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Molecular Autopsy:  
A Population-Based Case Series

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**The Importance of Variant Interpretation in Whole Exome Molecular Autopsy:  
A Population-Based Case Series**

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**ABSTRACT**

**Background:** Potentially lethal cardiac channelopathies/cardiomyopathies may underlie a substantial portion of sudden unexplained death in the young (SUDY). The whole exome molecular autopsy (WEMA) represents the latest approach to postmortem genetic testing for SUDY. However, proper variant adjudication in the setting of SUDY can be challenging.

**Methods:** From January 2012 through December 2013, 25 consecutive cases of SUDY aged from 1-40 years (average age at death  $27 \pm 5.7$  years; 13 white, 12 black) from Cook County, Illinois, were referred following a negative (n=16) or equivocal (n=9) conventional autopsy. A WEMA with analysis of 99 sudden death-susceptibility genes was performed. The predicted pathogenicity of ultra-rare, non-synonymous variants (NSVs) was determined using the American College of Medical Genetics (ACMG) guidelines.

**Results:** Overall, 27 ultra-rare NSVs were seen in 16/25 (64%) SUDY victims. Among black individuals, 9/12 (75%) had an ultra-rare NSV compared with 7/13 (54%) white individuals. Of the 27 variants, 10 were considered “pathogenic (P)” or “likely pathogenic (LP)” in 7/25 (28%) individuals in accordance with the ACMG guidelines. P / LP variants were identified in 5/16 (31%) of autopsy-negative cases and in 2/6 (33%) SUDY victims with equivocal findings of cardiomyopathy. Overall, 6 P/LP variants in 4/25 (16%) cases were congruent with the phenotypical findings at autopsy and therefore considered “clinically actionable”.

**Conclusions:** WEMA with gene-specific surveillance is an effective approach for the detection of potential pathogenic variants in SUDY cases. However, systematic variant adjudication is crucial to ensure accurate and proper care for surviving family members.

**KEY WORDS:** Genetics, Sudden Death, Channelopathies, Cardiomyopathies

## CLINICAL PERSPECTIVE

### What is new?

- The first population-based, case-series study involving the whole exome sequencing-based cardiac channelopathy/cardiomyopathy gene specific molecular autopsy of sudden unexplained death in the young (SUDY) cases within the United States.

### What are the clinical implications?

- While the ACMG guidelines are useful, careful evaluation of the decedent's autopsy findings and/or pre-mortem clinical phenotype remains critical before adjudicating a variant as the definitive cause of death.
- The goal of these investigative studies is to provide closure to families surrounding the loss of their loved one, but perhaps the only thing worse than no answer, is to give a false answer prematurely.

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## INTRODUCTION

Sudden cardiac death is a major world-wide public health burden with an estimated annual incidence ranging from 180,000 to 450,000<sup>1</sup> in the United States and as many as 3.7 million deaths globally<sup>2</sup>. The majority of these deaths are due to coronary artery disease among the elderly<sup>3</sup>. However, approximately 2,000 to 5,000 young people between 1 to 35 years of age die suddenly each year in the United States<sup>4</sup>. For many of these sudden deaths in the young, a comprehensive medico-legal investigation including a conventional autopsy examination elucidates a clear cause of death. However, in up to 40% of these cases, gross and microscopic inspection of the heart and other organs fails to reveal a definite cardiac/non-cardiac etiology<sup>5</sup>, and are categorized as autopsy negative sudden unexplained death in the young (SUDY).

Potentially lethal and heritable cardiac channelopathies like long QT syndrome (LQTS), catecholaminergic polymorphic ventricular tachycardia (CPVT), and Brugada syndrome (BrS), are associated typically with grossly and histologically normal hearts and may account for a significant portion of SUDY. Additionally, heritable cardiomyopathies, including hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), and arrhythmogenic cardiomyopathy (ACM), may present with a mild structural phenotype that could escape detection at autopsy. Together, these genetic heart diseases may be the underlying etiology for a significant percentage of SUDY cases.

Recently, guidelines for autopsy investigations of SUDY cases stipulate procurement and retention of tissue suitable for DNA extraction as a class I recommendation and advise that postmortem genetic testing (i.e. the molecular autopsy) be considered as the new standard of care in the decedent's evaluation<sup>6-8</sup>. With at least 99 sudden death-susceptibility genes to date, post-mortem genetic testing with whole exome sequencing (WES) and targeted gene analysis

represents a cost- and time-effective approach for performing the molecular autopsy. While there is increasing evidence to support the use of a whole exome molecular autopsy (WEMA)<sup>5, 9-15</sup>, standardization of the procedure for characterizing putative pathogenic mutations is crucial to enable proper counseling of surviving family members and accurate publishing for scientific progress. Recently, the American College of Medical Genetics (ACMG) has provided guidelines for the interpretation of sequence variants<sup>16</sup> which may be helpful in delineating the predicted pathogenicity of variants identified by WEMA in SUDY cases.

Here, we describe a cohort of 25 unrelated SUDY victims referred consecutively by the Office of the Medical Examiner, Cook County, Illinois. We performed a WEMA to determine the spectrum and prevalence of ultra-rare, nonsynonymous variants (NSVs) within sudden death-susceptibility genes and demonstrate use of both the ACMG guidelines and the necropsy-derived phenotype data for proper variant adjudication.

## **MATERIALS AND METHODS:**

The data, analytical methods, and study materials will not be made available to other researchers for the purposes of reproducing the results or replicating the procedure.

### **Study Subjects**

From January 2012 to December 2013, 25 consecutive, unrelated SUDY cases were referred to the Windland Smith Rice Sudden Death Genomics Laboratory at the Mayo Clinic in Rochester, Minnesota, to undergo WEMA following Mayo Clinic IRB approval. All 25 cases underwent a comprehensive autopsy by the Office of the Medical Examiner from Cook County, Illinois.

Enrollment criteria required sudden death of an individual between the ages of 1 and 40 years of age which remained unexplained or equivocal following a comprehensive autopsy.

### **Control Population**

973 European white control exomes (509 females, 464 males) from the ICR1000 UK exome series and the 1958 Birth Cohort study were included for a case:control subset analysis of genetic variation amidst these 99 genes between Caucasian decedents and these controls.<sup>17</sup> As previously reported, exome sequencing was performed using the Illumina TruSeq and Illumina instruments.<sup>17</sup>

### **DNA Isolation**

Genomic DNA was isolated from autopsy whole blood or frozen tissue using the Gentra Puregene Blood Kit (Qiagen, Maryland, US) following the manufacturer's protocol.

### **Whole Exome Next-Generation DNA Sequencing**

Genomic DNA samples were submitted to Mayo Clinic's Advanced Genomics Technology Center for WES. The Bravo liquid handler and Aligent's protocol was used to prepare paired-end libraries, and DNA was fragmented using a Covaris E210 sonicator. Agencourt AMPure SPRI beads were used to purify the constructs. SureSelect forward and Agilent SureSelect ILM Pre-Capture Indexing reverse primers were used to enrich the DNA fragment libraries, which were analyzed with Agilent Bioanalyzer DNA 1000 chip.

Exome capture was performed with the SureSelect XT Human All Exon V5 plus UTR Target Enrichment System (Agilent, Santa Clara, California). Dynal Dynabeads MyOne Streptavidin T1 captured the DNA:RNA hybrids, and Agencourt Ampure XZP beads eluted DNA from the beads, which were amplified with Agilent Sure Select Post-Capture Indexing forward and Index PCR reverse primers. Sequencing of the exome libraries was completed with

Illumina HiSeq 2000 platform (San Diego, California) and TruSeq SBS sequencing kit V3 reagents.

### **Variant Filtering and Pathogenicity Assessment**

Following WES, variants were filtered using Qiagen's Ingenuity® Variant Analysis™ software (Qiagen Bioinformatics, Redwood City, California). Variants were included only if they met the following filtering parameters: 1) had a high quality score (read depth > 10 reads, call quality > 20, genotype quality > 20, and present in genes outside the top 1% of exonically variable genes and top 5% of exonically variable 100 base windows), 2) were NSVs (i.e. missense, nonsense, frameshift insertion/deletion [INDEL], in-frame INDEL, or splice error), and 3) met our rarity threshold (minor allele frequency [MAF]  $\leq 0.00005$  in any ethnic group within Exome Aggregation Consortium [ExAC, n=60,706]<sup>18</sup>, 1,000 Genome Project [1KG, n=1,094]<sup>19</sup>, and the National Heart, Lung and Blood Institute Grand Opportunity Exome Sequencing Project [ESP, n=6,503] databases). Variants meeting the above criteria underwent a further gene-specific surveillance for all known cardiac channelopathy-, cardiomyopathy-, and sudden unexplained death in epilepsy (SUDEP)-susceptibility genes (N=99, **Supplemental Table 1**).

The ACMG guidelines for the interpretation of sequence variants were used to classify identified variants as pathogenic (P), likely pathogenic (LP), or variant of uncertain significance (VUS)<sup>16</sup>. To be considered “clinically actionable”, the variant had to meet ACMG guideline criteria for a P or LP variant designation and be congruent with the presence/absence of disease-gene suggested autopsy findings. Here in, we consider a “clinically actionable” variant as a LP or P variant that should immediately prompt the physician to perform mutation-specific cascade genetic testing besides the standard cardiology clinical evaluation in all first-degree relatives of the deceased. Further, among those relatives who test positive for the implicated variant,



periodic re-assessment of potential disease manifestation related to the identified disease-susceptibility variant should be performed.

Candidate disease-causing variants identified through WEMA were confirmed in the decedents' genomic DNA using standard polymerase chain reaction (PCR) and Sanger sequencing methods. PCR primers, conditions, and sequencing methods are available upon request.

### **Statistical Analysis**

Fisher's exact tests were performed to determine statistical significance between two groups. A  $p < 0.05$  was considered to be significant.

## **RESULTS:**

### **Sudden Unexplained Death in the Young Cohort**

The demographics for the 25 SUDY cases are summarized in **Table 1**. The average age at death was  $27 \pm 5.7$  years. There were 17 males (68%) and 8 (32%) females. Thirteen (52%) cases were white and 12 (48%) were black. Sixteen (64%) cases were autopsy negative and 9 (36%) cases had equivocal findings or inconclusive cardiac abnormalities with 6 (24%) having equivocal findings for a possible cardiomyopathy. The 25 SUDY victims died during the following circumstances: unwitnessed (10; 40%), sleep (9; 36%), nonspecific (3; 12%) and exertion or auditory trigger (3; 12%). Eleven (44%) SUDY cases had a personal history of either illicit drug use (24%) or mental illness (20%). A prior history of arrhythmia or syncope was noted in 2 (8%) cases. No cases had a known family history of arrhythmia, syncope, seizure, or cardiac arrest.

## **Yield of Ultra Rare-Non-Synonymous Variants (NSVs) in Sudden Death-Susceptibility Genes**

Following WEMA, we identified 27 ultra-rare (MAF < 0.00005), NSVs within the 99 sudden death-susceptibility genes in 16/25 (64%) individuals overall including 9/12 (75%) black and 7/13 (54%) white decedents (**Figure 1 and Table 2**). Compared to the 54% yield observed in white cases, ultra-rare NSVs were identified in 281/973 (28.9%,  $p=0.064$ ) European white controls (**Figure 2**). Interestingly, 4/13 (30.8%) white SUD cases hosted multiple ultra-rare NSVs amidst these 99 genes versus 43/973 (4.4%,  $p=0.002$ ) of the European controls (**Figure 2**). Of the 27 NSVs identified, 4 (p.R783H-MYH7, p.L567Q-SCN5A, p.Y462S-RYR2, and p.N4763S-RYR2) were present in “major” genes (i.e. strong evidence for disease association) for cardiac channelopathies and cardiomyopathies (*KCNQ1*, *KCNH2*, *SCN5A*, *RYR2*, *MYH7*, and *MYBPC3*) and 23 variants were in “minor” genes (i.e. limited evidence genes) (**Table 2 and Figure 3**). None of the variants were identified within any of the three SUDEP-susceptibility genes (*KCNA1*, *SCN1A*, and *SCN8A*).

### **Variant Adjudication with ACMG Guidelines**

Of the 27 ultra-rare NSVs, 10 NSVs (37%) were classified as pathogenic (P) variants or likely pathogenic (LP) variants based on the ACMG guideline criteria (**Figure 1 and Table 2**). These 10 NSVs were found in 7/25 (28%) individuals [5/13 (38%) white and 2/12 (17%) black]. Compared with the white SUDY cases, 36/973 (3.7%) white controls hosted an ultra-rare NSV in  $\geq 1$  of the 99 genes that would be graded as either a LP or P variant ( $p=0.000098$ ). Multiple LP or P variants were identified in 2/13 (15.4%) of the white sudden death victims versus 2/973 (0.21%,  $p=0.00094$ ) white controls (**Figure 2**). P or LP variants were identified in 5/16 (31%) autopsy negative SUDY victims and in 2/6 (33%) SUDY victims that had an equivocal finding

of cardiomyopathy at autopsy. There were 9 (36%) decedents who each hosted an ultra-rare variant of uncertain significance (VUS). These VUSs are potentially informative given their ultra-rare status. However, all 17 VUSs identified occurred in so called “minor” genes where the strength of evidence for disease association is limited (**Figure 3**). Furthermore, only 5 of the 17 VUS matched the phenotype at autopsy. Although there appears to be genotype-phenotype concordance potentially, these variants could not be considered the underlying cause of death until further evidence for pathogenicity is satisfied with either functional validation studies or other criteria in the ACMG guidelines. Twelve VUSs were discordant with the phenotype observed at autopsy and therefore were dismissed as likely benign (**Figure 3**).

### **Clinically Actionable Variants**

The ACMG has proposed that the use of the term “likely pathogenic” (and therefore also pathogenic) should mean that the variant of interest has a  $\geq 90\%$  certainty of being disease-causing <sup>16</sup>. However, some NSVs identified through WES that meet the ACMG criteria for a P or LP designation, may not be consistent with the autopsy findings for the SUDY. Of the 10 P/LP variants that were identified, 6 variants (p.R350Q-DES, p.R783H-MYH7, p.N4763S-RYR2, p.L567Q-SCN5A, p.V2736fs-TTN, and p.D22167fs-TTN) identified in 4/28 (14.3%) were congruent with the SUDY’s phenotype at autopsy and therefore deemed “clinically actionable” (**Table 2** and **Figure 3**).

The remaining 4 P/LP variants (p.Q1289X-DSP, p.K942X-MYH6, c.903+1 G>A-NEBL, and p.Y462S-RYR2) were not congruent (**Table 2** and **Figure 3**) with autopsy findings.

Although p.Q1289X-DSP, p.K942X-MYH6, c.903+1 G>A-NEBL, all satisfy the ACMG guideline criteria for P or LP designation based on being an “ultra-rare” and null variant (i.e. nonsense or splice-error), these variants in cardiomyopathy-associated genes were identified in

SUDY victims with no autopsy findings suggestive of a structurally abnormal heart. Therefore, these variants may require additional lines of evidence prior to assigning them as being disease-causing and relevant to surviving family members. The p.Y462S-RYR2 variant was identified in a patient with autopsy findings consistent with HCM. RYR2 mutations cause CPVT, a structurally normal heart-associated arrhythmia syndrome. This SUDY case also hosted a desmin (p.R350Q-DES) LP variant that would be consistent with his autopsy findings and most likely represents the pathogenic basis for the decedent's sudden death.

### **Case Summaries for SUDY Victims with a Clinically Actionable Variant**

Overall, 4/25 (16%) of the SUDY cases hosted at least one “clinically actionable” variant. A 14-year-old white male (Case 1, **Table 2**) who experienced an exertion-related autopsy negative sudden death, hosted a p.L567Q-SCN5A pathogenic variant and a p.N4763S-RYR2 likely pathogenic variant. *SCN5A* encodes a voltage-gated cardiac sodium channel, and mutations in the gene have been associated with LQT3<sup>20</sup> and BrS1<sup>21</sup>. Electrophysiological studies of the p.L567Q-SCN5A mutation have demonstrated a significant effect on sodium channel inactivation<sup>22</sup>, and the patients with this specific mutation are particularly prone to sudden death<sup>23</sup>. *RYR2* gene mutations cause CPVT, a structurally normal heart associated arrhythmia syndrome that often manifests during exertion.

A 27-year-old black female (Case 9, **Table 2**) who collapsed suddenly with seizure-like activity, had a p.R783H-MYH7 pathogenic variant and a p.V27236fs-TTN likely pathogenic variant. She had a history of a cardiac blood clot four years prior to death and her autopsy was equivocal for ischemic cardiomyopathy. The *MYH7* gene is associated with familial HCM and DCM. Although the p.R783H variant results in a conservative amino acid substitution, this specific variant has been observed previously in two individuals with cardiomyopathy<sup>24</sup>. *TTN*

frameshift mutations have been associated with DCM. The TTN frame-shift variant identified in this SUDY case localizes to the sequence segment encoding for the A-band region of the protein, where such frameshift mutations are overrepresented in cases of DCM compared to controls<sup>25</sup>.

A 28-year-old white male (Case 15, **Table 2**), found dead in bed, hosted both a p.R350Q-DES and a p.Y462S-RYR2 likely pathogenic variant. He had history of arrhythmias and was scheduled for cardioversion later that year. Additionally, his autopsy was equivocal for HCM with microscopic findings of myocyte hypertrophy and gross findings of an enlarged tricuspid valve with abnormal chordae, enlarged chambers, left ventricle hypertrophy, and fusion of right and non-coronary cusps of the aortic valve. His heart was enlarged, weighing 610g. His mother was diagnosed previously with HCM. *DES*-encoded desmin is a class III intermediate filament specific to muscle cells. Mutations in desmin have been associated with HCM, DCM, myofibrillar myopathy, and sudden death<sup>26-29</sup>. *RYR2* encodes the cardiac ryanodine receptor found in the sarcoplasmic reticulum of cardiac muscle. The receptor facilitates calcium release that is crucial for cardiac contraction.

A 36-year-old white female (Case 23, **Table 2**), who was found unresponsive in bed, hosted a p.D22167fs-TTN frameshift variant designated as likely pathogenic. She had history of heart murmur of an unknown type. Although originally considered autopsy negative, microscopic findings at autopsy included mild to moderate interstitial fibrosis as well as myocyte hypertrophy. The mutation results in a frameshift mutation within the region encoding for the A-band portion of the TTN protein where frame-shift mutations are overrepresented in DCM cases when compared to controls<sup>25</sup>.

## DISCUSSION

Since providing the first ever proof-of-principle case report of a WES-based comprehensive molecular autopsy of a previously healthy 16-year-old SUDY victim, where we identified a

pathogenic *MYH7* mutation<sup>30</sup>, the utility of the whole exome molecular autopsy (WEMA) has been recognized increasingly as an efficient and cost-effective method for comprehensive post-mortem genetic evaluation of SUDY cases<sup>5, 9-15</sup>.

Here, we present the first population-based study involving WEMA of SUDY cases within the United States. Using WES and a gene-specific analysis involving 99 cardiac channelopathy-, cardiomyopathy-, and SUDEP-associated genes, we identified an ultra-rare (MAF < 0.00005; 1 in 20,000 alleles), amino acid altering variant in a sudden death-susceptibility gene in 54% of our white and 75% of our black SUDY victims.

In 2016, Bagnall and colleagues reported on the genetic analysis of 59 cardiac channelopathy/cardiomyopathy genes in a similar SUDY cohort of 113 cases from Australia or New Zealand<sup>5</sup>. Using a MAF < 0.1% (i.e. 1 in 1000 alleles), 27% of their cases hosted what they termed a ‘clinically relevant’, ‘pathogenic’ or ‘likely pathogenic’ cardiac gene mutation. Of the 36 ‘clinically relevant’ variants identified, 30 had a MAF < 0.00005 (the threshold used in our study). Thus, 6 of the variants identified would not have been considered to be potentially disease-causing using our more stringent MAF cut-off. In fact, 4 of these variants have a higher prevalence in ExAC (1 in 500 to 1 in 1000 subjects) than the estimated disease prevalence (for example, 1 in 2000 for LQTS) that they would be associated with.

While both studies support the utility of WEMA to identify potential sudden death-causing variants within sudden death-susceptibility genes, the challenge of the WES-based molecular autopsy does not lie in the identification of variants, but rather, in the adjudication of their predicted pathogenicity. Accurate variant classification is crucial to enable proper counseling of surviving family members and accurate publishing for scientific progress.

Erroneously or prematurely adjudicating ambiguous variants as pathogenic has the potential to harm patients and their families. Tragically, this became a reality for one family described by Ackerman et al., as they dealt with the disastrous consequences of unnecessary treatment based on an erroneously interpreted variant in *KCNQ1*<sup>29</sup>. Our group and others have estimated previously that as much as 10% of the variants published as LQTS-associated mutations may be classified incorrectly<sup>31,32</sup>. This number is likely to be higher when accounting for all sudden death genes, as a recent study has indicated that as many as 30% of all disease-causing genetic variants in the literature may have been reported incorrectly<sup>33</sup>.

In order to assist in the interpretation of identified variants, the ACMG guidelines provide a framework for variant classification by incorporating a variety of weighted factors that lead to a final delineation of pathogenic (P), likely pathogenic (LP), benign, or variant of uncertain significance (VUS)<sup>16</sup>. Using strict criteria, variants are assessed for “very strong” (i.e. a null variant), “strong” (i.e. the same amino acid change as a previously established pathogenic variant, confirmed as de novo when there is no family history, or is associated with well-established functional studies demonstrating a deleterious effect), “moderate” (i.e. absence from large control populations like ExAC), or “supporting” (i.e. multiple lines of computational evidence predicting a deleterious effect) evidence for pathogenicity. Points earned in each category are combined in a variety of ways to reach a final variant classification.

Recently, following interrogation of 77 cardiac channelopathy/cardiomyopathy genes, Lahrouchi and colleagues reported a yield of ACMG guideline predicated “pathogenic” / “likely pathogenic” variants in 13% of their mostly European decent (88%) cohort of 302 SUDY cases without structural heart disease or nonspecific cardiovascular changes<sup>15</sup>. Congruent with a phenotype of negative-autopsy and suspected sudden cardiac arrhythmia death, 85% of the

“pathogenic” / “likely pathogenic” variants identified by Lahrochi and colleagues were in major cardiac channelopathy-susceptibility genes (i.e. *KCNQ1*, *KCNH2*, *SCN5A*, and *RYR2*)<sup>15</sup>.

While using their own interpretation for labeling variants as ‘pathogenic’ or ‘likely pathogenic’, Bagnall and colleagues reported finding such variants in 27% of their SUDY cases<sup>5</sup>. However, when applying the ACMG guideline criteria to their identified variants, this yield decreases to only 7.1% (8/113). In fact, based on the ACMG guidelines, 28 of their 36 ‘pathogenic’ or ‘likely pathogenic’ variants would be demoted to a VUS. The difference in variant interpretation stems largely from the over-calling of missense variants as being ‘likely pathogenic’. In their study, missense variants with a MAF < 0.1% that were predicted to be damaging by at least 2 out of 3 in silico tools (SIFT, Polyphen, Mutation Taster) and involving conserved nucleotides (GREP score  $\geq 2$ ) were considered as ‘likely pathogenic’<sup>5</sup>. However, according to ACMG, this level of evidence alone would be deemed insufficient to promote a variant to a “likely pathogenic” designation.

Based on ACMG guideline criteria, 28% of our overall cohort hosted at least one “pathogenic” / “likely pathogenic” variant. The yield of “pathogenic”/ “likely pathogenic” variants was about 30% for both the autopsy-negative and equivocal cases with cardiomyopathic findings at autopsy. This reflects a 20% higher yield of ACMG guideline predicated variants in our cohort than what was observed in the phenotypically and demographically similar cohort investigated by Bagnall and colleagues<sup>5</sup>. The large difference in yield may stem from differences in the next-generation sequencing platforms and bioinformatics-based variant annotation and analytical pipelines that were used as well our inclusion of 40 additional genes that were not included in the Bagnall study<sup>5</sup>. In fact, 3 of our 25 (12%) cases had either a TTN



frameshift (2 cases) or NEBL splice-error (1 case) “likely pathogenic” variant. However, these two genes were not included in the Bagnall study<sup>5</sup>.

However, while 28% of our SUDY cases hosted a “pathogenic” or “likely pathogenic” variant based on the ACMG guidelines, the decedent’s phenotype at autopsy was congruent with the genetic finding in only 14% of cases and therefore considered “clinically actionable”. These “clinically actionable” pathogenic/ likely pathogenic variants have sufficient genotype-phenotype evidence to warrant cascade genetic testing of surviving family members. However, other ultra-rare variants that don’t rise to our present consideration of “clinically actionable” might be deemed as nevertheless worthy of careful research/clinical-based investigations to see if the variant co-segregates with the disease phenotype which if it did, then those variants might be elevated to a “clinically actionable” status that would warrant cascade genetic testing for future family members.

Interestingly, three cardiomyopathy-associated genes variants that satisfied the ACMG guideline criteria for pathogenic or likely pathogenic designation based on being an “ultra-rare” and null variant (i.e. nonsense or splice-error) were identified in SUDY victims with no autopsy findings suggestive of a structurally abnormal heart. Although some cardiomyopathy-associated gene variants can provide a potentially lethal arrhythmic substrate prior to the development of overt cardiomyopathic changes, because these identified variants were not congruent with the observed phenotype, the designation of these variants, as ACMG guideline-predicated “pathogenic” or “likely pathogenic” variants, should be rendered with some residual skepticism.

It is noteworthy that 1/3 of the ultra-rare variants identified were categorized as "needs further functional study." These variants are potentially informative clinically given their presence in disease-associated genes and their ultra-rare status. However, they currently lack

sufficient clinical evidence (i.e. co-segregation with disease phenotype) and research-based evidence (i.e. in vitro functional validation assay) to be put forward as a disease-causing, clinically relevant variant.

## CONCLUSIONS

Despite 64% of this population-based cohort of consecutive, unrelated SUDY cases having ultra-rare, nonsynonymous variants within sudden death-susceptibility genes, 28% had a variant classified as either “pathogenic” or “likely pathogenic” based on the ACMG guidelines. Furthermore, 14% of cases had a congruent genotype-phenotype correlation enabling the variant to be clinically actionable for cascade genetic testing of surviving family members. The substantial number of VUSs demonstrates the necessity for further standardizing the adjudication of putative pathogenic variants. While the current ACMG guidelines are useful, careful evaluation of the decedent’s autopsy findings and/or pre-mortem clinical phenotype remains critical before adjudicating a variant as the definitive cause of death in SUDY cases. The goal of these investigative studies is always to provide closure to families surrounding the loss of their loved one, but perhaps the only thing worse than no answer, is to give a false answer prematurely.

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### **AUTHOR CONTRIBUTIONS**

MJA and DJT designed the study. DJT performed DNA extraction. GWS, JPA, and DJT performed variant filtering, and variant analysis. SMW performed conventional autopsies. GWS, JPA, and DJT wrote the initial drafts of the manuscript. GWS and DJT should be regarded as joint first authors.

### **DISCLOSURES**

MJA is a consultant for Audentes Therapeutics, Boston Scientific, Gilead Sciences, Invitae, Medtronic, MyoKardia, and St. Jude Medical. MJA and Mayo Clinic have an equity/royalty relationship with AliveCor, Blue Ox Health Corporation, and StemoniX. However, none of these entities were involved in this study in any way.

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**Table 1: SUDY Cohort Demographics**

<b>Cases</b>	25
<b>Age (years)</b>	27.0 ± 5.7
<b>Male</b>	17 (68%)
<b>Female</b>	8 (32%)
<b>White</b>	13 (52%)
<b>Black</b>	12 (48%)
<b>Autopsy Negative</b>	16 (64%)
<b>Equivocal</b>	9 (36%)
<b>Equivocal Cardiomyopathy</b>	6 (24%)
<b>Event: Unwitnessed</b>	10 (40%)
<b>Event: Sleep</b>	9 (36%)
<b>Event: Nonspecific</b>	3 (12%)
<b>Event: Exertion/Auditory</b>	3 (12%)
<b>Illicit drug use</b>	6 (24%)
<b>Mental Illness</b>	5 (20%)
<b>Arrhythmia or syncope</b>	2 (8%)

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**Table 2: SUDY Case Summary and Variant Adjudication**

Case	Sex	Age (years)	Race	Setting of SUDY	Autopsy Findings	Autopsy Classification	Equivocal Cause	Variant(s)	Associated Disease(s)	ACMG Criteria Met	ACMG Classification	Clinically Actionable?
1	M	14	White	Exertion	Contraction band necrosis	Autopsy negative	-	p.L567Q-SCN5A	LQTS, BrS, DCM	PS1, PS3, PS4, PM1, PM2, PP3	<b>Pathogenic</b>	<b>Yes</b>
								p.N4763S-RYR2	CPVT, ACM	PM1, PM2, PP2	<b>Likely pathogenic</b>	<b>Yes</b>
								p.Y332C-PDLIM3	DCM	PM2	VUS	No
								p.G28A-MYPN	HCM, DCM	PM2	VUS	No
2	M	19	Black	Auditory	Interventricular septum hypertrophy, right atrial enlargement with endocardial fibroelastosis, right ventricular elongated chordae	Autopsy negative	-	p.S688P-PKP2	ACM	PM2	VUS	No
3	F	19	White	Unwitnessed	None	Autopsy negative	-	p.I591V-ACTN2	HCM, DCM	PM2	VUS	No
4	F	19	White	Sleep	None	Autopsy negative	-	-	-	-	-	-
5	F	21	White	Unwitnessed	None	Autopsy negative	-	<b>c.903+1G&gt;A-NEBL</b>	DCM	PVS1, PM2	<b>Likely pathogenic</b>	No
6	F	23	White	Unwitnessed	None	Equivocal	Possible drug toxicity	-	-	-	-	-
7	M	25	Black	Sleep	Thin LV wall (0.6cm), scattered hypertrophic myocytes	Equivocal	Cardiomyopathy	p.K120T-KCNJ2	LQTS	PM2	VUS	No
8	M	26	White	Sleep	None	Autopsy negative	-	-	-	-	-	-



9	F	27	Black	Nonspecific	LV hypertrophy (1.8cm), LV posterior MI scarring	Equivocal	Ischemic cardiomyopathy	p.R783H-MYH7	HCM, DCM	PS1, PM1, PM2, PM5, PM6, PP2, PP4	<b>Pathogenic</b>	<b>Yes</b>
								p.V27236fs-TTN	HCM, DCM	PVS1, PM2	<b>Likely pathogenic</b>	<b>Yes</b>
								p.V190M-JUP	ACM	PM2	VUS	-
10	F	27	Black	Unwitnessed	Interstitial and perivascular fibrosis	Autopsy negative	-	p.R25W-LAMP2	HCM	PM2	VUS	-
11	M	27	Black	Unwitnessed	None	Equivocal	Possible sarcoidosis	-	-	-	-	-
12	M	27	White	Sleep	Focal perivascular fibrosis, probe-patent foramen ovale	Autopsy negative	-	p.K942X-MYH6	HCM, DCM	PVS1, PM2	<b>Likely pathogenic</b>	No
13	M	28	Black	Unwitnessed	Focal myocyte hypertrophy	Autopsy negative	-	p.E44Q-ILK	DCM	PM2	VUS	No
14	M	28	Black	Unwitnessed	Intraventricular septum hypertrophy (2.0cm)	Equivocal	Cardiomyopathy	p.A52P-MYOM1	HCM	PM2	VUS	No
15	M	28	White	Sleep	Enlarged heart (610g), LV hypertrophy, enlarged tricuspid valve with abnormal chordae, fusion of aortic valve cusps, myocyte hypertrophy	Equivocal	Hypertrophic cardiomyopathy	p.Y462S-RYR2	CPVT, ACM	PM1, PM2, PM5, PP2	<b>Likely pathogenic</b>	No
								p.R350Q-DES	DCM	PM1, PM2, PM5, PP2	<b>Likely pathogenic</b>	<b>Yes</b>
16	M	29	Black	Exertion	Focal interstitial and perivascular fibrosis, myocyte nuclear hypertrophy	Equivocal	Excited delirium (schizophrenia)	p.R1899H-MYH6	HCM, DCM	PM2	VUS	No
17	M	29	Black	Unwitnessed	Subendocardial fibrosis	Autopsy negative	-	p.Q1289X-DSP	ACM	PVS1, PM2, PP3	<b>Pathogenic</b>	No
								p.F1198L- LAMA4	DCM	PM2	VUS	No

								p.R3Q-CTF1	DCM	PM2	VUS	No
18	M	29	White	Unwitnessed	Focal interstitial and perivascular fibrosis	Equivocal	Cardiomyopathy	-	-	-	-	-
19	M	29	White	Sleep	None	Autopsy negative	-	p.G8D-SCN4B	LQTS	PM2	VUS	No
20	M	30	Black	Sleep	None	Autopsy negative	-	-	-	-	-	-
21	M	30	White	Nonspecific	None	Autopsy negative	-	-	-	-	-	-
22	M	30	White	Unwitnessed	None	Autopsy negative	-	-	-	-	-	-
23	F	36	White	Sleep	Interstitial fibrosis, myocyte hypertrophy	Autopsy negative	-	p.D22167fs-TTN	HCM, DCM	PVS1, PM2	Likely pathogenic	Yes
								p.V585M-CACNA1C	BrS, LQTS	PM2	VUS	No
24	M	37	Black	Sleep	Enlarged heart (625g), LV hypertrophy (2.2cm), intraventricular septum hypertrophy (2.0cm), myocyte fibrosis and disarray	Equivocal	Hypertrophic cardiomyopathy	p.W427R-RBM20	DCM	PM2	VUS	No
								p.A531T-MYLK2	HCM	PM2	VUS	No
								p.P486L-TBX1	HCM	PM2, PP2	VUS	No
25	F	39	Black	Nonspecific	Tunneling of LAD (1mm deep)	Autopsy negative	-	-	-	-	-	-

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## FIGURES

### **Figure 1: Yield of Ultra-Rare Nonsynonymous Variants and ACMG Guideline-Designated**

**Pathogenic/Likely Pathogenic Variants Identified in Sudden Unexplained Death in the Young** - Shown is a bar graph indicating the percent yield of “ultra-rare” (minor allele frequency < 0.005%) nonsynonymous variants and ACMG guideline predicated “pathogenic” / “likely pathogenic” variants detected among 99 cardiac channelopathy/cardiomyopathy/SUDEP-associated genes for our overall cohort and the ethnic-specific (white or black), autopsy negative, and autopsy equivocal subsets.

### **Figure 2: White Case: Control Comparative Analysis of Ultra-Rare Nonsynonymous Variants and ACMG Guideline-Designated Pathogenic/Likely Pathogenic Variants**

Shown is a bar graph indicating the percent yield of “ultra-rare” (minor allele frequency < 0.005%) nonsynonymous (NS) variants and ACMG guideline predicated “pathogenic” / “likely pathogenic” variants detected among 99 cardiac channelopathy/cardiomyopathy/SUDEP-associated genes for white sudden unexplained death in the young cases (n=13) and European white controls (n=973).

**Figure 3: Variant Interpretation Flow-Diagram** – Shown is a flow-diagram algorithm used to determine whether an identified “ultra-rare” non-synonymous variant was considered “clinically actionable”, “needs further functional study”, or presumed “likely benign”. Numbers in parenthesis indicate number of variants.

\*Major genes include *KCNQ1*, *KCNH2*, *SCN5A*, *RYR2*, *MYH7*, and *MYBPC3*. Minor genes include all other genes in 99 gene panel. VUS = variant of uncertain significance.





