Genotype-structure-phenotype relationships diverge in paralogs \textit{ATP1A1}, \textit{ATP1A2}, and \textit{ATP1A3}

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Abstract

Objective
We tested the assumption that closely related genes should have similar pathogenic variants by analyzing >200 pathogenic variants in a gene family with high neurologic impact and high sequence identity, the Na,K-ATPases \textit{ATP1A1}, \textit{ATP1A2}, and \textit{ATP1A3}.

Methods
Data sets of disease-associated variants were compared. Their equivalent positions in protein crystal structures were used for insights into pathogenicity and correlated with the phenotype and conservation of homology.

Results
Relatively few mutations affected the corresponding amino acids in 2 genes. In the membrane domain of \textit{ATP1A3} (primarily expressed in neurons), variants producing milder neurologic phenotypes had different structural positions than variants producing severe phenotypes. In \textit{ATP1A2} (primarily expressed in astrocytes), membrane domain variants characteristic of severe phenotypes in \textit{ATP1A3} were absent from patient data. The known variants in \textit{ATP1A1} fell into 2 distinct groups. Sequence conservation was an imperfect indicator: it varied among structural domains, and some variants with demonstrated pathogenicity were in low conservation sites.

Conclusions
Pathogenic variants varied between genes despite high sequence identity, and there is a genotype-structure-phenotype relationship in \textit{ATP1A3} that correlates with neurologic outcomes. The absence of “severe” pathogenic variants in \textit{ATP1A2} patients predicts that they will manifest either in a different tissue or by death in utero and that new \textit{ATP1A1} variants will produce additional phenotypes. It is important that some variants in poorly conserved amino acids are nonetheless pathogenic and could be incorrectly predicted to be benign.
Glossary

AHC = alternating hemiplegia of childhood; APA = aldosterone-producing adenoma; CAPOS = cerebellar atrophy, areflexia, pes cavus, optic nerve atrophy, and sensorineural deafness; CMT = Charcot-Marie-Tooth; RDP = rapid-onset dystonia–parkinsonism.

We expect that genes that diverged only slightly after past duplications should have similar pathogenic variants. The Na,K-ATPase gene family provides a good test data set because there are >200 different human mutations (in >800 patients or families), and the proteins and many mutations are well studied.1–4 All known pathogenic variants are de novo or have dominant inheritance.

Na,K-ATPase is the membrane protein responsible for producing sodium and potassium ion gradients. The 3 paralogs (isoforms) of the catalytic subunit (α) in the human brain are 87%–88% identical among α1, α2, and α3. Except at the N-terminus, their proteins align without insertions or deletions of more than 1 amino acid (figure e-1, links.lww.com/NXG/A134). In mice, knockouts are lethal in utero5 or at birth.6,7 ATP1A1 is expressed in most tissues, including neurons and glia in the brain. ATP1A2 is expressed in astrocytes; skeletal, cardiac, vascular, and smooth muscle; and adipocytes. ATP1A3 is not only in neurons but also in cardiac and several other tissues. Few pathogenic variants have been discovered in ATP1A1 so far in Charcot-Marie-Tooth neuropathy and aldosterone-producing adenomas. In contrast, there are multiple germline neurologic diseases of ATP1A2 and ATP1A3.

The Na,K-ATPase paralogs have the same fundamental ion transport mechanism.8 Sodium-bound and potassium-bound conformations are shown in different crystal structures (figure e-2, links.lww.com/NXG/A134). Variant locations in crystal structures can sometimes be related to specific enzyme functions. Mutations can produce effects on Na,K-ATPase activity, ion affinity, ion leakage, or biosynthesis. We investigated whether there are correlations between pathogenic variants (missense and 1-codon deletions), their positions in protein structure, evolutionary conservation, and the spectrum of disease phenotypes.

Methods

Sources of disease variants

The literature was searched for mutations by gene symbol. Disease variants in ATP1A1 were from aldosterone-producing adenomas (APAs)3 and cases of Marie-Charcot-Tooth type 2.4 Variants in ATP1A2 were from familial (FHM2), sporadic hemiplegic migraine, and epilepsy syndromes.2,9 Variants in ATP1A3 included alternating hemiplegia of childhood (AHC), rapid-onset dystonia–parkinsonism (RDP), and other milder and more severe neurologic manifestations found by genome sequencing of undiagnosed patients.10,11 Some variants in ATP1A3 were from our own patients, and a few alternative codons for known mutations were from ClinVar ncbi.nlm.nih.gov/clinvar/). The reference sequences were NM_000701.7 (ATP1A1); NM_000702.3 (ATP1A2); and NM_152296.4 (ATP1A3). Many suspected mutations have been tested in the laboratory for pathogenicity, but not all. The criteria for including variants in the analysis are detailed in supplementary information. All variants that met the criteria were included regardless of diagnosis.

Analysis of structure and conservation

Na,K-ATPase protein crystal structures in Na+-bound and K+-bound conformations at the highest available resolutions, 3WGU, 3KDP, and 2ZXE,12–14 were from the PDB database (rcsb.org). The positions of variants were studied in Swiss protocols approvals, registrations, and patient consents

No human experimentation was performed. Written informed consent was obtained from all patients or their guardians for cases not taken from the literature.

Data availability

All variants are listed in supplementary information tables links.lww.com/NXG/A134.

Results

Variant distributions in ATP1A2 and ATP1A3

The premise that high sequence conservation predicts variant pathogenicity implies that paralogs with high sequence identity should have similar variant distributions in the protein structure. ATP1A2 and ATP1A3 have the most disease-associated variants for comparison (figure 1A): 77 in ATP1A2 and 130 in ATP1A3 at this writing, including alternative substitutions (e.g., p.D801 to p.D801N and p.D801Y). There are striking differences, however, in the proportion of disease
variants found in different protein domains of ATP1A2 and ATP1A3 (figure 1B). Such differences would not be predicted by shared enzyme mechanisms and high sequence identity. Disease-causing variants in ATP1A2 and ATP1A3 should frequently appear at the corresponding amino acids. Instead, of 154 mutated amino acid positions in the 2 genes (68 in ATP1A2 and 86 in ATP1A3), only 18 mutated amino acids overlapped.

Although more such overlap should emerge with time, the low level of overlap is not simply that there are not enough cases yet: it contrasts sharply with the number of recurring variants. First, there were many cases in which alternative codons produced different substitutions of an amino acid: 25 amino acids in ATP1A3 had alternative substitutions (up to 6), and 13 amino acids in ATP1A2 had 2 alternative substitutions. Second, for ~600 independent occurrences of variants in ATP1A3 patients (a family is 1 occurrence), 62% have recurred twice or more, and of those, more than half have recurred 5 or more times. ATP1A2 has many fewer reported occurrences (~160), but 20% of their mutations recurred. Of the 18 pairs of variants overlapping in ATP1A2 and ATP1A3,
none involved highly recurrent mutations. These data suggest that there is little bias toward crucial residues shared by ATP1A2 and ATP1A3 but strong bias for other residues.

Singletons and families in the literature were ascertained by symptoms suspicious of a genetic origin. Separate clinical diagnoses such as RDP and AHC were independently linked to ATP1A3.\textsuperscript{10,16} The divergence in structure distribution suggests the existence of genotype-structure-phenotype relationships.

Additional phenotypes for each gene may yet be discovered.

**Structure-phenotype relationship for ATP1A3**

Human disease variants in ATP1A2 and ATP1A3 produce neurologic syndromes with onset at birth, in childhood, or in adulthood, including familial hemiplegic migraine (FHM2 [MIM:602481]),\textsuperscript{2} RDP (MIM:128235),\textsuperscript{1,29} AHC (MIM: 614820),\textsuperscript{1,2} and cerebellar ataxia, areflexia, pes cavus, optic nerve atrophy, and sensorineural deafness (CAPOS [MIM: 601338]).\textsuperscript{11} Disease variants in ATP1A2, expressed in astrocytes, produce FHM2 phenotypes associated with spreading depression or spreading excitation: migraine, hemiplegia, and seizures.\textsuperscript{2} Symptom severity has not been systematically studied for ATP1A2 mutations,\textsuperscript{2,17} but ATP1A3 disease variants can be divided by severity.\textsuperscript{10,11,18–20} The most severe are defined by onset in infancy and include AHC,\textsuperscript{1} severe infantile epilepsy,\textsuperscript{18,21} and severe hypotonia.\textsuperscript{22} Milder phenotypes have onset in children and adults and include RDP, CAPOS, relapsing encephalopathy and cerebellar ataxia\textsuperscript{19} also called fever-induced weakness and encephalopathy,\textsuperscript{23} and rapid-onset ataxia.\textsuperscript{20,24} Ages at symptom onset were taken from original reports.

The P domain, the site of ATP hydrolysis, is highly evolutionarily conserved. Figure 3A dissects this domain because its structure is otherwise difficult to appreciate. Two distant stretches of amino acids (P1 and P2) interdigitate in a twisted sheet of β-sheet strands encircled by α-helices. In figure 3A, the top row shows an end view of the β-sheet, the α-helices that surround it, and the 2 parts combined. The active site has an aqua magnesium ion. The second row shows a side view of the same structures. Figure 3B shows the distributions of mutated amino acids. In the β-sheet, all but 3 are clustered at the face of the active site. In the ring of α-helices, all mutations except 1, in magenta, are away from the active site. The helix mutations are confined to the central 5 of the 9 helices. The more distal helices and strands so far have no mutations.

In Figure 3, color distinguishes the variants found in ATP1A2, ATP1A3, or both. All have similar patterns of distribution in the P domain. Figure e-4 (links.lww.com/NXG/A134) compares the mild and severe phenotype variants of ATP1A3. Any suggestion of differences in location would need to be

**Figure 2 Structure-phenotype relationship in the membrane domain**

This figure shows the transmembrane α-helices, omitting the portions that extend into the extracellular and intracellular spaces. All the ATP1A2 membrane mutations are shown (left), whereas ATP1A3’s are divided into 2 groups: milder ATP1A3 phenotypes (middle) with onset from childhood to adult and severe ATP1A3 mutations (right) with onset in infancy. ATP1A2 and the milder ATP1A3 phenotypes have similar distributions that appear to exclude the residues right around the ions (pink = potassium ions). In contrast, the severe ATP1A3 mutations are usually close to the ions. Almost 70% of the severe mutations are in contiguous stretches in M4, M5, and M6 near the ions. In contrast, 100% of the mild mutations are not adjacent to any other mutations, and the few in M4, M5, and M6 are on either side of the contiguous stretches. The distributions highlight 2 unique features: that mild and severe ATP1A3 mutations have different distributions and that ATP1A2 mutations all look like “mild.” In fact, hemiplegic migraine seldom has onset in infancy.\textsuperscript{17} Equivalent “severe” mutations of ATP1A2 have not been found. Variants of 3 ATP1A3 residues in pale yellow can produce either mild or severe phenotypes. Fourteen ATP1A3 residues altogether have produced both RDP and mild AHC or an intermediate phenotype. Eleven of those were recurrent (with the same or different substitution) or appeared also in ATP1A2 or ATP1A3 patients. Here, 2 had different substitutions in patients with differing phenotypes (S137Y or S137F severe,\textsuperscript{11} S137del mild\textsuperscript{35}, Q140L severe,\textsuperscript{10} Q140H mild\textsuperscript{34}), and the third, D923N, reduces affinity for Na\textsuperscript{+} and presents as a continuum between AHC and RDP,\textsuperscript{36,37} severe or mild in the same family.\textsuperscript{38}
supported by further evidence. Figure e-5 compares the ATP1A2 and ATP1A3 variants in the S domain. On the whole, P domain and S domain mutation distributions do not differ systematically between ATP1A2 and ATP1A3.

Two types of variants in ATP1A1
Presumably because ATP1A1 is essential in early embryonic life, not many disease variants have been discovered. Those fall into 2 groups with different characteristics. Seven ATP1A1 putatively pathogenic variants found in Charcot-Marie-Tooth (CMT2) axonal sensorimotor neuropathy are in the cytoplasmic A, P, and N domains, and 1 is at an ion binding residue frequently mutated in ATP1A3 but with a less disruptive substitution (figure 4A). Adrenal adenomas, like many cancers, have a high somatic mutation rate, and APAs have distinctly different ATP1A1 variants (short deletions and missense), all in the membrane domain (figure 4B).28–31 Those tested for function showed reduced Na,K-ATPase activity, cell depolarization, or a modest inward leak current.28,29,32–34 The amino acids of the most recurrent

The P domain is the most conserved in the gene family. It is covalently phosphorylated during transport, and the Mg$^{2+}$ ion (aqua) is at the active site. (A) The P domain consists of 2 halves that are separated in the linear structure but that interdigitate in a complex way when the protein folds. The first half, P1 in figure 1B, is shown in pale yellow, and the second half, P2 in figure 1B, in pink. There is a twisted β-sheet (left views) surrounded by α-helices (middle views). Strands and helices are numbered in the order found in the sequence. (B) The known mutations are displayed with color coding as indicated. Stick view is used for mutations in the β-sheet to avoid overcrowding. Note how most of the side chains of the mutated residues extend down into the active site surface. Spacefill view of the mutations, without side chains, is used for the α-helices. Note how the mutations are confined to just 5 helices: helix 1, which is associated with the stalk domain, and 4 helices that form a belt around the middle of the P domain: 2, 3, 7, and 8. There are no mutations in the end strands (1 and 4) or the end helices (4, 5, 6, and 9), which may be less important to function. The magenta mutation in an α-helix is the only helix mutation near (but not in) the active site. It is ATP1A3 T613M, a recurrent RDP mutation. The data indicate that the ATP1A2 and ATP1A3 distributions of mutations are very similar in the P domain, but there are 2 types of mutations. Most of the mutations in the β-sheet are at the active site. Most of the ones in the α-helices are distant from the active site. There was no correlation with phenotype severity.
adenoma variants, p.Gly99Arg, p.Leu104Arg, and p.Val332Gly, had previously been shown to line the ion pathway or function as gates: their chemical modification blocked palytoxin-induced currents caused by the toxin’s ability to bind to Na,K-ATPase and wedge it open.35 In sum, the CMT2 ATP1A1 disease variants are likely to cause a mild loss of function, and the adenoma variants may cause a gain of function with limited currents leading to aberrant aldosterone production.

Pathogenic variants without evolutionary conservation

To determine whether mutations are in amino acids with high evolutionary conservation, we used ConSurf to estimate conservation based on 94 nonredundant homologous sequences from the P-type ATPase superfamily, from vertebrates to yeast and bacteria.36 Figure 5A shows the conservation scores of all residues as small blue symbols, and the bar shows the locations of domains as in figure 1B. The 2D presentation highlights that for much of the protein, high conservation and low conservation residues are closely interspersed in the linear sequence. Shaded boxes highlight 2 nonrandom features. First, there are 3 large stretches of conserved residues (tan) with practically no low conservation residues (off-white). In the right off-white box, at around amino acid 800, there is a vertical streak of less-conserved residues that correspond to a loop in the extracellular domain, which is magenta on the domain bar. Second, there are 2 stretches of amino acids that are depleted of high conservation residues (lavender boxes). Because conservation was calculated using sequences that are related but have divergent functions, we infer that these regions, rather than being of lesser importance, evolved for functions not shared by more distant homologs: regions of special interest for ATP1A1, ATP1A2, and ATP1A3.

The average conservation scores for missense variants found in patients were similar for all 3 genes at the 30th percentile (data not shown). Two-thirds of the mutated residues were in the highest 20%, and only 3% were in the lowest 20%. The conservation scores were graphed to look for patterns unique to ATP1A2 and mild-phenotype ATP1A3 (figure 5B) and severe ATP1A3 (figure 5C). A difference in the distribution of variants is clear both in the location and conservation score, with the severe-phenotype ATP1A3 variants being more restricted and all falling below zero on the graph (5C). Another difference that cannot be appreciated on the graphs is that 65% of the severe ATP1A3 variants are recurrent, whereas the proportion is 38% for mild ATP1A3 variants and 20% for ATP1A2. The region at the C-terminus with no very high conservation residues has many mutations (figure 5, B and C).
Human ATP1A1, ATP1A2, and ATP1A3 amino acid sequences were aligned with *Sus scrofa* ATP1A1 (the species used in protein crystal structures 3KDP and 3WGU), and each residue affected by a disease variant was assigned the conservation score calculated for ATP1A1. (A) The individual conservation scores of all aligning amino acids are shown with score on the Y axis and amino acid sequence (1–1,023) on the X axis. As seen in figure e-1 (links. lww.com/NXG/A134), there is no alignment at the N-terminus, so N-terminal residues do not appear. Each blue spot is one amino acid, and the Na,K-ATPase domains are illustrated in the bar at the bottom. Some patterns of conservation can be discerned, highlighted by transparent boxes: two regions with few or no highly conserved amino acids (lavender) and 3 regions of conserved residues (tan) with practically no low conservation residues (off-white). (B) The ATP1A2 variants (green) and the mild ATP1A3 variants (magenta) have similar distributions. There are 14 cases (~15%) where homology scores were in the less-conserved half. The C-terminal region has many pathogenic variants in both genes despite lacking the highest conservation scores. Residues E174 and R1008 from figure 6 are marked with arrowheads. (C) The severe ATP1A3 variants had a more restricted distribution. It can be seen that many align with the membrane spans. Although their conservation scores varied widely, none were in the less-conserved half. Of the points that do not lie in one of the 3 clusters of high conservation, 8 were shared by mild and severe ATP1A3 phenotypes (visible as 2 colors per symbol at high magnification). This suggests that intermediate phenotypes tend to have less restricted structure distributions than the most severe phenotypes. (D) All the synonymous (brown; n = 281) and missense (coral pink; n = 145) ATP1A3 variants from gnomAD (138,632 sequences) are plotted to compare to the clustering of mutations.
Normally, one would expect that variants with low conservation scores are benign. In the ATP1A2 and mild ATP1A3 variants in figure 5B, 14 amino acids have positive (poor) conservation scores. Of these, 10 were supported as pathogenic by laboratory testing of function, recurrence as an alternative amino acid, or occurrence in a second gene, and 3 of those were de novo. There was no pattern in their scores. Three others had insufficient data, and a fourth was published as possibly disease causing and is analyzed further.

The missense and synonymous variants in gnomAD gave no obvious pattern (figure 5D). More than half (58%) of the synonymous variants were in the higher half of the conservation score, whereas only 39.3% of missense variants were (p = 10^{-5}, t test, 2 tailed, equal variance) consistent with genetic constraint.

Structure data assist tricky predictions

ATP1A2 variant E174K has a conservation score of 2.077, putting it at the top of figure 5B. The variant appeared in a family that experiences migraine with side-changing paresthesias, which is on the FHM2 spectrum. Pedigree data were supportive but incomplete. When expressed in Xenopus oocytes, no reduction of pump activity was seen, but the ion concentrations used were high enough to saturate the enzyme. In later experiments using protein expressed in insect cells, it was learned that the enzyme had altered kinetics: reduced affinity for Na⁺ and increased affinity for K⁺. Because Na⁺ concentration is rate limiting for enzyme activity in vivo, this mimics reduction of activity. Figure 6, A and B illustrate how the position of E174 in the crystal structures explains the pathogenicity. E174 is at the surface of the A domain, which rotates. In the K⁺-bound structure, E174 faces the cytoplasm, and the side chain is in contact with nothing (figure 6A). In the Na⁺-bound form, however, it binds 2 amino acids in the N-domain (figure 6B), i.e., it is at a domain-domain interface where the change from negative to positive charge will be damaging. This explains the abnormal kinetics because the sodium conformation would be destabilized.

In this example, an amino acid with the worst conservation score nonetheless is legitimately pathogenic.
The second example is ATP1A2 variant R1008W, which was found together with a known pathogenic variant, R548C, in a compound heterozygous child with unusually early onset of severe epilepsy, hemiplegia, and migraine symptoms.\(^3\) The severity of the phenotype, not seen in a family with R548C alone\(^4\) or in the child’s mother who carried the R548C mutation, suggested that both variants contributed to disease severity. An adjacent arginine with the same substitution, R1007W, previously described in a family with hemiplegic migraine and epilepsy, was shown to be pathogenic by functional studies in *Xenopus* oocytes.\(^4\) Although R1007 has a conservation score in figure 5B of −0.967 while R1008 is +0.069, their identical substitutions suggested that the adjacent variants might both be pathogenic.\(^3\) However, their positions in the crystal structure tell a different story (figure 6C). R1007’s side chain faces into the protein and makes contacts with 2 amino acids in M10 and 2 in the S domain. One of those contacts is the protein kinase A site, and R1007 had been shown previously by mutagenesis to play a complex role in controlling Na,K-ATPase.\(^4\) In contrast, the adjacent R1008 side chain points away from the protein, where it may associate with phospholipid headgroups.\(^4\) Adjacent amino acids can have very different propensities to be pathogenic. In this example, a variant with some apparently credible features, nonetheless, may be benign.

**Discussion**

It was not expected that pathogenic variants seen in Na,K-ATPase paralogs would differ. The concepts of conservation of sequence homology and genetic constraint (intolerance of substitution) are tightly linked. The premise is that evolutionary conservation occurs because variants causing functional damage are eliminated by selection. This study documents that sets of paralogs can violate that logic at the disease level: sequence similarity between paralogs is not always a strong predictor for the discovery and interpretation of new human pathogenic variants. The Na,K-ATPase catalytic subunit gene family instead shows partial divergence in pathogenic variants. Ascertainment bias plays a role: patients must survive with the mutation, and symptoms must be uncommon enough to suggest monogenic disease. Mutations outside of these constraints would occur but be undetected.

It is fundamental that mutation phenotypes can appear in one tissue or cell type and not another because of differences in how physiology is affected. Partial loss of Na,K-ATPase activity may have more impact in a cell with high transport activity than one with low activity; in inhibitory rather than excitatory neurons; or presynaptically rather than postsynaptically. An elevation of intracellular Na\(^+\) due to a reduction of sodium affinity may have more effect on dendrites than on action potentials or have different secondary effects on transmitter reuptake. Similar mutations in astrocytes and neurons can have different impacts on intracellular Ca\(^2+\), pH, or Cl\(^-\) because of the expression of different sodium-dependent carriers. When comparing different mutations, however, variants that simply reduce activity should have a ceiling of physiologic impact; e.g., a mutation inactivating the ion binding site should have the same physiologic effect as a mutation inactivating the ATP hydrolysis site, provided both changes produce inert enzyme. Loss of function alone, however, does not explain why many ATP1A2 and ATP1A3 neurologic variants are biased toward specific mutations as suggested by their independent recurrence.

To understand the genotype-structure-phenotype differences displayed by ATP1A2 and ATP1A3 and by mild and severe ATP1A3 mutations, we postulate a gain-of-function defect in the severe mutations. Certain mutations of Na,K-ATPase may cause a greater perturbation of physiology than pump inactivation alone by alteration of ion selectivity or stoichiometry; by depolarizing inward currents carried by protons or Na\(^+\); by leaks through the ion pathway because of defective gating; or by protein instability or misfolding in the membrane.\(^2\) Small inward proton currents occur in wild-type enzyme and are sometimes blocked in mutants, but their physiologic impact is still uncertain.\(^2\) Larger leaks or an outward proton current would be disruptive for both neurons and glia and potentially toxic. The extreme toxicity of palytoxin is due to the opening of the ion pathway through Na,K-ATPase like a channel.\(^4\) Any leaks in surviving patients should be very slow relative to palytoxin and might manifest only at physiologic temperatures or higher. The ATP1A3 variants that are tightly clustered around the ion sites would be candidates. So would sensitive spots such as G358 on the P1 helix embedded in the S domain, where a G358V mutation produced fatal infantile epilepsy\(^5\) and G358C, S, and D produce AHC. So would the highly recurrent AHC mutations E815K and G947R that have dominant negative effects\(^6\) and are in a network of residues affecting the cytoplasmic ion pathway.\(^2\) The importance of pathogenic leaks is supported by temperature-sensitive mutations studied in Ca\(^{2+}\)-ATPases in *Drosophila*\(^7\) in addition to the aldosterone-secreting adenoma *ATP1A1* mutations.\(^8\) Many other mutations, such as in the P domain, N domain, or extracellular surface, may affect only biosynthesis or activity.

One significant observation is that the membrane domain pathogenic variants causing severe phenotypes in *ATP1A3* are entirely missing from the *ATP1A2* patient population. It is not that hemiplegic migraine *ATP1A2* mutations are never fully inactivating: a number had no detectable activity in laboratory studies.\(^8\) Even among the variants shared by *ATP1A2* and *ATP1A3*, however, none were close to the ion binding sites. The logical prediction is that severe variants of *ATP1A2* occur but have unrecognized disease consequences. For example, they may produce membrane depolarization that interferes with astrocyte roles in synapse development and remodeling,\(^9\) and this clinically supersedes the cortical spreading depression that contributes to migraine.\(^2\) Other possibilities are a phenotype in other *ATP1A2*-expressing tissues (skeletal,
vascular, smooth, or cardiac muscle; fat) or lethality, perinatally or in utero.

The variants of ATP1A3 that we have called “milder” nonetheless produce disabling neurologic disease with stable, progressive, or episodic motor symptoms, often with cognitive deficits and psychiatric manifestations.\textsuperscript{1,50,51} No pathogenic variants have been found yet, however, in the N-terminus, the A domain, the linkers to the A domain, or some of the peripheral membrane spans, suggesting that their functional impact is too small (or too great) to produce recognized diseases.

Genetically defined loss-of-function mutations (LoF: frame-shifts, premature stops, and deletions) are almost absent from gnomAD for ATP1A1 and ATP1A3 and reduced in ATP1A2. This is evidence of purifying selection, and that the genes are highly constrained, however, LoF mutations are also largely missing in the patient populations. Recessive variants are missing so far from the whole gene family. This is puzzling because partial reduction of activity of such important enzymes should have pathophysiological effects if homozygous. A possibility is that recessive variants will be found for phenotypes or risk factors that are not yet recognized. ATP1A1 recessive variants could manifest in any tissue with high ion transport. ATP1A3 variants might affect CNS functions already affected by dominant mutations in RDP and AHC, such as depression, behavioral and cognitive deficits, or psychosis.\textsuperscript{50–52}

This gene family's mutational landscape shows that the spectrum of pathologic variants varies among paralogs. Important pathologic variants can occur in evolutionarily recent specializations that are not conserved at all. The mutations in ATP1A2 and ATP1A3 and the germline mutations in ATP1A1 were overwhelmingly missense or deletions of 1 codon, which suggests that the mutated protein must be present for the known manifestations. It will be interesting to see whether recessive mutations have a structural distribution more representative of sequence identity in the gene family.

Author contributions
Design and execution of the study: K.J. Sweadner, E.A., J.T.P., and L.J.O. Contribution to genetic and phenotypic data and interpretation of data: K.J. Swoboda and A.B. Writing of the manuscript: K.J. Swoboda, E.A., and L.J.O. Revisions of the manuscript: all authors.

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