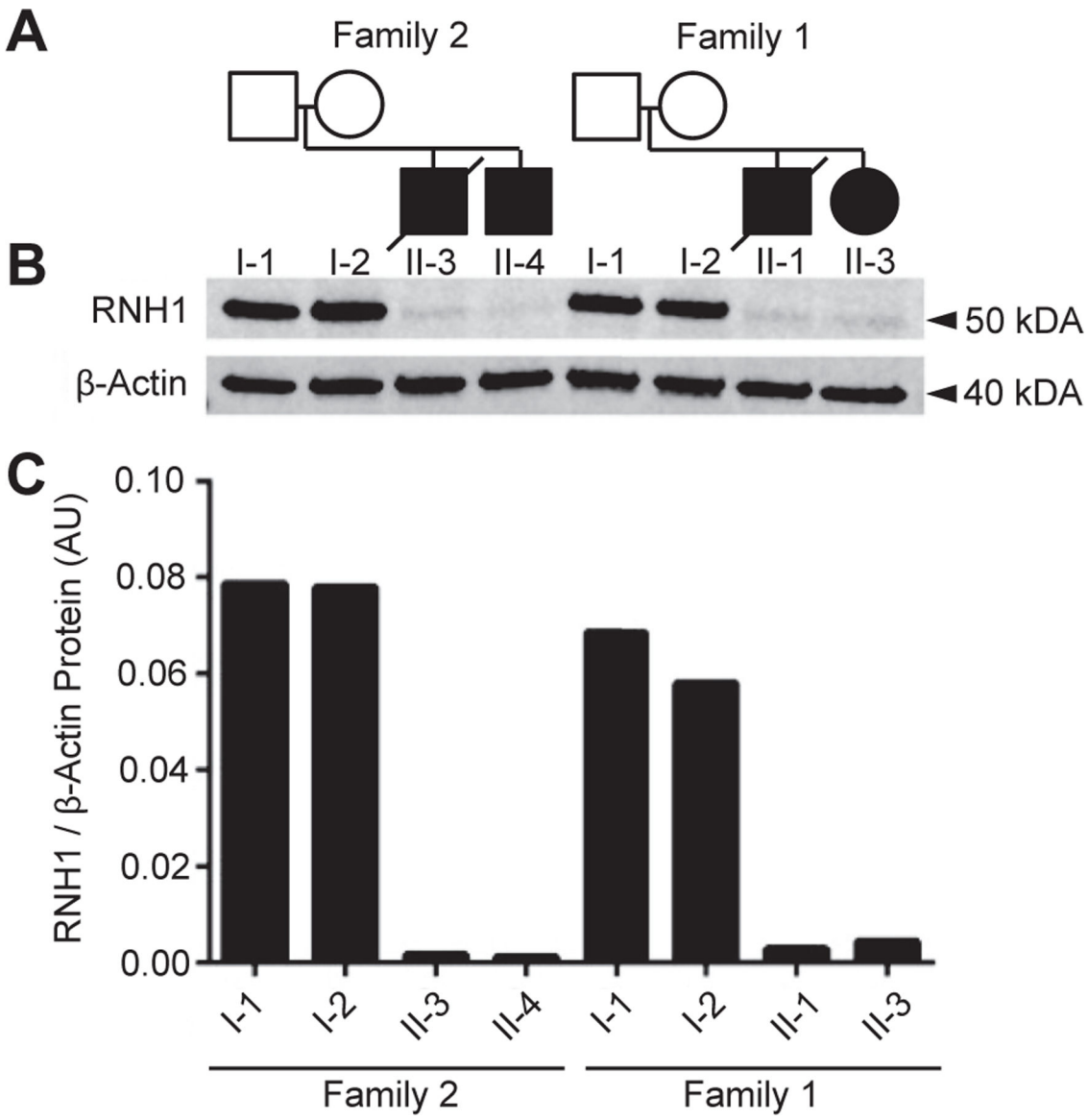

Figure 4. Missense variants in family 1 and family 2 result in RNH1 protein degradation

A. Pedigrees indicating individual of origin for skin punch biopsies that were used to establish primary dermal fibroblasts. See Figure 1 for further details. B. Immunoblotting of RNH1 in 20 μg/lane total protein harvested from primary fibroblast cultures. β-actin was used as a loading control. See Supplemental Figure 1 for full blot images. C. Quantification of signal shown in panel B. RNH1 quantity was normalized to β-actin and shown in terms of arbitrary units. Affected individuals have nearly undetectable RNH1 protein compared with heterozygous parents.

Table 1

Clinical features, genetic variants, and neuroradiological findings in the 8 subjects across 4 unrelated families with biallelic *RNH1* variants and acute encephalopathy

Family (Individual)	Family 1: (1-II-1)	Family 1: (1-II-3)	Family 2: (2-II-3)	Family 2: (2-II-4)	Family 3: (3-II-3)	Family 3: (3-II-5)	Family 4: (4-II-1)	Family 4: (4-II-2)
Age at most recent examination	3 y 9 mo (deceased)	10 y	5 y	3 mo (deceased)	25 mo (deceased)	9 y	22 y	16 y
Sex	Male	Female	Male	Male	Female	Male	Female	Male
Ancestry *RNH1* variant(s) (NM_203387.3)	Northern European (United States) c.279G>T; p.(Gln93His) c.626G>A; p.(Cys209Tyr)		Guatemalan c.887T>C; p.(Leu296Pro)		British/European (Canada) c.40G>T, p.(Glu14Ter) c.1117C>T, p.(Arg373Trp)		Northern European (United States) c.682_685delinsCTGGGCCTTGGGCA, p.(Ser228LeufsTer17) c.1117C>T, p.(Arg373Trp)	
Zygosity	Compound heterozygous		Homozygous		Compound heterozygous		Compound heterozygous	
Neonatal course and early development	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Age at initial acute presentation	2 y, 8 mo	6 mo	4 mo	3 mo	25 mo	6 y, 1 mo	1 y, 2 mo	8 mo
Precipitating factors (time until acute presentation)	Rhinovirus, afebrile (24–48 h)	Parainfluenza 3 and rhinovirus, afebrile (48 h)	Viral illness, afebrile (unknown)	Febrile illness with tactile fever, unconfirmed (48 h)	Influenza A, febrile illness (48 h)	Influenza A, febrile illness (24–48 h)	Febrile illness (24 h)	Febrile illness (24 h)
Acute MRI findings	DWI restriction and T2 abnormality in periventricular and deep white matter	T2 abnormality in thalami, basal ganglia, internal capsule, brainstem, dentate nuclei, without DWI restriction, but normal 11 d later	DWI restriction and T2 signal abnormality only in the spinal cord (conus) initially; 2 mo later also in periventricular and deep white matter with micro hemorrhages and enlarged thalami	No MRI (CT-diffuse edema, ventriculomegaly, hypoattenuation in thalami, basal ganglia, midbrain and deep white matter)	No MRI (cerebral and cerebellar edema on CT)	DWI restriction and T2 signal abnormality in periventricular and deep white matter, thalami, brainstem midbrain and dentate nuclei, hemorrhage in pons	DWI restriction and T2 signal abnormality in periventricular and deep white matter and dentate nuclei	DWI restriction and T2 signal abnormality in periventricular and deep white matter
EEG findings at initial acute presentation	Not available	Normal	Diffuse excessive beta activity, multifocal sharp waves	No electrical activity (at time of death)	Unavailable	Diffuse slowing	Diffuse slowing	Normal
Acute inflammatory and/or autoimmune markers	Not available	CRP elevated; IL-2 receptor and IL-13 elevated	CSF cytokines elevated	Not performed	Not performed	CRP normal. Anti-MOG normal. Cytokines IL-1RA and MCP-1 elevated	CRP elevated	CRP elevated

Family (Individual)	Family 1: (1-II-1)	Family 1: (1-II-3)	Family 2: (2-II-3)	Family 2: (2-II-4)	Family 3: (3-II-3)	Family 3: (3-II-5)	Family 4: (4-II-1)	Family 4: (4-II-2)
Diagnosis at initial presentation	ADEM, followed by atypical HUS	ADEM	Myelitis; ANE 2 mo later	Seizures, cerebral edema	ANE	ANE	ADEM	ADEM
Treatment of initial acute encephalopathy	High-dose pulse corticosteroids levetiracetam IVIG eculizumab	High-dose corticosteroids Broad-spectrum antibiotics IVIG lorazepam levetiracetam acyclovir	High-dose pulse corticosteroids IVIG	Corticosteroids	Acyclovir phenytoin Broad-spectrum antibiotics	High-dose corticosteroids IVIG plasmapheresis anakinra colchicine acyclovir broad-spectrum antibiotics	High-dose pulse corticosteroids	High-dose pulse corticosteroids
Recurrent acute presentation (s), precipitating factors, diagnoses	None	None	None	n/a	n/a	None	3.5 y Febrile illness ADEM	4.5 y Febrile illness ADEM
Current neurologic status	Deceased (3 y 9 mo)	Typical neurodevelopment, receiving ongoing IVIG treatment (age = 10 y)	Profound neurologic and intellectual disabilities (age = 5 y)	Deceased (infancy)	Deceased (age = 2 y)	Typical neurodevelopment, emotional lability (age = 6 y)	Profound neurologic and intellectual disabilities (age = 22 y)	Profound neurologic intellectual disabilities (age = 16 y)
Chronic MRI findings (timing after initial acute presentation)	Extensive cavitation of previously affected areas (3 y, 3 y 6 mo, 3 y 8 mo)	Not done	Extensive cavitation of previously affected areas; chronic internal hemorrhage (17 mo, 2 and 3 y)	n/a	Not available	Not available	Extensive cavitation of previously affected areas (17 mo, 3, 4, and 17 y)	Extensive cavitation of previously affected areas (4, 12, and 16 y)
Other acute/ chronic findings	None	Chronic hepatitis (elevated transaminases)	Acute intermittent hepatitis (elevated transaminases)	n/a	Autopsy Necrosis of thalami, pontine tegmentum dentate nuclei	None	Chronic pigmentary retinopathy	Chronic pigmentary retinopathy

ADEM, acute disseminated encephalomyelitis; *ALT*, alanine transaminase; *ANE*, acute necrotizing encephalopathy; *anti-MOG*, anti-myelin oligodendrocyte glycoprotein; *AST*, aspartate aminotransferase; *CRP*, C-reactive protein; *CSF*, cerebrospinal fluid; *CT*, computed tomography; *DWI*, diffusion-weighted imaging; *EEG*, electroencephalography; *HUS*, hemolytic uremic syndrome; *IVIG*, intravenous immune globulin; *MRI*, magnetic resonance imaging; *n/a*, not available; *GA*, gestational age.