ATP1A3 Variants are Associated with Variably Penetrant Short QT and Lethal Ventricular Arrhythmias

Short Title: Multicenter registry: *ATP1A3* and short QT

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Sources of Funding:
JEE was supported by the NIH Clinical and Translational Science Award UL1TR002553. SW receives funding from FWO-FKM (1861419N). SB and SMS were supported by Epilepsy Society, UK and the Muir Maxwell Trust (SB). MAM receives support from Duke University fund (3912274) and from a donation from the CureAHC Foundation. APL is supported by National Institutes of Health (K08-HL136839 and R01-HL160654), Doris Duke Charitable Foundation (CSDA-2020098), Y.T. and Alice Chen Pediatric Genetics and Genomics Research Center.

Disclosures:
MAM has a provisional patent for treatment of alternating hemiplegia of childhood. All other authors have no conflicts or disclosures.

Date of the revision: August 20, 2024.

Total manuscript word count: 3,000

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Meeting Presentation Statement: this work was presented at the American Heart Association in Chicago, IL and virtual November 5, 2022.

Acknowledgements:
We thank the following AHC patient organizations for their support to the IAHCRC-CLOUD Platform, and to the data collection by their national centers participating in the study: USA (CureAHC Foundation), French (AFHA), Icelandic (AHC Samtökin), Dutch (AHC Vereniging Nederland), Spanish (AESHA), UK (AHC-UK), German (AHC18+ e.V.), Polish (AHC-PL) and Italian (AISEA) Associations. Dr. Andrew Landstrom had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

KEY POINTS

Question: What is the association between presence of *ATP1A3* variant and pathologically shortened QTc?

Finding: In this multicenter study that included 123 patients with a disease-causing variant in *ATP1A3*, patients with a pathogenic variant in *ATP1A3* had a shorter QTc when compared to patients without a disease-causing variant in *ATP1A3*, and when compared to age and sex-matched healthy controls. Patients with the ATP1A3-D801N variant had highly penetrant short QTc phenotype.

Meaning: Patients with a variant in *ATP1A3* are at risk for having a shortened QTc which implies that this disease state could serve as a model for studying short QT.

Question: What is the association between presence of *ATP1A3* variant and ventricular arrhythmia predisposition?

Finding: Compared to patients with other pathogenic *ATP1A3* variants, patients with the ATP1A3-D801N variant were more likely to experience the composite cardiac outcome comprised of ventricular tachycardia/ventricular fibrillation arrest, sudden cardiac arrest, cardiogenic syncope, or appropriate ICD discharge.

Meaning: Patients with the highly penetrant short QTc phenotype ATP1A3-D801N were more likely to experience a major cardiac event compared to other patients with less penetrant *ATP1A3* variants.

Question: How does the location of *ATP1A3* variants and in vitro studies contribute to the understanding of the genotypic impact on shortened QTc phenotype and predisposition to ventricular arrhythmias?

Finding: Individuals with highly penetrant short QT phenotypes were found to have variants which localized to the potassium binding domain unlike individuals with low penetrant QT phenotypes. Induced pluripotent stem cell cardiomyocytes derived from an ATP1A3-D801N patient showed evidence for pro-arrhythmic phenotype.

Meaning: Cryo-EM structure mapping and in vitro cellular modeling indicates a pro-arrhythmic phenotype which may be exacerbated in variants located in the potassium binding domain of ATP1A3.

Social Media Post: International registry study finds the potential reason behind the sudden death events of patients with AHA and ATP1A3 mutations: @mary_moyamendez @APLandstrom @MBidzimou at @Duke_Childrens find D801N associated with short QT interval, ventricular arrhythmias, and cardiac arrest

ABSTRACT

Importance: Alternating hemiplegia of childhood (AHC) is a disorder that can result from pathogenic variants in the *ATP1A3*-encoded sodium-potassium ATPase alpha 3 (ATP1A3). While AHC is primarily a neurologic disease, some individuals experience sudden unexplained death (SUD) potentially linked with cardiac arrhythmias.

Objective: Given the rarity of variants in *ATP1A3*, we established an international, multicenter study to determine the impact of *ATP1A3* variants on cardiac electrophysiology and whether lethal ventricular arrhythmias may account for SUD in patients with AHC.

Design: Blinded, manual measurements of QT intervals and corrected QT interval (QTc) were performed independently by 2 pediatric cardiac electrophysiologists. Induced pluripotent stem cell cardiomyocytes were derived from ATP1A3-D801N positive AHC patients (iPSC-CM^{D801N}).

Setting: Multicenter.

Participants: Patients with AHC were grouped as: *ATP1A3* variant status (positive vs. negative) and subgroups of the most common AHC variants (D801N vs. E815K vs. G947R vs. other). A healthy control cohort was established for comparison.

Exposure: Presence of *ATP1A3* variant.

Main Outcome and Exposure: Outcomes, including survival, were abstracted and variants were mapped on cryo-EM structure maps. iPSC-CM^{D801N} were used to validate ventricular repolarization and arrhythmic susceptibility *in vitro*.

Results: Our cohort consisted of 148 largely unrelated probands from 12 centers across 10 countries. Of these, 123 individuals were *ATP1A3* genotype positive. Probands with the ATP1A3-D801N variant had significantly shorter QTcs compared to ATP1A3-E815K, ATP1A3-

G947R, all other *ATP1A3* variants, and healthy controls (*P*=0.001, *P*=0.02, *P*=0.0008, *P*<0.0001, respectively). Three D801N-positive individuals had a major cardiac event (P=0.02). ATP1A3-D801N as well as 4 rare variants (D805N, P323S, S772R, C333F) found in individuals with the shortest QTcs in our cohort localized to the potassium binding domain of ATP1A3. IPSC-CM^{D801N} had shortened action potential duration, higher mean diastolic potential, and exhibited delayed after depolarizations compared to healthy controls.

Conclusions and Relevance: In the largest study to date on the impact of *ATP1A3* on the heart, ~70% of individuals with ATP1A3-D801N variants have short QTcs (<370 msec) and an association between ventricular arrhythmias and cardiac arrest. This may underlie the SUD etiology in AHC.

Keywords: alternating hemiplegia of childhood; *ATP1A3*; short QT; sodium potassium ATPase, sudden death.

Abbreviations: AHC, alternating hemiplegia of childhood; ICD, implantable cardioverter-defibrillator; SUD, sudden unexplained or unexpected death; ECG, electrocardiogram; QTc, corrected QT interval; VT, ventricular tachycardia; VF, ventricular fibrillation.

INTRODUCTION

The gene *ATP1A3* encodes the sodium-potassium ATPase isoform 3 (ATP1A3) is critical for maintaining the electrochemical Na^+/K^+ gradient in neurons while its respective role in the heart remains unclear.[1-3] Variants in *ATP1A3* are known to cause several neurological diseases, including a condition affecting approximately 1 in 1,000,000 individuals known as alternating hemiplegia of childhood (AHC).[4] The spectrum of disease manifestations in AHC includes neurological, psychological, behavioral, gastrointestinal, and speech abnormalities.[5] The Aicardi diagnostic criteria for AHC includes sudden onset of episodes consisting of hemiplegia, dystonia, quadriparesis, seizures/seizure-like events, and oculomotor abnormalities prior to 18 months of age.[6,7] In addition, individuals with AHC have a predisposition to sudden unexplained death (SUD).[8,9]

It has been previously postulated that SUD in patients with AHC is due to cardiac arrhythmias.[10,11] This remains unproven, and the mechanistic interplay between brain function and cardiac repolarization in individuals with a variant in *ATP1A3* is not fully understood. Previous work analyzing ECGs from patients with *ATP1A3* variants found that 60% contained resting abnormalities, such as T wave abnormalities and conduction delay.[12] Our previous work identified a link between the ATP1A3-D801N genotype and shorter corrected QT interval (QTc), a known arrhythmia substrate, when compared to all other *ATP1A3* variants.[13] Two probands with the ATP1A3-D801N variant experienced ventricular fibrillation (VF) following sedation-induced bradycardia which was fatal for one of these individuals. It is critical to further explore the association of the ATP1A3-D801N variant with markedly short QTc measurements as these individuals may be at increased risk for ventricular arrhythmias. Given that variants in *ATP1A3* are rare, a multicenter registry affords the opportunity to define this association. In this study, we

collaborated with international centers to further study the association between *ATP1A3* genotype and QTc, arrhythmia predisposition, and survival.

METHODS

Study Approval

This study was approved by Duke University Hospital System Institutional Review Board (IRB) (Pro00037150 & Pro00094341). This collaborative study was carried out by member centers of the IAHCRC, the International Consortium for Research on AHC and all the ATP1A3 Rare Diseases (www.iahcrc.net), according to the collaboration and data sharing rules defined in the IAHCRC Charter.

Study Cohorts

Inclusion criteria for this study were: 1) availability of clinical genetic test results for the major genes associated with AHC defined by the Aicardi criteria[14]; 2) at least 1 ECG with original tracings available for review. A control group of healthy pediatric individuals was established which included infants, children, and adolescents presenting to Duke University Hospitals from 2021-2023 who received a diagnostic ECG and demonstrated no evidence of cardiovascular disease on evaluation.

Study Parameters and ECG Evaluation

This study used the same parameters as previously reported[13]. QT and QTc analysis was performed from diagnostic ECGs blinded to genotype and correlated to genotype. Outcome analysis was conducted with a primary outcome composition measure of sudden cardiac arrest,

suspected cardiogenic syncope, documented ventricular tachycardia (VT)/ventricular fibrillation (VF) arrest, or implantable cardioverter-defibrillator (ICD) appropriately discharged.

Mapping Variants to the ATP1A3 Crystal Structure

The crystal structure of the human alpha3 Na^+/K^+ ATPase in its K^+ occluded state (8D3X)[15] was used to create the visual representation of the cryogenic electron microscopy (EM).[16]

Human Induced Pluripotent Stem Cells

Blood was collected from healthy controls and patients with AHC. Peripheral blood mononuclear cells were isolated, reprogrammed to iPSCs, and then re-differentiated into cardiac myocytes (iPSC-CMs) using established methods.[17]

Action Potential Recordings

Action potentials (AP) were recorded in whole cell current-clamp mode using single cell patch clamp of iPSC-CMs. AP morphology and AP parameters were analyzed as previously described.[18,19]

Statistical Analysis

Analysis was completed according to ATP1A3 sub-group: *ATP1A3* variant status (positive vs. negative) and subgroups of the most common AHC variants (D801N vs. E815K vs. G947R vs. loss of function (LOF) vs. all other).

Additional details can be found in the **Supplemental Materials.**

RESULTS

Cohort characteristics

A total of 148 individuals met inclusion criteria. Fifty-one percent were female and the average age at time of diagnostic ECG was 11.5±10.5 years. Of these, 123 individuals (83%) were *ATP1A3* genotype positive, and 25 (17%) *ATP1A3* genotype negative. All variants were deemed likely pathogenic/pathogenic for AHC by ACMG criteria.[20] Of the 123 *ATP1A3* positive individuals, 35 (28%) carried the ATP1A3-D801N variant, 21 (17%) carried the ATP1A3-E815K variant, 8 (7%) carried the ATP1A3-G947R variant, and 8 (7%) had a loss of function (LOF) variant in *ATP1A3* (**Figure 1**). There were no statistically significant differences in demographics between these subgroups. The Bland-Altman analysis indicated acceptable agreement between the two raters who performed the blinded, manual QT/QTc re-measurements (**Supplemental Figure 1**). A list of participating centers is in **Supplemental Table 1.** For comparison, we established a healthy control cohort of 74 children with an average age of 10.5±7.7 years and similar demographic profile to the *ATP1A3* positive cohort. **Supplemental Table 2** summarizes these findings.

The ATP1A3-D801N genotype is associated with a highly penetrant short QT phenotype while -E815K and -G947R are low penetrant short QT alleles

Given previous literature that ATP1A3-D801N variants have abnormal cardiac repolarization[13], we assessed the manually remeasured QTcs using the largest cohort to date. Individuals who were *ATP1A3* genotype positive had a significantly shorter QTc compared to *ATP1A3* genotype negative (381.8±36.6 msec vs. 405.7±24.2 msec, respectively; P=0.002).

There was no sex difference in QTcs when comparing genotype negative females and males (406.9±22.5 msec vs. 408.1±25.7 msec, respectively; *P*=0.9) (**Supplemental Figure 2**) or genotype positive females and males (384.2±36.4 msec vs. 377.8±37.1 msec, respectively; *P*=0.33).

We next compared QTcs of individuals with the most common *ATP1A3* variants, ATP1A3-D801N, ATP1A3-E815K, and ATP1A3-G947R.[6,21] Individuals with the ATP1A3-D801N variant had a significantly shorter QTc when compared to individuals with the ATP1A3-E815K variant (362.9±26.6 msec vs. 393.6±43.1 msec, respectively; *P*=0.001), the ATP1A3-G947R variant (362.9±26.6 msec vs. 388.4±26.5 msec, respectively; *P*=0.02), and all other *ATP1A3* variants in the cohort (362.9±26.6 msec vs. 387.8±37.1 msec, respectively; *P*=0.0008). ATP1A3-D801N individuals had a significantly shorter QTc when compared to patients with a LOF variant in *ATP1A3* (362.9±26.6 msec vs. 403.0±33.5 msec, respectively; *P*=0.0007). Finally, ATP1A3-D801N individuals had a significantly shorter QTc than healthy controls (362.9±26.6 msec vs 415.4±20.95 msec, respectively; *P*<0.0001; **Figure 2, Supplemental Figure 3**). There were no statistically significant differences in QTc when comparing individuals with the ATP1A3-E815K, ATP1A3-G947R, ATP1A3-LOF, and all other *ATP1A3* variants to each other. Example ECGs from each subgroup can be found in **Supplemental Figures 4-8**.

Among individuals with the ATP1A3-D801N variant, 24/35 (69%) had QTcs below this <370 msec threshold[22] (**Supplemental Figure 9**). Conversely, we found ATP1A3-E815K, ATP1A3-G947R and ATP1A3-LOF individuals demonstrated a low penetrance (4/21, 19%; 1/8, 13%; 1/8, 13%; respectively). As expected, none of the healthy controls had a QTc <370 msec. Of the 123 individuals with *ATP1A3* variants, 75 had epilepsy/seizures as part of their diagnosis. There was no significant association between the presence of epilepsy/seizures and borderline or

diagnostic short QTc (N=25, P>0.99; N=13, P=0.81; respectively). Most individuals experienced either a persistently short QTc or a normal QTc over time with little significant fluctuation in QTc (**Supplemental Results, Supplemental Figure 10**). When comparing *ATP1A3* genotype negative versus positive individuals, and *ATP1A3* variant sub-groups, there was no significant difference in type or quantity of QT modulatory or anti-seizure medications. There was no compound effect of anti-arrhythmic medications as no individuals were taking cardiac sodium channel, L-type calcium channel, or cardiac potassium channel blockers in this cohort. There was minimal influence from concurrent QT prolonging medication use. That is, only 3/148 individuals (2.0% of the cohort) were taking medications known to prolong the QT interval. Likewise, while many patients were taking flunarizine, commonly used in AHC to improve the neurologic symptoms, we saw no significant effect of this medication on QTc (**Supplemental Results**). Finally, there was no cardiomyopathy, heart failure, or significant congenital heart disease present in the cohort (**Supplemental Results**). Taken together, these findings suggest that individuals with a pathogenic variant in *ATP1A3* have a shorter QTc when compared to individuals without a pathogenic *ATP1A3* variant. Moreover, the D801N variant is associated with a persistently short QTc (<370 msec) in a highly penetrant fashion, while all other *ATP1A3* variant sub-groups are associated with low penetrant short QTc.

Individuals with the ATP1A3-D801N variant are predisposed to arrhythmias preceded by bradycardia

To draw a link between short QT interval and arrhythmia predisposition, we examined the occurrence of ventricular arrhythmic events. We previously reported 2 ATP1A3-D801N probands who experienced ventricular arrhythmias, which was fatal in one case, proceeded by

sedation-induced bradycardia in the setting of receiving chronotropic suppressing medications.[13,23,24] In this larger cohort, another ATP1A3-D801N proband had a persistently short QTc (range: 300-368 msec) and had an implantable cardioverter-defibrillator (ICD) placed after exercise induced syncope. We derived an *a priori* composite cardiac outcome of ventricular tachycardia (VT)/VF arrest, sudden cardiac arrest, suspected cardiogenic syncope, or appropriate ICD discharge. **Figure 3** shows that ATP1A3-D801N individuals were the only patients in our cohort to experience any of these outcomes (P=0.02). No events in this composite cardiac outcome occurred during an AHC spell. Further, we calculated the frequency of these events, and found that the ATP1A3-D801N individuals experienced one of the composite cardiac outcomes at a rate of 0.6 events/100 life years. Additionally, since ATP1A3-D801N individuals appear to be vulnerable to ventricular arrhythmias during times of sedation, we examined events as a function of total anesthesia exposures which revealed 2 events in separate individuals out of 26 anesthesia exposures (frequency of ~8%/anesthesia exposure). There were no other anesthesia events in all other *ATP1A3* variants despite comparable anesthesia exposures (**Figure 3**). Individual cases meeting the composite endpoint are further detailed in the **Supplemental Results**.

Variants localizing to the potassium binding domain of ATP1A3 result in shortened QTc

We next determined whether variants associated with QT shortening localize to a common functional domain of ATP1A3. We mapped the 3 variants which account for 60% of all AHC cases[25], as well as variants with the shortest QTcs in our cohort along the 3D structure of ATP1A3 using a visual representation of the cryogenic EM. Our analysis revealed 4 variants in *ATP1A3*, each found only once in our cohort (P323S, S772R, C333F, D805N) and represented

the shortest QTcs in the cohort (290 msec, 322 msec, 328 msec, 333 msec; respectively). We investigated the molecular similarity of QT-shortening, missense-causing variants by mapping these positions on the crystal structure of human ATP1A3[15]. As shown in **Figure 4**, the amino acid positions with the highest penetrance are clustered in the potassium binding pocket of ATP1A3 within the transmembrane domains M4, M5, M6, and M8. Taken together, these findings suggest that *ATP1A3* variants which specifically impact the function of the potassium binding domain may be associated with QT shortening and can predispose to ventricular arrhythmias.

AHC patient derived iPSC-CM^{D801N} have a shorter short action potential duration and predisposition to delayed after depolarizations

To determine the mechanism of ATP1A3-D801N-mediated alterations to cardiac repolarization and arrhythmogenesis, we derived induced pluripotent stem cell (iPSC) lines from an ATP1A3-D801N female with a short QTc (iPSC-CM^{D801N}), and from a genotype negative, healthy individual with normal cardiac evaluation (iPSC-CM^{WT}) (**Figure 5A-B, Supplemental Figure 4 & 11)**. Each iPSC line was validated via karyotyping and flow cytometry (**Supplemental Figure 12-15**). IPSCs from each line were differentiated into cardiomyocytes. and validated with qPCR and immunofluorescence (**Supplemental Figure 16-18**). Using whole cell patch clamp, we found that iPSC-CM^{D801N} displayed a significantly shortened action potential duration at 50% and 90% of the amplitude (APD_{50} and APD_{90}, respectfully) compared to iPSC-CM^{WT} (**Figure 5C-E**). iPSC-CM^{D801N} also had increased maximum diastolic potential (MDP) compared to iPSC-CM^{WT} (**Figure 5F**). Additionally, 27% of iPSC-CM^{D801N} displayed delayed after depolarizations (DADs), while iPSC-CM^{WT} displayed none (**Figure 5G-H**). Taken

together, these findings demonstrate abnormally fast repolarization and an arrhythmic substrate in ATP1A3-D801N human cardiac myocytes *in vitro*.

DISCUSSION:

Classically described as a primarily neurologic disease, individuals with AHC have a well-known risk of SUD.[26] Individuals with AHC, both with and without a history of seizures, have an increased risk of mortality of 3.2 deaths per 1000 people per year, which increases to 4.5% by 29 years of age.[6,10] Despite this, the cause of SUD in this disease remains unknown. Exploring the link between short QT and arrhythmias is critical to understanding the role of the heart in AHC so that individuals at risk can be identified and therapies conceived. To this end, we report a large, multicenter, international registry to establish this association.

We find that individuals with the ATP1A3-D801N variant have a shortened QTc compared to all other *ATP1A3* variants. Moreover, we find that the allele is highly penetrant, and expressivity of disease is present early in life and without change across lifespan. Three probands with the ATP1A3-D801N variant experienced VT/VF arrest, cardiac death, or required an ICD implantation for exercise-induced syncope. None of these events occurred during an AHC spell which suggests that these sudden cardiac events were not due to a cardiopulmonary failure secondary to the neurologic manifestations of AHC. This work is in agreement with our previous experience showing that individuals with the ATP1A3-D801N variant are at risk of ventricular arrhythmias during periods bradycardia outside of classic AHC spells.[13] Our previous work also examined an independent cohort utilizing de-identified referrals for syncope, sudden cardiac arrest, VT/VF, or abnormal QT interval. Out of 6,437 individuals, 8 (0.12%) carried rare, ACMG-classified variants of unknown significance in *ATP1A3* and were otherwise negative for

pathologic/likely pathologic variants in sudden cardiac death genes.[13] Taken together, these findings suggest a "high risk" genotype among individuals with AHC and supports the hypothesis that *ATP1A3* should be evaluated further as a short QTc candidate gene.

Short QT syndrome (SQTS) is characterized by an abnormally short cardiac repolarization time which can cause sudden cardiac death.[27] It is a rare disease with an estimated prevalence of 0.02-0.1% in adults and 0.05% in pediatric patients[27,28]. Diagnosis is reliant upon evaluation of an individual's QTc with a measurement <370 msec being considered borderline and a measurement <350 msec to be diagnostic.[22] To date, >30 rare variants have been identified in genes associated with cardiac calcium and potassium channels as causing SQTS.[29] While patients with SQTS are at risk of lethal ventricular arrhythmias at all ages, pediatric patients are particularly at risk with a cardiac arrest rate of 4%.[30] One study followed pediatric patients with SQTS for an average of 5.9 years and found that nearly half of the patients required ICD placement.[31] Currently, the majority of individuals with short QT and SQTS do not have a known genetic cause.[32,33] Despite this, diagnostic genetic testing is recommended when the disease is suspected with the goal of potentially identifying the proband to facilitate cascade familial screening.[34,35]

The mechanism behind how *ATP1A3* variants result in short QT remains unknown. Here, we model the location of the 7 variants, 3 of which account for 60% of AHC cases[36] and 4 of which are rare but have the shortest QTcs in our cohort. Both D801N and D805N probands have shortened QTc, highlighting the role of these aspartate residues in ionic transfer for successful potassium binding.[37] The S772 hydroxyl group is within the accepted distance required for hydrogen bonding[38] from the D805 carboxyl, suggesting that in this conformation S772 is important for stability and/or protonation of D805.[39,40] We hypothesize that C333F

affects the contribution of its closest hydrogen binding residue, S765, regarding the overall shape of the transmembrane region. Finally, the ATP1A3-P323S variant has the shortest QTc in our cohort suggesting that P323S affects the helical shape of M4, and consequently affects this transmembrane region[41,42].

Our study reveals that individuals with certain *ATP1A3* variants demonstrate a short QTc and a predisposition to fatal ventricular arrhythmias. This is supported by our *in vitro* experiments showing that iPSC-CM^{D801N} have higher MDP and shortened APD. The MDP elevation is likely due to reduced ATP1A3 function, which would increase $[Na^+]_i$. We also found that iPSC-CM^{D801N} have a predisposition to DADs, which are a known substrate for arrhythmia development.[43] Therefore, we hypothesize that *ATP1A3* is a new causative gene for SQTS. We suspect that a dysfunctional ATP1A3 protein increases $[Na^+]_i$ and biases the sodium-calcium exchanger towards Ca^{2+} influx, ultimately leading to increased $[Ca^{2+}]_i$ which favors faster repolarization time and a pro-arrhythmic phenotype. We demonstrate that individuals with the ATP1A3-D801N variant and *ATP1A3* probands with the shortest QTcs in our cohort have variants in the potassium binding site of the ion pump. Most studies to date have been performed in neurons and show that variants in *ATP1A3* can lead to nonsense mediated decay or protein misfolding with resultant decreased protein trafficking and haploinsufficiency.[44-46] The finding that the ATP1A3-D801N variant results in cardiac repolarization abnormalities, while other variants, including predicted null alleles, are not associated with a highly penetrant short QT, would suggest a dominant negative impact on *ATP1A3* as observed in neuronal models.[47] While this is the largest study to date on this topic, there remains a need for more investigation into the mechanism of short QTc pathogenesis in patients with variants in *ATP1A3*.

Finally, our findings suggest that patients with *ATP1A3* variants, particularly those hosting the D801N variant, are at-risk of ventricular arrhythmias and sudden death and that there may be an exacerbating influence involving medication-triggered bradycardia[13]. Additional study is needed to further explore the relationship between heart rate, QTc dynamics, and arrhythmias risk, as well as the potential for chronotropic suppressing medications to compound this risk. Despite this, given our findings, it would highlight the need for these patients to be evaluated and followed by pediatric electrophysiologists, trained in this risk assessment, and to be supported by a multi-disciplinary team to provide optimal care.

LIMITATIONS

Although this is the largest *ATP1A3* cohort to date, AHC is rare making a retrospective study design essential. Nonetheless, this approach is prone to referral bias and the findings are associative. Additionally, while medications at the time of ECG were recorded, the impact of a heterogenous medical approach precludes clear associations between our findings and potential medication impact. The dynamics of QT at variable heart rates is unknown and deserves further evaluation. The link between sinus node dysfunction and ventricular arrythmias is relatively unexplored, and the interplay between sinus node dysfunction, medications, and life-threatening cardiac arrhythmias could not be fully assessed in this study given the limitations of multi-center retrospective data. Further, the role of medications as an inciting trigger for sudden death events is not well defined. Thus, further work is needed to determine what role, if any, heart rate slowing may play in ATP1A3-mediated disease. Additionally, sinus arrhythmia and bradycardia is a known feature of AHC, whether this is due to the central nervous system versus a primary nodal disease remains, or both, remains unknown. In addition, the composite cardiac outcome

analysis in our study is limited by survival bias. Finally, the full mechanism by which ATP1A3 mutations may cause QT shortening, or nodal cell dysfunction, remains undefined at this time and future work is needed to determine the cellular mechanisms of disease.

CONCLUSIONS:

We describe repolarization abnormalities, namely QT shortening, in patients ATP1A3-D801N. Further, we describe an association of QTc shortening observed in these patients with an increased likelihood of experiencing ventricular arrhythmias, cardiogenic syncope, and sudden cardiac arrest for the first time in an international, multicenter study. Patients with AHC should receive a cardiac evaluation to assess their risk for experiencing ventricular arrhythmias as well as receive preventative strategies during care, such as avoidance of medications with QT modulatory effects and bradycardia. This study posits the emergence of *ATP1A3* as a gene associated with SQTS and sudden death and therefore, could serve as a model for studying sudden cardiac death in research endeavors.

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FIGURE LEGENDS

Figure 1. Design of multicenter, international study. A) Flow chart showing the design of this study and the inclusion and exclusion criteria used to establish the patient cohort for this study. B) Pie chart showing distribution of genotype-positive versus genotype-negative individuals within the study cohort. C) Pie chart demonstrated the variant breakdown of gene-positive individuals. Numbers representing the number of individuals present within each group. AHC, alternating hemiplegia of childhood; QTc, corrected QT; Pos, genotype-positive; Neg, genotype-negative; LOF; Loss of Function.

Figure 2. Comparison of QTc measurements divided by genotype and *ATP1A3* variant status. A) Representative ECG tracings taken from lead V5 of representative probands from various *ATP1A3* genotypes. B) Graph with QTc (mean±SEM) for genotype positive versus negative individuals (**P*=0.002). C) Graph with QTc (mean±SEM) for each major variant in *ATP1A3* (D801N variant versus E815K variant versus G947R versus LOF versus all other *ATP1A3* variants versus healthy controls) (*P=0.01; **P=0.02; ***P=0.0007; ****P=0.002; *****P<0.0001). D) and E) Graph showing penetrance with percentage of individuals with QTc <370 msec and <350 msec, respectively for each major variant in *ATP1A3* (D801N variant versus E815K variant versus G947R versus LOF versus all other *ATP1A3* variants versus healthy

controls). Msec: millisecond; Genotype Pos, genotype-positive; Genotype Neg; genotype-negative; LOF; Loss of Function.

Figure 3. ATP1A3-D801N individuals are susceptible to experiencing the composite cardiac outcome of ventricular tachycardia/ventricular fibrillation arrest, sudden cardiac arrest or suspected cardiogenic syncope or ICD appropriately discharged. A) Bar graph of the percentage of events that met this composite outcome with only ATP1A3-D801N individuals experiencing cardiac outcomes in our cohort with the incidence of cardiac events per 100 life years displayed at the bottom. B) Bar graph of cardiac events as a function of total anesthesia exposures. C) Kaplan-Meyer curve depicting freedom from the composite cardiac outcome showing that patients with the ATP1A3-D801N variant (red line) are significantly more likely to experience one of the composite cardiac outcomes compared to all other *ATP1A3* variants (black line, P=0.02). Grey shading depicts 95% confidence intervals.

Figure 4: Structural mapping of amino acid positions whose missenses are associated with variable penetrance of shortened QTc on the crystal structure of human alpha 3 Na/K ATPase in the K^+ occluded state. A) Illustrates the crystal structure with beta 1 subunit in blue, FXYD in purple, and alpha 3 subunit in gray. The shortened QTc associated amino acids D801, D805, P323, S772, and C333 are colored red. B) Illustrates a closer view of the region of interest on the human alpha 3 Na/K ATPase. C) and D) illustrates the transmembrane region in from an anterior and posterior view, respectively which is where the variants resulting in shortened QTc with variable penetrance are located.

Figure 5: AHC patient derived iPSC-CM^{D801N} have shorter action potential duration and predisposition to delayed after depolarizations. A) Pedigree of a 4-year-old proband with *de novo* inherited missense variant ATP1A3-D801N. B) Sanger sequencing of iPSCs from ostensibly healthy individual (ATP1A3-WT) and proband (ATP1A3-D801N). C) Representative action potential tracing and (D-F) action potential duration at 50% repolarization (APD50) and 90% repolarization time (APD90) and mean diastolic potential (MDP) from iPSC-CM^{WT} and iPSC-CM^{D801N}, respectively. G) Representative action potential tracing of spontaneously depolarizing PSC-CM^{WT} and iPSC-CM^{D801N} with black arrow indicating delayed after depolarizations (DADs). H) Percentage of cells with delayed after depolarizations (DADs) in iPSC-CM^{WT} (0/24 cells, 0%; 95% CI 0.0-10.7%) and iPSC-CM^{D801N} (3/11 cells, 27%; 95% CI 9.7-56.5%). Mann Whitney test was conducted to assess statistical significance. *, $P<0.001$; **, $P<0.0001$.