Impact of Variant Reclassification in the Clinical Setting of Cardiovascular Genetics

THESIS

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Abstract

**Introduction:** Genetic testing for cardiovascular disease (CVD) is a powerful tool that enables clinicians to identify genetic forms of CVD and predict the risk for CVD in at-risk family members. Cardiovascular genetic testing has advanced over the past ten years, but these advancements have posed new challenges mainly in the field of variant classification. To address these challenges, the American College of Medical Genetics and Genomics (ACMG) published guidelines for the interpretation of sequence variant interpretation in 2015.

**Goal:** The goal of this study was to determine what impact the ACMG guidelines have on variant classification in clinical cardiovascular genetics.

**Methods:** We performed a retrospective chart review to identify patients who underwent clinical genetic testing and were found to have a variant identified in a gene associated with CVD. For each variant, systematic evidence review was performed by collecting information from both public and private variant databases and PubMed. We applied the ACMG guidelines to each variant for classification, which were compared to classifications provided on patients’ genetic test reports.

**Results:** This study identified 223 unique variants in 237 patients. Eighty (36%) of the variants resulted in classifications that differed from their clinical reports. Twenty-seven (34%) of these reclassifications were determined to be clinically significant. In total, these variant classifications affected 101 patients in a single clinical setting. For 39
patients (39% of 101), these reclassifications would result in changes in medical management recommendations for their at-risk relatives.

**Conclusion:** Application of the ACMG guidelines resulted in a change of classification for approximately one-third of the variants in this study. Clinical genetic counselors can have a more active role in the process of variant classification. It is important for variant classifications to be updated over time in the clinical CVD setting due to the impact reclassifications can have on clinical screening recommendations.
Dedication

This document is dedicated to my parents, sister, and fiancé.
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BACKGROUND

Genetic testing for cardiovascular disease (CVD) has enabled clinicians to diagnose genetic CVD and to presymptomatically test individuals who may have a genetic variant leading to an increased risk of developing a one of these conditions, such as the heritable cardiomyopathies, channelopathies, familial hypercholesterolemia, and others. By testing before the onset of symptoms, these individuals and their family members are able to receive screening and medical management that will help prevent morbidity and mortality, including sudden cardiac death (SCD). Due to the genetic heterogeneity of these conditions, most clinical genetic testing for these diseases is done by gene panels utilizing next generation sequencing technology, which allows for the simultaneous testing of multiple genes (Ackerman, 2015).

Gene panel testing allows us to identify the genetic cause of cardiovascular disease in more cases by providing the opportunity to incorporate genes not traditionally associated with the clinical phenotype. Previous studies have shown that a significant number of pathogenic variants not associated with the clinical phenotype are identified by multi-disease panel genetic testing (Pugh et al., 2014). For example, a patient may present with an enlarged left ventricle, low ejection fraction, and heart failure. This clinical phenotype would be consistent with dilated cardiomyopathy (DCM). However, gene panel testing may reveal a pathogenic variant in the DSC2 gene, which is typically associated with arrhythmogenic right ventricular cardiomyopathy (ARVC). The results
of this gene panel test would not only demonstrate that the \textit{DSC2} gene may also be associated with DCM, it would also allow us to provide genetic testing for at-risk family members. If testing had only focused on the genes mainly known to be associated with DCM, results may have been negative, and risk stratification for the patient’s family members would not have been possible.

While cardiovascular genetic testing has advanced over the past ten years, these advancements have posed new challenges, mainly around variant classification. To illustrate the issue, in the past, genetic testing was performed via single gene tests that were chosen based on the patient’s clinical phenotype. Thus, for example, a patient with QT prolongation, broad based T waves, syncope triggered by swimming, and abnormal QT response to epinephrine would undergo clinical genetic testing for the \textit{KCNQ1} gene because this presentation is associated with the type of LQTS caused by this gene. If a novel or rare variant were found in this gene on the genetic test, the likelihood for this variant to be classified as a pathogenic result would have been high, even if it was a novel variant with no previous information reported in the medical literature. However, based on current variant classification guidelines, simply finding a variant in the suspected gene would not be enough evidence to classify this variant as pathogenic because it could also be a rare benign variant. Therefore, additional evidence would still have been needed to classify this variant as pathogenic. Previously, because our clinical genetic testing was performed in a gene known to be consistent with the clinical phenotype, there was a greater chance that any variants found in the \textit{KCNQ1} gene could have been contributing to the LQTS in the patient (Ackerman, 2015). Prompted by new insights, the field has
now departed from this type of variant classification, leading to changes and discrepancies in variant classification, an area that merits study (Balmana et al., 2016).

**Cardiovascular Genetic Phenotypes**

Cardiomyopathies are disorders of the heart muscle where enlarged, thick, or rigid heart muscle causes the heartbeat to become irregular. Cardiomyopathies can be acquired or inherited. The inherited cardiomyopathies are caused by pathogenic genetic variants in genes that encode the cytoskeletal, sarcomeric, and desmosomal proteins (Campuzano et al., 2015). The role of cytoskeleton proteins in the body are to hold the inner structures of a cell in their place and stabilize the cell’s structure as it undergoes stress by providing an anchor to the cellular membrane. Sarcomeric proteins in muscle cells are what allow the muscles to contract and relax. Finally, desmosomal proteins hold our muscle cells together and give our muscle tissues stability by providing a bridge structure between them (Bezzina, Lahrouchi, & Priori, 2015). The cardiomyopathies include diseases such as dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), and arrhythmogenic right ventricular cardiomyopathy (ARVC) (Campuzano et al., 2015).

Dilated cardiomyopathy is diagnosed in individuals who have an enlarged left ventricle and a low ejection fraction. Patients with DCM usually present with symptoms of heart failure (e.g. Swelling of the legs/feet, shortness of breath, fatigue), abnormal heart rhythm, or cardiac arrest (Hershberger & Morales, 2007). The phenotype of DCM overlaps with the phenotypes of other cardiomyopathies such as HCM and ARVC making the process of clinical diagnosis more difficult in some cases (Pugh et al., 2014).
Genetic forms of DCM are typically caused by variants that disrupt the normal function of genes involved in the sarcomere or desmosome (Yacoub, 2014). There are currently more than 40 genes (including both “strong evidence” and “limited evidence” genes) that have been found in association with DCM (Pugh et al., 2014). “Strong evidence” genes have definitive evidence either in the primary literature or from expert consensus that pathogenic variants in these genes are associated with increased risk for the specific disease in question. “Limited evidence” genes are also selected from the primary literature and from expert consensus, however, there is only early evidence of a relationship between variants in these genes and the risk for specific diseases. Various types of genetic variants (e.g. missense, nonsense, splice site, deletion variants) have been reported as pathogenic in DCM. The types of genetic variant expected to cause disease depends on the mechanism of disease associated with each DCM gene. As an example, truncating variants found in the \textit{MYBPC3} gene are known to cause disease (Carrier, Mearini, Stathopoulou, & Cuello, 2015). DCM is mostly inherited in an autosomal dominant manner; however, some are inherited in an autosomal recessive or X-linked manner. This disorder also has reduced penetrance (meaning not everyone with a genetic diagnosis of DCM will develop clinical symptoms of DCM) and variable expressivity (meaning that not every person who develop symptoms of DCM will develop the same symptoms or have the same severity of symptoms) (Hershberger & Morales, 2007). Patients with a genetic diagnosis of DCM require increased screening through the use of echocardiogram and electrocardiogram (Hershberger et al., 2009).
Hypertrophic cardiomyopathy is diagnosed in patients who have thickening of the left ventricular wall (IVSd ≥ 1.5cm). Heart failure, abnormal heart rhythm, cardiac arrest, chest pain, and syncope are often the symptoms of HCM (Cirino & Ho, 1993). HCM is typically caused by genetic variants that disrupt the normal function of genes involved in the sarcomere (Yacoub, 2014) and there are currently more than 20 genes that have been found in association with HCM. Similar to DCM, the type of variants that cause abnormal protein function depend on the mechanism of disease associated with each HCM gene. In the MYH7 gene, for example, disease-causing variants are those that result in disruption of the function of the wild type allele in the same cell (dominant-negative effect). HCM is mostly inherited in an autosomal dominant manner, and this disorder also has reduced penetrance and variable expressivity (Cirino & Ho, 1993). Patients with a genetic diagnosis of HCM require increased screening through the use of echocardiogram, Holter monitoring, and electrocardiogram (Hershberger et al., 2009).

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is characterized by loss of muscle cells, ventricular wall thinning, and replacement of muscle cells with fatty tissue. Typically, these findings all occur in the right ventricle, however, left ventricular involvement is possible. The presentation of ARVC include heart failure, abnormal heart rhythm, and sudden cardiac death (McNally, MacLeod, & Dellefave-Castillo, 1993). ARVC is typically caused by genetic variants that disrupt the normal function of genes involved in the desmosome (Yacoub, 2014). There are currently more than 10 genes that have been found in association with ARVC and it is mostly inherited in an autosomal dominant manner, although autosomal recessive inheritance is also possible. Typically,
any type of variant that leads to either loss of protein function or gain of protein function (depending on the gene) causes ARVC (Awad, Calkins, & Judge, 2008). This disorder also has reduced penetrance and variable expressivity (McNally et al., 1993). Patients with a genetic diagnosis of ARVC require increased screening involving echocardiogram, Holter monitoring, electrocardiogram, and magnetic resonance imaging (MRI) (Hershberger et al., 2009).

Channelopathies are disorders where the structure of the heart is normal, but the heart beat becomes irregular due to ion instability. The channelopathies are caused by pathogenic genetic variants in genes that encode the ion channels (Campuzano et al., 2015). Ion channels are gates in the cell membrane responsible for allowing the flow of ions (e.g. Na⁺, K⁺, Cl⁻) across the cell membrane in order to regulate the heartbeat. The channelopathies include long QT syndrome (LQTS), Brugada syndrome (BrS), catecholaminergic polymorphic ventricular tachycardia (CPVT), and short QT syndrome (SQTS) (Campuzano et al., 2015).

Long QT syndrome is diagnosed in people who have a prolongation of their QT interval (QTc >480 ms) on an electrocardiogram. Patients with long QT syndrome usually present with episodes of syncope, cardiac arrest, or sudden cardiac death. These episodes can be triggered by exertion, emotional stimuli, or auditory stimuli. This disorder also has reduced penetrance and variable expressivity. Variants causing loss of protein function, gain of protein function, and dominant-negative effects have all been reported in LQTS patients. The mechanism of disease is gene specific. As an example, variants in the KCNH2 gene that cause loss of protein function contribute to LQTS
whereas variants in the *SCN5A* gene that cause gain of protein function contribute to LQTS. There are approximately 15 genes that have been identified in association with LQTS and it is mostly inherited in an autosomal dominant manner. The three most common genes associated with LQTS are *KCNQ1*, *KCNH2*, and *SCN5A* (Campuzano et al., 2015). Patients with a genetic diagnosis of LQTS require medical interventions such as the avoidance of QT prolonging medications, the use of beta blockers, and in some cases, implantable cardioverter defibrillator ICD placement (Priori et al., 2013).

Brugada syndrome (BrS) is suspected in patients with an elevated ST segment on an electrocardiogram. The presentation of BrS includes episodes of syncope, palpitations, chest discomfort, and sudden cardiac death, and these episodes usually occur during rest or sleep. Various types of variants have been reported in patients with BrS, but the mechanism of disease is gene specific. While gain-of-function variants in the *SCN5A* gene are known to cause LQTS, loss-of-function variants in this gene cause BrS. There are approximately 10 genes that have been identified in association with BrS, and it is mostly inherited in an autosomal dominant manner. The most common gene associated with BrS is *SCN5A* (Campuzano et al., 2015). This disorder also has reduced penetrance and variable expressivity, and patients with BrS require the avoidance of ST elevating medications, avoidance of fevers, and ICD placement (Priori et al., 2013).

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is characterized by severe arrhythmias that occur under adrenergic stimulation. The electrocardiogram is usually normal at rest. Episodes of syncope, seizures, and sudden cardiac death occur in patients with CPVT. These episodes are triggered by adrenaline during times of exercise.
or emotional stress. There is some overlap between the symptom triggers in CPVT and LQTS causing some CPVT patients to be misdiagnosed as LQTS patients. There are approximately 5 genes that have been identified in association with CPVT. The most common gene associated with CPVT is \textit{RYR2} (Campuzano et al., 2015). Mostly variants causing gain of protein function are reported in the CPVT genes. CPVT is inherited in an autosomal dominant or autosomal recessive manner, and it also has reduced penetrance and variable expressivity. It is recommended that patients with a diagnosis of CPVT avoid sports or exercise, use beta blockers, and in some cases, have an ICD placed (Priori et al., 2013).

Short QT syndrome (SQTS) is diagnosed in individuals who have a short QT interval on an electrocardiogram. Patients with SQTS usually have episodes of syncope, palpitations, and sudden cardiac death typically occurring during childhood (Campuzano et al., 2015). Gain-of-function genetic variants in potassium channel genes are known to cause SQTS. These three genes are \textit{KCNQ1}, \textit{KCNH2}, and \textit{KCNJ2}. Loss-of-function variants in these three genes lead to SQTS. SQTS is inherited in an autosomal dominant manner, and patients with a genetic diagnosis of SQTS require ICD placement (Priori et al., 2013).

Familial Hypercholesterolemia (FH), a genetic disorder characterized by high cholesterol levels especially involving the low-density lipoprotein (LDL), results in early-onset coronary artery disease when untreated. FH is caused primarily by pathogenic variants in the low-density lipoprotein receptor (\textit{LDLR}) gene. The LDL receptor monitors the levels of LDL-cholesterol in the bloodstream and binds to excess LDL-
cholesterol triggering its breakdown in the lysosomes. In addition to highly elevated LDL cholesterol level, FH is characterized by xanthomas which can occur in the feet, knees, elbows, and hands. Missense variants, nonsense variants, deletions, frameshifts, and splice site variants have all been reported in association with FH. Three genes have been identified in association with FH: \textit{APOB}, \textit{LDLR}, and \textit{PCSK9}. FH is inherited in an autosomal dominant manner with homozygous or compound heterozygous variants having an additive effect, causing a more severe clinical presentation than heterozygous variants. Patients with FH require the use of statins to lower cholesterol and lifestyle modifications to lower the risk for coronary artery disease (Watts et al., 2015).

\textit{Variant Classification}

Genetic testing is a powerful tool in diagnosing cardiovascular genetic disorders and helping to predict risk in at-risk family members. The downside to this new technology is that our ability to perform large-scale sequencing has outpaced our ability to interpret this information in the clinical setting (Ackerman, 2015). When a variant is identified, clinical genetic test reports contain a list of genetic variants that differ from the reference sequence (i.e. standardized human genome sequence). The reports also contain a clinical classification of the variant such as pathogenic, likely pathogenic, uncertain significance, likely benign, and benign. In May of 2015, the American College of Medical Genetics and Genomics published “Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular
Pathology” in order to help standardize the classification of genetic variants across clinical genetic testing laboratories (Richards et al., 2015).

Pathogenic variants have a negative impact on the protein and cause the function of that protein to change in a way that leads to disease. Variants are classified as pathogenic based on the available evidence demonstrating that the variant causes abnormal or no protein to be produced. For example, in the KCNQ1 gene, the variant c.1781G>A is known to be pathogenic. Therefore, people who have this variant receive a diagnosis of LQTS and will be managed accordingly. The pathogenic classification for this variant is based on different lines of evidence. First, there are multiple functional studies (in vivo or in vitro) that show that when this variant is present in the gene, there is a decrease in the number of ion channels present in the cell membrane. Not having enough ion channels present in the cell membrane causes the abnormal heart rhythm seen in LQTS. Also, this variant is predicted to have a damaging effect by in silico prediction programs such as SIFT and PolyPhen. Another pathogenic variant in the KCNQ1 gene at this same amino acid position has been shown to segregate with LQTS in an affected family. All of this evidence supports a pathogenic classification for this variant. It is important to know there are many different types of evidence that can support a pathogenic classification, and this variant is just one example.

Benign variants do not negatively impact the protein and the protein is able to function normally with the presence of the variant. A benign classification is also based on many different types of evidence showing that a variant does not have a negative impact on a protein. As an example, in the DSG2 gene, the variant c.2318G>A is
classified as benign. The presence of this variant has been reported in approximately 26% of the general population, which is an extremely high allele frequency supporting a benign classification. This variant was also predicted to be benign by in silico prediction programs. This evidence supports a benign classification for this variant, however, there are other types of evidence that can support a benign classification as well.

Sometimes, a variant may have some evidence supporting a pathogenic classification, but not enough evidence to classify the variant as pathogenic. These variants typically receive a classification of “likely pathogenic.” Similarly, a classification of “likely benign” is given to variants that have some evidence supporting a benign classification, but not enough evidence to assign the variant a benign classification. In these situations, the type of evidence needed to classify a variant as likely pathogenic or likely benign is the same type of evidence needed for a pathogenic and benign classification. It is the amount of evidence that determines whether a variant receives a classification of likely pathogenic vs. pathogenic and likely benign vs. benign.

The last category of classification is called a variant of uncertain significance (VUS). Variants of uncertain significance are genetic variants for which enough evidence to determine the classification is not available. When classifying variants, each variant begins as a VUS. The evidence is then used to move the classification towards pathogenic or towards benign. If there is not enough evidence to change the classification from VUS, the variant will remain a VUS. Also, if there is evidence in favor of both a benign and pathogenic classification, then these classifications conflict with each other, and the variant will default to being a VUS. For example, if a variant
has evidence supporting a likely pathogenic classification and evidence supporting a likely benign classification, then the final classification of this variant would be a VUS.

In about 75% of LQTS cases, a variant of any type is detected through clinical genetic testing. Many of these variants are classified as pathogenic or likely pathogenic and are considered the underlying genetic etiology that clinicians hope to identify for diagnostic and predictive purposes. However, sometimes a VUS is detected which pose challenges for clinicians (Ackerman, 2015). Unfortunately, the more genes we analyze, the more variants we detect and the higher chance of a VUS to be found. Many of the variants classified as VUS may have little or no clinical significance, and they may represent "normal genetic background noise" that exists in the healthy population. The important component of genetic variant classification is distinguishing potentially pathogenic or uncertain variants from normal genetic background noise (Giudicessi & Ackerman, 2013). This distinction is important because the results obtained from genetic testing are used to make medical management decisions for the patients as well as their family members.

When a pathogenic variant is identified by clinical genetic testing, that information is used to determine the medical management in a family. For example, if a patient who receives a diagnosis of DCM has a pathogenic variant found by genetic testing consistent with this diagnosis, the family members are offered a site-specific genetic test to determine whether they also carry the same pathogenic variant or not. Individuals who have the pathogenic variant would be at risk for developing DCM, and would be recommended to undergo clinical screening consisting of physical examination,
electrocardiogram, and echocardiogram yearly in childhood and every 1-3 years in adulthood (Hershberger et al., 2009). For family members who do not have the pathogenic variant, screening is not indicated because they would not be at risk to develop the genetic form of DCM observed in the family.

When a pathogenic variant is not identified on a clinical genetic test, the patient and family members are managed based on the family history. As an example, if a patient presented with a clinical diagnosis of DCM and a pathogenic variant was not identified on clinical genetic testing, the genetic cause for DCM in this patient was not identified. In this situation, each of the patient’s first degree relatives (i.e. parent, child, and/or sibling) would be treated as though they are at a 50% risk to develop DCM based on the fact that most genetic forms of DCM are inherited in an autosomal dominant fashion. Therefore, at-risk family members would be recommended to undergo clinical screening consisting of physical exam, electrocardiogram and echocardiogram every 3-5 years beginning in childhood (Hershberger et al., 2009). Without a pathogenic variant being identified, risk stratification of family members is not possible.

Finally, when a VUS is identified on a clinical genetic test, it is unknown whether the variant causes disease, therefore, medical management decisions based on this result are not possible and at-risk first degree relatives are managed accordingly. However, it is possible for the laboratory to continue to gather information about the variant in order to help further clarify the role of a VUS in disease, such as functional information and supporting evidence as they become available in the medical literature, as well as the variants segregation data if the patient’s family members are also tested. Also, additional
genetic testing may be considered to determine whether there is another genetic explanation for the disease observed in the family. Therefore, the diagnostic odyssey continues until further clarification can be made about a VUS.

Providing patients with the information that a genetic variant is pathogenic when it is in fact benign can have many adverse effects for families. Patients typically change their course of medical management with some involving invasive surgical procedures (e.g. placement of an ICD) based on the pathogenic variant result. These increased monitoring and/or procedures may not be if the variant is actually benign and not contributing to the disease despite being reported as pathogenic by a laboratory. Also, family members who test positive for a pathogenic variant are determined to be at an increased risk to develop the disorder, even if asymptomatic. These risks may not be accurate if the variant is benign rather than pathogenic. Finally, family members who test negative for a pathogenic variant would not require a change in medical management. If the reported pathogenic variant is actually benign, family members may be falsely reassured, and screening or management that is needed in the family members may be missed. Also, when variant classifications are changed and patients are contacted, the patients may experience confusion and distrust in medicine (Manrai et al., 2016). Therefore, it is important to be as accurate as possible when classifying genetic variants in order to properly manage patients and in order to not dismiss family members who may also need interventions.

To determine the significance of a variant, laboratories collect and assess the evidence surrounding each variant including but not limited to functional studies,
population frequencies, conservation data, phenotype data, *in silico* analysis, and familial segregation data. Ideally, different clinical genetic testing laboratories would come to the same conclusion regarding variant classification for a particular variant by using the same resources, data, and tools. However, this is not always the case. In a study done by the Collagen Diagnostic Laboratory (CDL) at the University of Washington, upon review of variant classifications performed by other genetic testing laboratories, the CDL was only concordant on their classification of the same genetic variants in 29% of cases and had a significant discrepancy in classification (defined as a difference of two or more steps in clinical significance such as VUS vs. benign) in 42% of cases. These differences in classification were due to a variety of reasons, including the use of CDL-private data not available to outside laboratories or the outside laboratories not referencing publicly available data (Pepin et al., 2016). Another study using data reported in the Prospective Registry of Multiplex Testing (PROMPT) online genetic registry evaluated conflicting classifications of variants in cancer genes excluding *BRCA1* and *BRCA2*. This study reported that 26% of 603 genetic variants had conflicting classifications among genetic testing laboratories. Of even more concern, 36% of these variants had conflicting classifications that would change the medical management patients would receive based on these results. When examining what led to these conflicting classifications, they discovered that the discrepancies resulted in how the evidence was being interpreted and used in assessment rather than what evidence was being considered (Balmana et al., 2016). Conflicting classifications of genetic variants among laboratories poses problems in the clinical setting because clinicians are making decisions on the medical
management the patient and their family members receive based on the variant classification. Therefore, it is possible that family members tested at different laboratories who carry identical genetic variants would receive different diagnoses or be given different medical management recommendations due to a difference in variant classification among the clinical genetic testing laboratories.

Assessing the significance of a genetic variant is a complex and challenging task. Most variation in the human genome, including rare variation, is not likely to contribute substantially to human disease (Amendola et al., 2016). Also, genes in which variation has been associated with a susceptibility to disease such as cardiomyopathy or channelopathy, are often prematurely incorporated into genetic testing panels before there is substantial evidence demonstrating the strength of the association. In such cases, it is even more difficult to determine the clinical significance of a variant in these genes since there is very little evidence available to be used in the classification process (Balmana et al., 2016). As the efforts of genetic variant data sharing improve, more information will likely become available that will aid our ability to determine pathogenic from benign genetic variants. The current challenge in clinical variant classification indicates that there is a critical need for consistent and methodical reclassification of genetic variants (Pugh et al., 2014). The previous version of the American College of Medical Genetics and Genomics (ACMG) guidelines for variant classification did not address how to weigh the evidence for variant classification, so each clinical genetic testing laboratory developed their own methods of variant classification (Richards et al., 2008). It has been a challenge deciding how to categorize and weigh evidence in variant classification and
guidance has been needed. Studies have shown that typically, variant reviewers at the laboratory overestimate pathogenicity (Amendola et al., 2016). Minimizing the number of variants being classified as pathogenic without strong supporting evidence is one issue the updated ACMG guidelines for variant interpretation sought to address (Richards et al., 2015).

The ACMG's revised guidelines are based on evaluating evidence supporting a pathogenic classification or a benign classification of a variant. For a pathogenic classification, these guidelines weigh evidence-based criterion as very strong, strong, moderate, or supporting. For a benign classification, each evidence-based criterion is weighted as stand-alone, strong, or supporting. The criteria are then combined to determine classification of the variant. The evidence needed to determine whether or not each criterion is met can be obtained from literature searches, as well as a variety of databases, some of which are publicly available, and some of which require paid access. Examples of such databases include ClinVar, Human Gene Mutation Database (HGMD), ExAC, and 1000 Genomes. These databases are used by clinical genetic testing laboratories, genetic counselors, and other healthcare providers because they contain useful information for variant classification. As an example, ClinVar contains classifications of each variant as reported by clinical laboratories and supporting evidence for variant classifications. HGMD contains classification of each variant, prediction program results, functional studies, and supporting literature. Finally, databases such as ExAC and 1000 Genomes contain population frequency information. The evidence
found in these tools can be used for variant classification combined with the ACMG guidelines.

A study by Amendola et al. set out to determine how the updated 2015 ACMG guidelines for variant classification compared to individual laboratory methods of classification and to determine the variance in use and interpretation of these classification criteria. This study showed that for 79% of genetic variants classified, there was no difference in classification using the ACMG guidelines compared to using the laboratory’s guidelines for classification. For the variants that did have a difference in classification, the ACMG guidelines were more likely to classify a variant as a VUS where the laboratory’s guidelines classified those variants as likely benign or benign. However, implementation of the ACMG guidelines did not significantly increase laboratory concordance of variant classification. Classifications among clinical laboratories were only concordant for 33 of 99 (34%) variants. After much discussion among laboratories either via email or conference calls regarding these classifications and the evidence used to determine them, concordance was increased to 70 of 99 (71%) genetic variants. Most of the discordance was due to the subjectivity in deciding when certain criteria for classification applied (Amendola et al., 2016). Therefore, this study demonstrated that while the ACMG guidelines were created to assist with standardizing variant classification, there was still much subjectivity in determining how these guidelines were applied.

The goal of this study was to evaluate the impact of the updated ACMG guidelines on the classification of genetic variants detected on clinical cardiogenetic
testing. This study aimed to determine whether applying the updated ACMG guidelines would result in clinically relevant changes in variant classification. It also wanted to determine what other factors, if any factors, play a role in variant reclassification such as whether the gene is “strong evidence” or “limited evidence” and the number of years passed since the gene was discovered. Finally, we propose recommendations regarding reclassification of genetic variants in a clinical cardiovascular genetic setting.
METHODS

This study was reviewed and approved by the Institutional Review Board at The Ohio State University. To identify the patient cohort, a database query was performed for all patients seen by Amy Sturm, MS, LGC, a genetic counselor specializing in the adult cardiovascular disorders at The Ohio State University Wexner Medical Center, for genetic counseling between January 1, 2004 and December 31, 2015. Only patients who had previously undergone clinical genetic testing or who underwent clinical genetic testing after receiving genetic counseling to test for the presence of a variant(s) in genes associated with cardiovascular disease (channelopathy, cardiomyopathy, familial hypercholesterolemia) were used for this retrospective chart review.

For each patient, the electronic medical record (EMR) or paper chart was reviewed for the laboratory report, pedigree, and phenotype information. From each laboratory report the following information was recorded: the name of the clinical genetic testing laboratory that performed the genetic testing, variant(s) identified in the gene the gene including the nucleotide and protein nomenclature, gene transcript the laboratory used for each gene on the report, and classification of the variant as reported by the laboratory. From each pedigree, the following was recorded: segregation of the variant in the family (relatives who underwent testing, whether they tested positive or negative, whether they were clinically affected or unaffected), maternal ancestry.
information, and paternal ancestry information. The problem lists, progress notes, and letters were reviewed to determine each patient’s clinical phenotype.

First, each variant was categorized by type of variant, such as missense, nonsense, frameshift, splice site, deletion, duplication, insertion, synonymous, etc. Multiple sources were then used to identify supporting evidence for each variant classification such as ClinVar, Human Genome Mutation Database (HGMD), ExAC, 1000 Genomes Project, and the primary literature.

For each genetic variant, we first used Mutalyzer along with the reference gene transcript provided by the reporting laboratory (if applicable) to convert the variant into genomic coordinates as well as to identify the variant nomenclature for other gene transcripts. If the reference gene transcript was not available on the original laboratory report, we used the most common gene transcript as identified in ClinVar and HGMD. This information helped us ensure that we were searching for the correct variant in all of our different sources. Next we searched for the variant in ClinVar. If the variant was present in ClinVar, we recorded the following information: clinical significance (a combination of all submitters’ classifications), star level of evidence, submitters’ names, submitters’ classifications (the classification as determined by each laboratory who submits to ClinVar), dbSNP ID, relevant literature, and other reported pathogenic missense variants at the same amino acid position. We noted if the variant was not found in ClinVar as well. For variants not in ClinVar, we searched dbSNP (a publicly accessible database of reported single nucleotide polymorphism, or SNPs) for the dbSNP ID (the unique ID number assigned to each SNP in the dbSNP database). Next, we
searched Uniprot which is a publicly accessible database containing protein and functional information for each gene. For all variants, we recorded the amino acid location in the protein (e.g. N-terminus, C-terminus, Actin-binding domain) as well as other pathogenic variants reported at the same amino acid position. For variants that were reported in Uniprot, we recorded molecular differences between the amino acids.

For each variant, we used four different sources to determine allele frequency in the general population. First, we searched for each variant in 1000 Genomes either through ClinVar or through Ensembl (for variants that were not present in ClinVar). If the variant was present in 1000 Genomes, the frequency was recorded. Next, we used genomic coordinates or dbSNP ID to search for each variant in the Exome Variant Server (EVS). If the variant was present in EVS, the frequency was recorded. Then, we used genomic coordinates to search for each variant in ExAC. While EVS variants are also present in ExAC, read coverage was generally better in EVS than ExAC for some variants that were “not present” in ExAC. If the variant was present in ExAC, frequency was recorded. If a variant that was present in ExAC had poor read coverage, this information was also noted. If the variant was not present, the read coverage was recorded for reference. Constraint data (a measure of the gene’s intolerance for variation) for missense variants in each gene was also recorded from ExAC. Finally, genomic coordinates were used to search for each variant in gnomAD (a publicly accessible database containing both exome and genome sequencing data collected from a variety of sequencing projects). If the variant was present in gnomAD, the frequency was recorded. If the variant was not present, coverage data was recorded for reference.
Each variant was then searched for in the Human Genome Mutation Database (HGMD). If the variant was present in HGMD, the following information was collected: HGMD classification, relevant literature, functional studies, SIFT and PolyPhen predictions, conservation information, other reported pathogenic variants at this same amino acid position, most common type(s) of disease-causing variants identified in the gene. Screenshots of the HGMD information were taken for reference. If the variant was not present in HGMD, in silico information was taken from ExAC, if available. If the variant was not present in ExAC, in silico information was taken from gnomAD, if applicable.

Online Mendelian Inheritance in Man (OMIM) was used to identify and record the following information for each gene in this study: reported phenotypes, year of discovery, mechanism for disease if known, and important functional domains/hotspots if known.

PubMed was then searched for any additional necessary information for variant classification. We searched PubMed for functional studies relevant to each variant first using the one letter amino acid nomenclature (e.g. A566L), then using the three-letter amino acid nomenclature (e.g. Ala566Leu). We also searched for gene-specific literature to identify the mechanism for disease, any important functional domains/hotspots, and spectrum of pathogenic variants. We used the following search methods for these searches: search by gene symbol (e.g. ABCC9) and filter for review articles only, search by gene symbol and “domain” (e.g. ABCC9 AND domain), and search by gene symbol and “function OR functional” (e.g. ABCC9 AND [function OR functional]). Each gene
was then classified as “strong evidence” or “limited evidence.” This classification was initially determined using the gene list provided by a clinical genetic testing laboratory and then confirmed based on the amount of information that is known about each gene reported in the primary literature. As an example, the AKAP9 gene was reported in the “limited evidence” category by a clinical genetic testing laboratory. After review of the primary literature, this gene has only been shown to have a possible association with Long QT Syndrome. This information confirms the “limited evidence” classification for this gene.

Upon completion of evidence collection, each variant was reclassified according to the ACMG Standards and Guidelines for Sequence Variant Interpretation (Richards, 2015). The following tables show these criteria for variant classification as well as our application of these criteria in this study.

<table>
<thead>
<tr>
<th>ACMG guideline criteria for pathogenic classification</th>
<th>ACMG guideline for application</th>
<th>Application of criterion in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Criterion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVS1</td>
<td>Null variant (nonsense, frameshift canonical +/- 1 or 2 splice sites initiation codon, singe or multi-exon deletion) in a gene where loss of function (LOF) is a known mechanism of disease</td>
<td>Applied as stated</td>
</tr>
<tr>
<td>PS1</td>
<td>Same amino acid change as a previously established pathogenic variant regardless of nucleotide change</td>
<td>Applied as stated</td>
</tr>
<tr>
<td>PS2</td>
<td>De novo (both maternity and paternity confirmed) in a patient with the disease and no family history</td>
<td>Applied as stated</td>
</tr>
</tbody>
</table>

Table 1: ACMG guideline criteria for pathogenic classification
| PS3 | Well-established *in vitro or in vivo* functional studies supportive of a damaging effect on the gene or gene product | If there were one or more functional studies demonstrating a pathogenic effect for the variant, this criterion was applied |
| PS4 | The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls (RR or OR as obtained from case-control studies is >5.0 and the confidence interval around the estimate of RR or OR does not include 1.0) | Applied as stated |
| PM1 | Located in a mutational hot spot and/or critical and well-established functional domain without benign variation | If the variant was located in a hotspot or important functional domain as identified in the primary literature, this criterion was applied |
| PM2 | Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes, or ExAC | If the variant was not found to be present in 1000 Genomes, EVS, ExAC, and gnomAD, this criterion was applied |
| PM3 | For recessive disorders, detected in *trans* with a pathogenic variant | Applied as stated |
| PM4 | Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants | Applied as stated |
| PM5 | Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before | Applied as stated |
| PM6 | Assumed *de novo*, but without confirmation of paternity and maternity | Applied as stated |
| Extra PM criterion | For very rare variants where case-control studies may not reach statistical significance the prior observation of the variant in multiple unrelated patients with the same phenotype, and its absence in controls, may be used as moderate level of evidence | If the variant was seen in three or more unrelated individuals with the same phenotype, the variant was identified in a gene associated with the phenotype, and the variant was absent in controls, an extra moderate criterion was applied to replace criterion PS4 |
Table 1 continued

<table>
<thead>
<tr>
<th>PP1</th>
<th>Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease</th>
<th>Applied as stated</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP2</td>
<td>Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease</td>
<td>If missense variants were known to be a common mechanism of disease in the gene, and the ExAC constraint data showed that the tolerance for missense variant in the gene had a standard deviation greater than 2 ((Z&gt;2)), this criterion was applied</td>
</tr>
<tr>
<td>PP3</td>
<td>Multiple lines of computational evidence support a deleterious effect on the gene or gene product</td>
<td>If all \textit{in silico} predictions were in favor of pathogenicity, this criterion was applied</td>
</tr>
<tr>
<td>PP4</td>
<td>Patient’s phenotype or family history is highly specific for a disease with a single genetic etiology</td>
<td>This criterion did not apply to the cardiovascular diseases in this study because they are all heterogeneous disorders</td>
</tr>
<tr>
<td>PP5</td>
<td>Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation</td>
<td>We chose not to use this criterion during our classification of these variants because we are doing our own assessment of the relevant evidence</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Criterion</th>
<th>ACMG guideline for application</th>
<th>Application of criterion in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA1</td>
<td>Allele frequency is above 5% in Exome Sequencing Project, 1000 Genomes, or ExAC</td>
<td>If the allele frequency in 1000 Genomes, EVS, ExAC, or gnomAD was found to be higher than 5%, this criterion was applied</td>
</tr>
<tr>
<td>BS1</td>
<td>Allele frequency is greater than expected for disorder</td>
<td>If the allele frequency in 1000 Genomes, EVS, ExAC, or gnomAD was found to be higher than 0.1%, this criterion was applied</td>
</tr>
</tbody>
</table>

Table 2: ACMG guideline criteria for benign classification continued
Table 2 continued

<table>
<thead>
<tr>
<th>BS2</th>
<th>Observed in a healthy adult individual for a recessive, dominant, or X-linked disorder with full penetrance expected at an early age</th>
<th>This criterion did not apply to the cardiovascular diseases in this study because they were all variable expressivity or incomplete penetrance</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS3</td>
<td>Well-established <em>in vitro</em> or <em>in vitro</em> functional studies shows no damaging effect on protein function or splicing</td>
<td>If there were one or more functional studies showing that the variant had no effect on the protein, this criterion was applied</td>
</tr>
<tr>
<td>BS4</td>
<td>Lack of segregation in affected members of a family</td>
<td>Applied as stated</td>
</tr>
<tr>
<td>BP1</td>
<td>Missense variant in a gene for which primarily truncating variants are known to cause disease</td>
<td>Applied as stated</td>
</tr>
<tr>
<td>BP2</td>
<td>Observed in <em>trans</em> with a pathogenic variant for a fully penetrant dominant gene/disorder, or observed in <em>cis</em> with a pathogenic variant in any inheritance pattern</td>
<td>Applied as stated</td>
</tr>
<tr>
<td>BP3</td>
<td>In-frame deletions/insertions in a repetitive region without a known function</td>
<td>Applied as stated</td>
</tr>
<tr>
<td>BP4</td>
<td>Multiple lines of computational evidence suggest no impact on gene or gene product</td>
<td>If all <em>in silico</em> predictions were in favor of benign, this criterion was applied</td>
</tr>
<tr>
<td>BP5</td>
<td>Variant found in a case with an alternate molecular basis for disease</td>
<td>Applied as stated</td>
</tr>
<tr>
<td>BP6</td>
<td>Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation</td>
<td>We chose not to use this criterion during our classification of these variants because we are doing our own assessment of the relevant evidence</td>
</tr>
<tr>
<td>BP7</td>
<td>A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved</td>
<td>Applied as stated</td>
</tr>
</tbody>
</table>
There were some modifications made to the guidelines as listed in Tables 1 and 2 to establish consistency in this study. First, a functional study that demonstrates a pathogenic effect was defined as a functional study that demonstrated the variant resulted in any type of function that was different from the function of the wild type protein. This definition was used frequently in the primary literature evaluating variant classifications. Oppositely, a functional study demonstrating no effect on the protein was defined as a functional study that demonstrated the variant resulted in function similar to the wild type protein. *In silico* prediction models were in favor of pathogenic if SIFT predicted a “damaging” or “deleterious” effect and PolyPhen predicted a “probably damaging” or “possibly damaging” effect. The models were in favor of benign if SIFT predicted the variant to be “tolerated” and PolyPhen predicted the variant to be “benign.” For the extra PM criterion listed in Table 1, multiple unrelated individuals was defined as 3 or more unrelated individuals by committee consensus. The method for evaluating tolerance of missense variation using ExAC constraint data was informed by a member of the ClinGen Cardiovascular Clinical Domain Working Group who is a variant scientist and clinical molecular genetics fellow. A standard deviation of 2 for this method was determined by committee consensus because above this level of standard deviation is typically considered statistically significant. There currently are not well established disease incidences for the cardiovascular phenotypes for use of criterion BS1 (allele frequency is greater than expected for disorder). Because pathogenic allele frequency is determined by available normal population databases with >1000 alleles, in the event there is no available frequency for extremely rare dominant disorders (incidence
≤1:1000), then a frequency of 0.1% can be utilized. Therefore, this frequency was used in this study as recommended by the experts at Nationwide Children’s Hospital ChildLab who use the ACMG guidelines for their variant classification.

The methods for variant reclassification in this study and the modifications made to the guidelines as stated above were reviewed by a committee and established by committee consensus. Prior to evaluating the 223 variants identified in this study, a smaller set of variants was used to create and review the methodology. All of the variants identified in the KCNQ1 gene (n=14) were used for this initial test set. A single interpreter performed the evidence collection and reclassification for each of variants in the initial KCNQ1 set. Following reclassification, the 14 variants were reviewed by a committee. Discrepancies in interpretation of the evidence and guidelines were discussed and consensus was reached. The agreed upon methods were then applied in the remaining variants identified. All potential discrepancies that arose during the reclassification of the remaining variants were again reviewed by the committee until consensus was reached.

To determine the reclassification for each genetic variant, the pathogenicity classification and benign classification were both evaluated. If these two classifications were conflicting (e.g. Likely pathogenic and Likely benign), a classification of VUS was used. For each variant that received an updated classification, we determined the number of steps by which the classification changed: one step (e.g. VUS→Likely benign), two steps (e.g. VUS→Pathogenic), three steps (e.g. Likely pathogenic→Benign), or four steps (e.g. Pathogenic→Benign). We also determined whether or not the reclassification
would cause a change in the medical management of the patient. For all variants that had a reclassification, the original test reports were referenced to determine the evidence used by the clinical genetic testing laboratory to classify the variant on the original report. We assessed whether changes in classification were due to changes in evidence or changes in how the evidence is weighted. Descriptive statistics were used to evaluate the data collected in this study.
RESULTS

A total of 223 unique variants were identified on the test reports of 237 patients. These patients came from 164 different families (Supplemental Table 1). As shown in Table 3 below, 32 of the variants were not present in ClinVar. Of the 223 variants identified, 80 (36%) received a different classification than the one the laboratory reported on the original clinical genetic test report. Forty of the reclassified variants (50%) were originally classified at the same genetic testing laboratory. For the 80 variants that received a different classification, 60 of the variants (75%) were reclassified by one step of clinical significance. Of these 60 variants, 41 variants (68%) were downgraded in clinical significance and 19 of the variants (32%) were upgraded in clinical significance. Nineteen of the 80 variants that received a different classification (24%) were reclassified by two steps of clinical significance. Of these 19 variants, 15 variants (79%) were downgraded in clinical significance and 4 of the variants (21%) were upgraded in clinical significance. One of the 80 reclassified variants (1%) was reclassified by three steps of clinical significance and this reclassification was downgraded in clinical significance. None of the variants were reclassified by four steps of clinical significance. A detailed breakdown of the number of variants that underwent each type of classification in the above categories is available in Table 4.
<table>
<thead>
<tr>
<th>Gene Transcript</th>
<th>Gene</th>
<th>Variant</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_000384</td>
<td>APOB</td>
<td>c.10508C&gt;T</td>
<td>p.Ser3503Leu</td>
</tr>
<tr>
<td>NM_000719.6*</td>
<td>CACNA1C</td>
<td>c.1174G&gt;A</td>
<td>p.Gly392Arg</td>
</tr>
<tr>
<td>NM_000719.6*</td>
<td>CACNA1C</td>
<td>c.5603T&gt;C</td>
<td>p.Leu1868Pro</td>
</tr>
<tr>
<td>NM_000719.6*</td>
<td>CACNA1C</td>
<td>c.5605G&gt;A</td>
<td>p.Val1869Met</td>
</tr>
<tr>
<td>NM_201590</td>
<td>CACNB2</td>
<td>c.253G&gt;T</td>
<td>p.Ala855Ser</td>
</tr>
<tr>
<td>NM_003476.3</td>
<td>CSRP3</td>
<td>c.364C&gt;T</td>
<td>p.Arg122Ter</td>
</tr>
<tr>
<td>NM_004006</td>
<td>DMD</td>
<td>c.2884C&gt;G</td>
<td>p.Leu962Val</td>
</tr>
<tr>
<td>NM_001943.3*</td>
<td>DSG2</td>
<td>c.1439C&gt;T</td>
<td>p.Thr480Ile</td>
</tr>
<tr>
<td>NM_004415.2</td>
<td>DSP</td>
<td>c.2702A&gt;G</td>
<td>p.Lys901Arg</td>
</tr>
<tr>
<td>NM_000238.2</td>
<td>KCNH2</td>
<td>c.1277C&gt;T</td>
<td>p.Leu1664Arg</td>
</tr>
<tr>
<td>NM_000238.2</td>
<td>KCNH2</td>
<td>c.1859G&gt;A</td>
<td>p.Pro426Leu</td>
</tr>
<tr>
<td>NM_000238.3</td>
<td>KCNH2</td>
<td>c.2145+1G&gt;A, c.IVS8+1G&gt;A</td>
<td>p.Ser620Asn</td>
</tr>
<tr>
<td>NM_00218.2*</td>
<td>KCNQ1</td>
<td>c.107DelT</td>
<td>p.Ser37fs</td>
</tr>
<tr>
<td>NM_00218.2*</td>
<td>KCNQ1</td>
<td>c.108InsT</td>
<td>p.Phe36fs+247X</td>
</tr>
<tr>
<td>NM_020433</td>
<td>JPH2</td>
<td>c.1990G&gt;A</td>
<td>p.Ala664Thr</td>
</tr>
<tr>
<td>NM_000117.2</td>
<td>EMD</td>
<td>c.106A&gt;C</td>
<td>p.Lys36Gln</td>
</tr>
<tr>
<td>NM_001079802</td>
<td>FKTN</td>
<td>c.625C&gt;T</td>
<td>p.Arg209Cys</td>
</tr>
<tr>
<td>NM_007078.2</td>
<td>LDB3</td>
<td>c.1253C&gt;G</td>
<td>p.Pro418Arg</td>
</tr>
<tr>
<td>NM_007078</td>
<td>LDB3</td>
<td>c.1288A&gt;G</td>
<td>p.Leu1664Arg</td>
</tr>
<tr>
<td>NM_000527</td>
<td>LDLR</td>
<td>c.269A&gt;C</td>
<td>p.Pro418Arg</td>
</tr>
<tr>
<td>NC_012920.1</td>
<td>MT-TK</td>
<td>m.13153A&gt;G</td>
<td>p.Ile273Val</td>
</tr>
<tr>
<td>NM_000257.2</td>
<td>MYH7</td>
<td>c.225G&gt;A</td>
<td>p.Gln75Gln</td>
</tr>
<tr>
<td>NM_004572.3</td>
<td>PKP2</td>
<td>c.1378+2T&gt;A</td>
<td>p.Glu8299A</td>
</tr>
<tr>
<td>NM_001035.2</td>
<td>RYR2</td>
<td>c.5557G&gt;A</td>
<td>p.Glu1853Lys</td>
</tr>
<tr>
<td>NM_001035.2*</td>
<td>RYR2</td>
<td>c.6430C&gt;T</td>
<td>p.Arg2144Cys</td>
</tr>
<tr>
<td>NM_001035</td>
<td>RYR2</td>
<td>c.EX30_32del</td>
<td></td>
</tr>
<tr>
<td>NM_198056.2</td>
<td>SCN5A</td>
<td>c.2398C&gt;T</td>
<td>p.Arg800Cys</td>
</tr>
<tr>
<td>NM_001001430.2</td>
<td>TNNT2</td>
<td>c.821+5G&gt;A, c.IVS15+5G&gt;A</td>
<td>p.R800C</td>
</tr>
<tr>
<td>NM_003319.4</td>
<td>TTN</td>
<td>c.EX120_122del</td>
<td></td>
</tr>
</tbody>
</table>

*Gene transcript was not available on the original genetic test report

Table 3: Variants not present in ClinVar
Variants reclassified by 1 step of clinical significance

<table>
<thead>
<tr>
<th>Reclassification</th>
<th>Number of variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogenic → Likely pathogenic</td>
<td>16</td>
</tr>
<tr>
<td>VUS → Likely benign</td>
<td>16</td>
</tr>
<tr>
<td>Likely pathogenic → VUS</td>
<td>7</td>
</tr>
<tr>
<td>Likely pathogenic → Pathogenic</td>
<td>6</td>
</tr>
<tr>
<td>VUS → Likely pathogenic</td>
<td>6</td>
</tr>
<tr>
<td>Benign → Likely benign</td>
<td>4</td>
</tr>
<tr>
<td>Likely benign → VUS</td>
<td>3</td>
</tr>
<tr>
<td>Likely benign → Benign</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4: Breakdown of reclassified variants

Of the 80 variants that were reclassified, 27 would result in a change in medical management for the patient’s at-risk family members. These 27 variants resulting in a change in medical management are 34% of the total number of variants that were reclassified (n=80) and 12% of the total variants evaluated in this study (n=223). For 20 of these variants, the reclassification involved the downgrading of a previously classified
pathogenic or likely pathogenic variant. Therefore, the reclassification would indicate that these variants may not have been the genetic risk factor for development of disease in these families. At-risk family members who tested negative for these pathogenic or likely pathogenic variants would not currently be undergoing clinical screening for cardiovascular diseases. These family members may actually be at risk to develop cardiovascular disease and require clinical screening as a result of the reclassification. At-risk family members who tested positive for these pathogenic/likely pathogenic variants would be undergoing clinical screening at frequent intervals to monitor for the development of disease. These family members may be able to undergo less frequent screening as a result of the reclassification. The patient may also want to consider additional genetic testing if applicable to identify a genetic etiology for their disease. For the remaining 7 variants, the reclassification involved upgrading a VUS to a likely pathogenic or pathogenic variant. As a result of these reclassifications, at-risk family members may now be able to have single-site genetic testing to determine whether or not they are at risk to develop cardiovascular disease and require clinical screening. Therefore, family members may be able to be discharged from clinical screening with a negative genetic test result.

These 80 variants with reclassifications were then evaluated by patient. Of the 237 patients in this research cohort, 101 patients (43%) had at least one variant that received a different classification. These reclassifications would result in a change in the clinical management for the at-risk family members of 39 patients. These 39 patients are 16% of our total patient population (n=237) and 39% of our patients who had a variant
reclassified (n=101). As showing in Table 5, of these 39 patients, most have DCM (n=15) and LQTS (n=11). Table 6 shows the breakdown of which genes had variants reclassified in each of the below disorders.

<table>
<thead>
<tr>
<th>Cardiovascular disorder</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCM</td>
<td>14</td>
</tr>
<tr>
<td>LQTS</td>
<td>11</td>
</tr>
<tr>
<td>HCM</td>
<td>8</td>
</tr>
<tr>
<td>ARVC</td>
<td>3</td>
</tr>
<tr>
<td>FH</td>
<td>1</td>
</tr>
<tr>
<td>Sinus node disease</td>
<td>1</td>
</tr>
</tbody>
</table>

Legend:
- **DCM** – Dilated Cardiomyopathy
- **LQTS** – Long QT Syndrome
- **HCM** – Hypertrophic Cardiomyopathy
- **ARVC** – Arrhythmogenic Right Ventricular Cardiomyopathy
- **FH** – Familial Hypercholesterolemia

Table 5: Distribution of CVD among 39 patients who had a clinically significant varaint reclassification

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCM</td>
<td>LMNA</td>
</tr>
<tr>
<td></td>
<td>MYBPC3</td>
</tr>
<tr>
<td></td>
<td>MYH7</td>
</tr>
<tr>
<td></td>
<td>RBM20</td>
</tr>
<tr>
<td></td>
<td>TNN13</td>
</tr>
<tr>
<td></td>
<td>TTN</td>
</tr>
<tr>
<td>LQTS</td>
<td>KCNH2</td>
</tr>
<tr>
<td></td>
<td>KCNQ1</td>
</tr>
<tr>
<td></td>
<td>SCN5A</td>
</tr>
</tbody>
</table>

Table 6: Breakdown of genes with variants reclassified
Of the 223 variants identified in the clinical genetic test reports analyzed in this study, 18 variants (8%) were identified in both patients of European ethnicity and patients of non-European ethnicities. Of these 18 variants, 7 variants (39%) were reclassified.

One hundred nineteen variants (53%) came from patients who reported European ethnicity. Of these 119 variants, 44 variants (37%) were reclassified. There were 25 variants (11%) identified in patients who reported non-European ethnicities. Of these 25 variants, 7 variants (28%) were reclassified. For 61 of the variants (27%), the ethnic background of the patients was unavailable. Of these 61 variants, 22 variants (36%) were reclassified. There was no evidence of a difference in the proportion of variants reclassified in patients of European and non-European ethnic background (p=0.85).

Of the 223 variants, 197 variants (88%) were identified in “strong evidence” genes and 26 variants (12%) were identified in “limited evidence” genes. Of the 197 variants identified in “strong evidence” genes, 74 variants (38%) were reclassified. Of the 26 variants identified in “limited evidence” genes, 6 variants (23%) were reclassified. The 74 reclassified variants from “strong evidence” genes represented 93% of the 80 total
reclassified variants. The six reclassified variants from “limited evidence” genes represented the remaining 7%. There was no evidence of a difference in the proportion of variants reclassified between “strong evidence” genes and “weak evidence” genes (p=0.15).

The variants identified in this study came from 58 unique genes. Of these 58 genes, 43 genes (74%) were “strong evidence” genes and 15 genes (26%) were “limited evidence genes.” Twenty-nine (50%) of the genes had only one variant identified in this study population. The mean number of variants per gene was 3.8 with a standard deviation of 5. The average number of years since discovery of the gene was 23.5 with a standard deviation of 6. On average, the number of variants in each gene that were reclassified was 0.275 of the total number of variants in the gene. Twenty-nine (50%) of the genes had at least one variant reclassified. The rate at which the variants in a gene were reclassified was not statistically different based on the years since discovery of the gene. The rate of reclassification for a gene that was discovered 10 or more years ago was 0.96 times that of a gene discovered less than 10 years ago (95% CI:0.8 to 1.14, p=0.66).

The original test reports were also reviewed to determine what led to the difference in classification between this study and the reporting laboratory. For 26 of the variants, the reclassification was due to a difference in evidence being used. An example of a difference in the evidence used is updated population data that is currently available or the ACMG guidelines not accounting for the type of data that the laboratory referenced. As an example, information regarding the amino acid change and
conservation is only accounted for in the ACMG guidelines if an *in silico* model has been used. If *in silico* predictions have not been done, this information cannot be used to apply criteria PP2 (multiple lines of computational evidence support a deleterious effect on the gene or gene product) or BP4 (multiple lines of computational evidence suggest no impact on gene or gene product). For 21 of the variants, the reclassification was due to how the ACMG guidelines weighted the evidence that was reported by the genetic testing laboratory (e.g. splice site variants predicted to cause abnormal protein splicing). Seventeen of the variants were reclassified due to a combination of the evidence being used and the weight the ACMG guidelines assign to each type of evidence. For 16 of the variants, the reason for reclassification could not be determined due to a lack of evidence referenced on the original genetic testing report.

On average, the amount of time spent researching and reclassifying each variant was 1.75 hours (105 minutes) with a range of 30 minutes to 4 hours (240 minutes). The amount of time spent was dependent upon the amount of information that existed in the databases and published literature for each variant and gene with those that were extensively studied taking the longest amount of time.

Multiple lines of computational evidence (*in silico* predictions) were unable to be obtained for many of the variants in this study in order to thoroughly assess for criteria PP3 (multiple lines of computational evidence support a deleterious effect on the gene or gene product) and BP4 (multiple lines of computational evidence suggest no impact on gene or gene product). Only 83 of the 223 variants (37%) had predictions for both SIFT and PolyPhen available in the databases we used for evidence collection. Forty-five of
the variants (20%) only had a prediction for PolyPhen available and one variant (1%) only had a prediction for SIFT available. For 94 of the variants (42%) there were no *in silico* predictions available for SIFT or PolyPhen in the databases we used to obtain this evidence. There were also no predictions available from splicing prediction programs for the evaluation of synonymous variants present in this dataset, which may have impacted the interpretation of these variants due to the inability to assess for criterion BP7 (a synonymous variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved).

Multiple criteria from the ACMG Standards and Guidelines for Sequence Variant Interpretation (Richards, 2015) were not used in the variant reclassifications (Table 7).
### Criteria not used during variant reclassification

<table>
<thead>
<tr>
<th>Criterion</th>
<th>ACMG guideline for application</th>
<th>Reason for not using</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP4</td>
<td>Patient’s phenotype or family history is highly specific for a disease with a single genetic etiology</td>
<td>Not applicable in this study because the cardiovascular diseases in this study are all heterogeneous disorders that display both locus and allelic heterogeneity</td>
</tr>
<tr>
<td>PP5</td>
<td>Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation</td>
<td>Not applicable in this study because only the published medical literature, publicly available data, and evidence referenced on the genetic test reports was used for variant classification in this study. Classifications without supporting evidence available were not taken into consideration.</td>
</tr>
<tr>
<td>BS2</td>
<td>Observed in a healthy adult individual for a recessive, dominant, or X-linked disorder with full penetrance expected at an early age</td>
<td>Not applicable due to the cardiovascular diseases having variable expressivity and incomplete penetrance</td>
</tr>
<tr>
<td>BP6</td>
<td>Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation</td>
<td>Not applied in this study for the same reason that criterion PP5 was not applied</td>
</tr>
</tbody>
</table>

Table 7: Criteria not used during variant reclassification
DISCUSSION

In 2015, the American College of Medical Genetics and Genomics (ACMG) published the “Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology” in order to establish consistency in variant classification among clinical genetic testing laboratories. The results of this study demonstrate how the use of these ACMG guidelines can lead to changes in variant classification. They also show how different interpretations of the ACMG guidelines may lead to discordant variant classifications among interpreters. These results also demonstrate that frequent and consistent review of new evidence is important for reclassification of existing variants. The implementation of the ACMG guidelines for variant classification has only begun to address the current issues in the field of variant classification for clinical genetic testing. There are still many improvements that need to be made in order to establish consistency in assessment of evidence during the variant classification process.

The most significant reclassification in this study was for the missense variant c.2759G>A (p.Arg920Gln) in the KCNH2 gene, which demonstrates how implementation of the guidelines and updates in evidence have impacted variant reclassification. This variant was reclassified from disease causing to likely benign by this study, which is a 3-step difference in clinical significance. Originally, the clinical
genetic testing laboratory reported this variant as disease causing in 2010 based on the following evidence:

- This variant has been previously published in one patient with LQTS (Kapplinger et.al., 2009)
- Not detected in the general population
- Located in the C-terminal region of the protein
- Results in a semi-conservative amino acid change
- The amino acid position is conserved through species
- A different variant, Arg920Trp, at the same amino acid position has been reported as pathogenic
- Pathogenic variants in nearby codons support functional importance of this region of the protein

If the ACMG guidelines are used to evaluate the above evidence referenced by the clinical laboratory on the genetic test report, the classification would be VUS rather than disease causing. The only criterion that would be applicable is criterion PM2 (absent from controls in Exome Sequencing Project, 1000 Genomes, or ExAC). Interestingly, this variant has also been reported as pathogenic in HGMD and ClinVar. Both HGMD and the research institute who submitted the pathogenic classification to ClinVar use the same paper referenced by the genetic testing laboratory above identifying this variant in one patient with LQTS as support for this classification.

When the evidence was re-evaluated by this study, the reclassification for this variant was likely benign. This variant is reported in the general population (1/44266 European
alleles, 1/21236 South Asian alleles, and 4/24954 Latino alleles). Therefore, this variant is not absent in the general population and criterion PM2 (absent from controls in Exome Sequencing Project, 1000 Genomes, or ExAC) was not applied. The Arg920Trp variant at the same amino acid position as this variant has been reported as a disease causing mutation by HGMD which meets the guidelines for criterion PM5 (novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before). This variant is a missense variant in a gene that was determined to have a low rate of benign missense variation using the ExAC constraint data for missense variation in the KCNH2 gene ($Z=4.8$), and where missense variants are a common mechanism of disease according to HGMD. These two pieces of evidence meet criterion PP2 (missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease). Both SIFT and PolyPhen predict this variant to have no impact on the protein which meets criterion BP4 (multiple lines of computational evidence suggest no impact on gene or gene product). Finally, this variant was identified in a patient in this study who also had a likely pathogenic variant in the KCNH2 gene which is an alternate molecular explanation for the disease in the patient. Therefore, criterion BP5 (variant found in a case with an alternate molecular basis for disease) was applied. The combination of the pathogenic criteria result in a VUS classification and the combination of the benign criteria result in a likely benign classification. Therefore, this study reclassified this variant as likely benign.
In addition to this evidence, the Arg920Trp variant at the same amino acid position has been reported to ClinVar by a clinical genetic testing laboratory as a VUS, which further decreases the possibility for the Arg920Gln variant to be pathogenic. The c.2759G>A (p.Arg920Gln) variant in the KCNH2 gene shows how the ACMG guidelines require more evidence to support a pathogenic classification than what clinical laboratories may have used in the past. This variant also demonstrates the impact of updated evidence in variant reclassification.

**Variant reclassification due to updated evidence**

The c.40393_40397dupAGCTC (p.Arg13467AlafsX71) variant in the TTN gene demonstrates how new evidence can change the classification of a variant. This variant was originally reported in 2014 as a VUS by the genetic testing laboratory. The evidence the laboratory used for this classification was the following:

- This variant has not been previously reported in a patient
- Variant causes a shift in the reading frame and a premature stop codon
- Likely results in a truncated protein
- Not observed in ESP (Exome Sequencing Project)
- Herman et al., 2012 reported that TTN truncating variants are present in 3% of control alleles and most truncating variants associated with DCM are located in the A-band region of the protein
- This variant is not located in the A-band variant of the protein
When the ACMG guidelines are applied to the above evidence referenced by the clinical laboratory, the resulting classification is a VUS. According to the above list of evidence used by the laboratory, this variant was not observed in the general population which would allow for the application of criterion PM2 (absent from controls in Exome Sequencing Project, 1000 Genomes, or ExAC). However, using the Herman et al. paper, the clinical laboratory predicts that this truncating variant will not have a negative impact on the protein. Therefore, criterion PVS1 (null variant in a gene where LOF is a known mechanism of disease) would not be applied.

In this study, this variant was reclassified as likely pathogenic based on the following evidence. This variant was not seen in 1000 Genomes, EVS, ExAC, or gnomAD. Therefore, criterion PM2 (absent from controls in Exome Sequencing Project, 1000 Genomes, or ExAC) still applies. The variant results in a shift in reading frame, likely leading to a truncated protein. While Herman et al. states that TTN truncating variants causing DCM are mostly present in the A-band region, there are reports of truncating variants in other regions of the TTN gene leading to cardiovascular disease according to the following website: cardiodb.org/titin/index.php. In 2015, one year after this variant was originally reported by the clinical laboratory, Roberts et al. published a paper which discussed that the likelihood for a truncating variant in TTN to be pathogenic is 93% when the variant is located in a highly expressed exon. This literature would not have been available to the lab when they were classifying this variant because it had not yet been published. Per this website - cardiodb.org/titin/index.php – the position of this variant is in a highly expressed exon which increases its probability of being a pathogenic
truncating variant. Therefore, criterion PVS1 (null variant in a gene where LOF is a known mechanism of disease) was applied during the reclassification of this variant in this study. The combination of criteria PVS1 and PM2 result in a reclassification of likely pathogenic.

The differences in classification of this variant shows how new evidence and updated literature can contribute to the understanding of the effect of genetic variants. It is therefore important to develop a plan for consistent variant reclassification based on new evidence to be evaluated and incorporated into the assessment.

Variant reclassification due to weight given to evidence by ACMG guidelines

The ACMG guidelines sought to create consistency in how certain types of evidence were weighted in the process of variant reclassification. The splice variant c.1378+2T>A identified in the PKP2 gene was originally classified by the clinical genetic testing laboratory in 2014 as disease causing. The evidence the laboratory referenced in the report included the following:

- This variant has not previously been reported as disease causing or benign
- This variant destroys the canonical splice donor site and is predicted to cause abnormal gene splicing
- Other splice-site variants have been reported in association with ARVC

When the ACMG guidelines are used to assess the above evidence, the only applicable criterion is PVS1 (null variant in a gene where loss of function is a known mechanism of disease). Alone, this criterion does not give a classification of disease
causing, but rather a classification of VUS. A reclassification of VUS for this variant was obtained in this study, and the supporting evidence did not differ from the evidence used by the genetic testing laboratory.

**Variant reclassification due to updated evidence and weight given to evidence by ACMG guidelines**

The missense variant c.694G>A (p.Asp232Asn) in the *LDB3* gene was originally classified as a likely benign variant by the clinical genetic testing laboratory in 2013. The evidence the laboratory used in this classification was the following:

- This variant has been reported in association with cardiomyopathy
- The variant has been seen in 1% of individuals of African American ethnicity in the general population

If the ACMG guidelines were applied to the above referenced evidence, the only applicable criterion would be criterion BS1 (allele frequency is greater than expected for disorder). Alone, criterion BS1 would give this variant a classification of VUS rather than a classification of likely benign.

In this study, this variant was reclassified as a benign variant. This variant was reported in Xi et al. as D117N (p.Asp117Asn) using an alternate gene transcript. This paper showed that this variant in the *LDB3* gene has a negative functional effect on the protein complex by causing loss of function of the sodium ion channel (*SCN5A*) when the two proteins interact. Therefore, we applied criterion PS3 (*in vivo or in vitro* functional studies support a deleterious effect) in the reclassification of this variant. However, this
variant was observed in the general population at a frequency of 0.78% in 1000 Genomes; 0.68% in EVS; 0.46% in ExAC; and 0.47% in gnomAD which allowed for the application of criterion BS1 (allele frequency is greater than expected for disorder). Levitas et al. showed that this variant did not segregate with disease in two families with DCM. Specifically, in one large family the variant was not present in multiple affected family members as shown in Figure 1. Therefore, criterion BS4 (lack of segregation in affected members of a family) was applied. PolyPhen and SIFT both predict that this variant does not have an effect on the protein which meets the guidelines for criterion BP4 (multiple lines of computational evidence suggest no impact on gene or gene product). The combination of criteria PS3, BS1, BS4, and BP4 give this variant a reclassification of benign.

In this case, the c.694G>A variant in the LDB3 gene would have undergone a reclassification if on the ACMG guidelines were applied to the evidence referenced in the original genetic test report. However, there was also additional evidence that was published after the release of the original genetic test report (2013) which allowed this variant to be reclassified as benign.
Figure 1: Segregation of variant in family

**Examples of difference in interpretation of the ACMG guidelines**

The variant c.215G>T in the *LMNA* gene is an example of how implementation of the ACMG guidelines and different interpretations of these guidelines led to a difference in variant classification between the clinical genetic testing laboratory and this study. Originally, in 2010, this variant was reported as a “VUS, likely disease causing.” The laboratory used the following evidence for this classification:

- This is a novel variant
- The variant results in a non-conservative amino acid change
- *In silico* programs predict a damaging effect on the protein
- Pathogenic variants in nearby codons support functional evidence of this region of the protein
- Absent from controls and is not present in the general population
- This variant being novel prevents the lab from calling it disease causing
After the release of the original test report in 2010, this variant was subsequently reclassified in 2013 by the genetic testing laboratory to a “disease causing” variant based on published data. After the updated ACMG guidelines were published in 2015, the laboratory again reclassified this variant as a VUS based on the following evidence:

- This variant has been reported in patients with DCM as well as a woman with ARVC
- It is reported to co-segregate with disease in a family
- Not observed in 666 control individuals
- Results in a non-conservative amino acid substitution
- The amino acid position is highly conserved across species
- *In silico* programs predict a probably damaging effect on the protein
- There is a reportedly pathogenic variant located at this same amino acid position
- This variant is not present in ESP
- Another laboratory reported this variant as a VUS in ClinVar

Using the ACMG guidelines, this evidence results in a classification of VUS according to the clinical genetic testing laboratory. However, this study used similar evidence and reclassified this variant as a likely pathogenic variant.

This variant is located in the rod domain which is a well-established functional domain for the *LMNA* gene as identified in the primary literature. Therefore, criterion PM1 (located in a mutational hot spot and/or critical and well-established functional domain without benign variation) was applied. The variant was not present in 1000 Genomes, EVS, ExAC, or gnomAD which allowed for application for criterion PM2.
(absent from controls in Exome Sequencing Project, 1000 Genomes, or ExAC). The variant p.Arg72Cys at the same amino acid position has been reported as pathogenic in HGMD and criterion PM5 (novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before) was applied. Lakdawala et al. reported that this variant was identified in one patient and two of the patient’s affected family members and which was defined as co-segregating with disease in this family. Therefore, criterion PP1 (co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease) was applied in this reclassification. This variant is a missense variant in a gene that was determined to have a low rate of benign missense variation using the ExAC constraint data for missense variation in the LMNA gene (s=3.4), and where missense variants are a common mechanism of disease according to HGMD. These two pieces of evidence meet criterion PP2 (missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease). SIFT and PolyPhen both predict this variant to have a damaging effect on the protein, and criterion PP3 (multiple lines of computational evidence support a deleterious effect on the gene or gene product) was applied. The combination of these criteria give this variant a reclassification of likely pathogenic.

This discordance between the reclassification of the clinical laboratory and this study is likely due to a difference in the interpretation of the evidence and guidelines between laboratories’ methods and the methods in this study. Therefore, while the guidelines were created to try and create a more uniform method of variant classification among
clinical genetic testing laboratories, the LMNA c.215G>T variant demonstrates how differing interpretations of the ACMG guidelines can lead to discordant variant classification.

**Conflicting variant classifications among laboratories and medical management**

One of the goals of the ACMG guidelines was to improve the variant classification concordance among clinical genetic testing laboratories. Conflicting classifications among laboratories for identical variants can lead to discrepancies in how at-risk family members are managed. The c.13G>C variant in the MYBPC3 gene is an example of a variant with conflicting classifications.

The original genetic testing laboratory, Laboratory A, reported this variant in 2014 as pathogenic using the following evidence in their interpretation:

- Variant has previously been reported in association with cardiomyopathy
- Absent in controls
- Not observed in the general population
- Non-conservative amino acid change
- This amino acid position is highly conserved across species

Despite reporting this variant as pathogenic on the original test report in 2014, Laboratory A submitted this variant to ClinVar in 2016 as a VUS referencing the following evidence for their classification:

- This variant has been previously published in association with multiple types of cardiomyopathy
• Absent from 1,800 control individuals
• Observed in .1% of alleles in 1000 Genomes and ESP
• Non-conservative amino acid substitution
• Amino acids with similar properties are tolerated at this position across species
• In silico predictions are inconsistent
• Two missense variants in nearby amino acid positions have been reported as pathogenic

Over time, Laboratory A downgraded this variant from pathogenic to VUS based on additional evidence. However, using the ACMG guidelines, the original evidence cited at the time of genetic testing in 2014 was not enough to support a pathogenic variant classification because only criterion BS1 (allele frequency is greater than expected for disorder) would have been applicable resulting in a classification of VUS.

Another laboratory, Laboratory B, also submitted this variant to ClinVar in 2015 as a VUS, however this laboratory used different evidence than Laboratory A to support their classification:

• This variant was reported in ten individuals with various cardiomyopathies
• Three of the above individuals carried pathogenic variants in the same gene
• This variant was present in ExAC at a frequency of 0.04%
• This amino acid position is not conserved across species
• Computational tools predict that this variant is benign
Although Laboratory B classified this variant as a VUS, the ACMG guidelines would give this variant a classification of likely benign based on this evidence. The likely benign classification would result from the application of criteria BP4 (multiple lines of computational evidence suggest no impact on gene or gene product) and BP5 (variant found in a case with an alternate molecular basis for disease). Laboratory B submitted this variant to ClinVar in August of 2015 which was after the ACMG guidelines were published. However, it is difficult to determine what led to the discordance between Laboratory B’s classification and the ACMG guidelines’ classification for this variant. It is possible that Laboratory B classified this variant prior to the publication of the ACMG guidelines in May of 2015 then submitted to ClinVar at a later date.

Three additional genetic testing laboratories, Laboratory C; Laboratory D; and Laboratory E, submitted this variant to ClinVar with a classification of pathogenic (submitted 2015), likely pathogenic (submitted 2013), and VUS (submitted 2012), respectively. The evidence supporting these classifications was unavailable. Finally, a research study submitted this variant to ClinVar in 2014 with a classification of VUS. The evidence supporting this classification was also unavailable. HGMD reported this variant as “disease causing mutation?” citing multiple different papers from the literature that report this variant as either pathogenic or VUS.

In this study, this variant was reclassified as a VUS due to conflicting evidence. Ratti et al. reported that this variant mildly reduces chemical shift perturbations which has the potential to impact the binding site for the protein. Therefore, this variant fulfills criterion PS3 (well-established in vitro or in vivo functional studies supportive of a
damaging effect on the gene or gene product). This variant is located in the CO domain of the \textit{MYBPC3} gene which has been reported as an important functional domain in the literature allowing for the application of criterion PM1 (located in a mutational hot spot and/or critical and well-established functional domain without benign variation). This variant is a missense variant in a gene for which truncating variants have been reported as the primary cause of disease which meets the guidelines for criterion BP1 (missense variant in a gene for which primarily truncating variants are known to cause disease). PolyPhen predicts this variant to be benign, so criterion BP4 (multiple lines of computational evidence suggest no impact on gene or gene product) was applied. This variant was also observed in a patient in this study who carried a likely pathogenic \textit{MYBPC3} variant which is another possible cause of the disease. Therefore, criterion BP5 (variant found in a case with an alternate molecular basis for disease) was applied. The combination of criteria PS3 and PM1 give this variant a classification of likely pathogenic according to the ACMG guidelines. However, the combination of criteria BP1, BP4, and BP5 result in a classification of likely benign according to the guidelines. When the two classifications are conflicting, the ACMG guidelines state that the variant classification defaults to VUS which was the reclassification for this variant assigned in this study.

This variant is an example of how different evidence, different interpretations of evidence, and conflicting evidence can result in different classifications among laboratories. Conflicting interpretations can result in patients with an identical genetic variant being managed differently, leading to inconsistent medical management for the
same genetic result. For example, a patient tested at Laboratory B and a patient tested at Laboratory C who carry the same c.13G>C variant in the \textit{MYBPC3} gene would have been given different medical management recommendations for their at-risk family members due to differences in the variant classification. This discordance in family members’ medical management is a problem, especially if members of the same family receive different classifications for the same variant due to different laboratories performing their tests. It is important to increase concordance among genetic testing laboratories in order to ensure that clinicians are managing at-risk patients in a consistent manner.

The variant c.1309G>A in the \textit{MYBPC3} gene, which also has conflicting interpretations among laboratories, is another example of this problem. The genetic testing laboratory, Laboratory A, originally classified this variant as “likely disease causing” at the time the patient was tested in 2009. Laboratory A used the following evidence to support this classification:

- This variant is a novel missense change
- The amino acid position is highly conserved across species
- Not detected in control population

In ClinVar, Laboratory A submitted this variant as a VUS in 2015. The following evidence was used to support this classification:

- This variant was reported in the literature in one individual with DCM
- Observed in multiple other unrelated patients tested at Laboratory A due to DCM
- Conservative amino acid substitution

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• The amino acid position is mostly conserved across species
• Pathogenic variants have been reported in nearby amino acid positions supporting the functional importance of this region
• Not observed in ESP

If the ACMG guidelines are applied to the above evidence, the resulting classification is also a VUS with the application of criterion PM2 (absent from controls in Exome Sequencing Project, 1000 Genomes, or ExAC) and an extra moderate criterion (for very rare variants where case-control studies may not reach statistical significance the prior observation of the variant in multiple unrelated patients with the same phenotype, and its absence in controls, may be used as moderate level of evidence).

Another clinical genetic testing laboratory, Laboratory B, also submitted this variant to ClinVar as a VUS in 2016 referencing the following evidence:
• Amino acid position is highly conserved across species
• This variant is present at a frequency of 0.02% in ExAC
• Reported in the literature in one individual with DCM
• Reported in other patients in ClinVar
• Computational evidence all predict that this variant is tolerated

The ACMG guidelines would apply to Laboratory B’s evidence similarly to Laboratory A’s evidence for this classification.

In this study, this variant was reclassified as likely benign. SIFT and PolyPhen both report this variant as being tolerated, which allowed for application of criterion BP4
(multiple lines of computational evidence suggest no impact on gene or gene product).
Also, this variant is a missense variant in a gene for which truncating variants are reported to be the primary cause of disease in the literature. Therefore, criterion BP1 (missense variant in a gene for which primarily truncating variants are known to cause disease) was applied. The combination of criteria BP1 and BP4 gave this variant a reclassification of likely benign.

This variant demonstrates how the use of different evidence and different methods for variant classification can lead to laboratories having different classifications from each other as well as different classifications over time. As an example, Laboratory A originally reported this variant as “likely disease causing,” but subsequently determined that the evidence did not support this classification and downgraded the classification to a VUS (submitted to ClinVar many years after the original report). This variant is another example of the issues the ACMG guidelines intended to address such as increasing clinical laboratory concordance.

**ACMG guideline criteria not applicable in the setting of cardiovascular disorders**

The pathogenic criterion PP4 (patient’s phenotype or family history is highly specific for a disease with a single genetic etiology) was not applicable in this study due to the heterogenous genetic etiology of cardiovascular diseases. Because there are many genes associated with the cardiovascular phenotypes (for example, more than 40 genes are known to be associated with DCM), it can be argued that finding a variant in a gene known to be associated with the phenotype does not contribute to the classification of the
variant. Therefore, this criterion should not be used when evaluating for a likely pathogenic or pathogenic classification of variants in the genes associated with cardiovascular diseases.

The benign criterion BS2 (observed in a healthy adult individual for a recessive, dominant, or X-linked disorder with full penetrance expected at an early age) was not applicable in this study due to the hereditary cardiovascular diseases having reduced penetrance and variable expressivity. Because of reduced penetrance, it is possible for patients with pathogenic variants to never develop symptoms of a disorder. Also, due to variable expressivity, the severity of symptoms in affected patients can vary even within the same family, and affected patients may appear asymptomatic due to only having very mild features. It is also possible for patients with the same genetic variant to develop disease at different ages. Due to reduced penetrance, variable expressivity, and widely variable age of onset for hereditary cardiovascular disorders, this criterion should not be used when classifying variants in the genes known to cause hereditary cardiovascular disease.

The pathogenic criterion PP5 (reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation) and the benign criterion BP6 (reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation) were not applied during variant reclassification in this study. The current ACMG guidelines do not define what type of sources are considered “reputable.” In general, these criteria are also difficult to apply due to the possibility for conflicting
variant classifications among “reputable” sources. Therefore, it would be helpful for this criterion to be updated to provide additional guidance, such as by defining what types of sources are considered “reputable” as well as specifying how many of those sources need to be concordant in their classification of the variant in order for these criteria to be met. Including the use of evidence that is not publicly accessible, such as variant classification made based on a laboratory's proprietary internal variant database, poses challenges for promoting inter-laboratory concordance of variant classification since each individual laboratory cannot independently assess the source of evidence, and various laboratories may not agree on the same source as being reputable or not.

Other suggested improvements to the guidelines used for variant classification

Many of the criteria in the ACMG guidelines do not provide specific information on when these criteria are met or not. Therefore, there is much room for interpretation regarding when the evidence supports the application of the criterion. Differences in interpretation of each criterion can lead to discordance in variant classification among laboratories even with the use of the ACMG guidelines.

Criterion PP1, which evaluates for segregation of a variant in the family, was also difficult to apply for cardiovascular diseases. The guidelines do not provide specific guidance on when it is appropriate to apply this criterion as supporting evidence, such as number of family members that must be evaluated to meet this criterion, number of affected family members with the variant that would be needed to meet this criterion, and number of generations or degrees of relationships that must be evaluated. To further
complicate the segregation analysis, the genes that are being evaluated in the patients with cardiovascular disorders typically have variable expressivity, reduced penetrance, and variable age of onset. Therefore, it was difficult to evaluate intra-family variant segregation in this patient population because asymptomatic family members who carry the variant in question could suggest that the variant is not contributing to the disease, or they could represent non-penetrance where individual will not develop disease in a lifetime even if the variant is contributing to disease, or they could represent mild expressivity where symptoms are so mild that the individual falsely appears as asymptomatic, or they may not yet have developed symptoms due to variable age of onset. Therefore, it was difficult to determine when a variant was segregating at the supporting evidence level as opposed to when it was not. The ACMG guidelines could be improved by providing information on how many affected individuals need to test positive in a family for the same variant and the number of meiosis cycles needed in order for a variant to be considered to segregate with disease. Providing this specific information would be especially helpful for disorders that demonstrate reduced penetrance, variable expressivity, and variable age of onset, such as hereditary cardiovascular diseases.

For criterion PP2 (missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease), the guidelines do not state how to determine whether a gene has a low rate of benign missense variation. Therefore, there is much room for interpretation on when this criterion is met. For this study, we used ExAC constraint data to determine whether a
gene was tolerant of missense variation. However, even with this method, it was difficult to determine at what level of standard deviation a gene could be considered “not tolerant” of missense variation. Therefore, the criterion could be improved by specifying how to determine when a gene has a low rate of benign missense variation. It would also be useful to specify what type of source is acceptable to determine whether missense variants are a common mechanism of disease. Defining more specifically when this criterion is applicable for a variant or gene, can reduce the subjective interpretation in the application of this criterion.

Some types of evidence could not be used in the reclassifications performed by this study because there was no criterion in the guidelines that accounted for this type of evidence. As an example, a study by Kapa et.al. evaluated the type, frequency, and location of variants identified in the $KCNH2$ gene and the authors found that all variants identified in the N-terminus and C-terminus of this gene had been reported as benign variants. Some of the variants identified in the $KCNH2$ gene in this current study were located in these portions of the gene. However, the current ACMG guidelines provide no way to account for this type of evidence. The guidelines provide a criterion for evaluating the pathogenic nature of a variant if it is present in a well-established functional domain or hotspot, but they do not provide a criterion for evaluating the benign nature of a variant if it is present in a location that has been established as an area tolerant of genetic variation. The ACMG guidelines could be improved with the addition of a benign criterion that took into consideration that some portions of a gene are tolerant of variation.
Recommendations for cardiovascular genetic variant classification based on the results of this study

The purpose of the ACMG guidelines was to provide genetic testing laboratories with a set of guidelines that would help laboratories achieve more concordance in variant classification as well as provide recommendations for what types and what amount of evidence is needed to classify a variant as pathogenic or benign. However, as discussed above, these guidelines are not easy to apply in every clinical genetics setting due to their limitations in disorders that have heterogenous genetic etiology, reduced penetrance, and variable expressivity, and variable age of onset. Also, the guidelines leave much room for interpretation of the evidence as well as room for interpretation of when a criterion is applicable or not. Therefore, these guidelines may not increase concordance as much as intended.

In order to address the limitations of the current ACMG guidelines, revised guidelines should be developed in order to increase concordance and assist with the classification of genetic variants. The revised guidelines will need to be gene or disease specific in order to address some of the issues that the current guidelines do not. Also, these new guidelines will need to be able to account for additional types of evidence that may aid in the classification of genetic variants. For example, if there are domains of the protein that are tolerant of change, then a benign criterion would be needed to account for this supporting evidence of a benign classification. By creating gene or disease specific guidelines for interpretations, all of the knowledge regarding each gene and disease (e.g.
protein domains, penetrance, types of variation) can be accounted for to ensure that each variant is thoroughly assessed for pathogenic and benign nature.

There also needs to be an established method for reclassifying variants in light of new evidence. To ensure that evidence is being re-evaluated using current evidence and classifications are current, there needs to be a systematic method for variant reclassification. As an example, each laboratory may be required to review the classification of a variant and the supporting evidence every two years to assess for changes. This systematic method for reclassification is important, so that patients and their family members are being managed properly. As an example, four patients in this study are from the same family and all of them had the variant c.5546A>G in the SCN5A gene. Three of them were tested at Laboratory A, and the variant was classified as a “VUS, likely pathogenic.” The fourth family member was tested many years later at Laboratory A, and the variant was classified as likely pathogenic. Therefore, it is possible that multiple family members were mismanaged for years because the variant was not re-evaluated before the fourth family member was tested. If there was a system for re-evaluating variant classifications, this family may have been able to receive more appropriate medical management earlier.

Evaluation of evidence for updates and reclassification is difficult when genetic testing laboratories do not report the evidence they used in their classification process on the genetic test report. There were 16 variants in this study that received a reclassification, and the genetic testing laboratory did not report what evidence was used in determining their reported classification. This lack of provided evidence prevented
this study from performing needed evaluations to determine what led to the discordance in variant classification. Therefore, laboratories should be required to report their supporting evidence for all reported variants on a clinical genetic testing report, which will enable the clinicians receiving the report to independently evaluate evidence and variant classifications. It would also be helpful if this was provided in a manner that was standardized across genetic testing laboratories so that clinicians could easily review the supporting evidence as needed for patient care.

*Role of genetic counselors in variant reclassification*

In order to ensure that patients and their family members are receiving the best care based on accurate genetic test results, genetic counselors should have a more active role in the variant reclassification process. Clinical genetic testing laboratories do a thorough review of the available evidence for each variant identified to determine classification. To completely apply the ACMG guidelines, however, laboratories would need to have additional information and/or evidence that is not always available. Therefore, it is important for the genetic counselors to collaborate with laboratories to aid in proper variant classification for each family. For transparency, laboratories should justify their classifications using standard reporting procedures. Likewise, it is the responsibility of genetic counselors to supply laboratories with detailed information regarding phenotype, family history and variant segregation within the family.

The amount of information provided by the clinical genetic testing laboratory can inhibit or aid in the process of variant reclassification for genetic counselors. Therefore,
it is critical for genetic counselors to evaluate the methods of each clinical genetic testing laboratory when choosing from which laboratory to order genetic testing. Genetic counselors may want to know what methods the laboratory uses for their initial variant classification. Does the laboratory use the ACMG guidelines, an internal method, or some combination of the two? Also, genetic counselors may want to know if the variant classifications are reviewed by gene or phenotype experts at the laboratory. Finally, what amount of information does the laboratory provide on their genetic test report and what is the format of the report? This information can allow for genetic counselors to work with laboratories who supply them with the necessary information and tools that they require to re-evaluate genetic variants.

It is important that all variants reported on a clinical genetic test report prior to the publication of the updated ACMG guidelines in May of 2015 be re-evaluated using those guidelines. The guidelines had a large impact on the reclassification of many variants in this study which demonstrates the importance of this re-evaluation using the current guidelines. Also, all variants identified on a genetic test report should be re-evaluated periodically, such as every 2 years, for potential updates in evidence since the previous classification. Therefore, new evidence that may result in the reclassification of a variant may be incorporated in a timely fashion.

Genetic counselors should be trained on the process of variant classification in order to have an active role in this process. Genetic Counseling Training Programs should incorporate this information into their curriculum so that genetic counselors are knowledgeable on these methods. By actively engaging genetic counselors in the variant
assessment process, genetic variants may be re-evaluated more often allowing for proper changes in medical management to be incorporated into patient care as needed. Genetic counselors should have the role of re-evaluating variants so that they are able to perform frequent variant reclassifications and incorporate these findings into the care of their patients and risk assessment of the patient’s family members. Periodic re-evaluation for each family's variant will become an essential component of the modern clinical care, as the variant classification guidelines will change, and new knowledge of genes and variants will become available over time.

Conclusion

This study demonstrated that the application of the “Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomic and the Association for Molecular Pathology” did result in a change in classification for approximately one-third of the variants in this study. However, application of these guidelines was difficult for the genes encountered in the clinical cardiovascular genetics setting. The results of this study demonstrate the need for gene or disease specific variant classification guidelines due to the uniqueness of each gene and cardiovascular phenotype. With the implementation of more specific classification guidelines and a system for consistent variant reclassification, families may be better managed, laboratory concordance in classifications may be increased, and the number of variants being classified as pathogenic when there is not sufficient supporting evidence may be decreased. If these goals can be accomplished,
clinicians will be able to provide better care and management to the patients and families evaluated in the cardiovascular genetics clinics.
LIMITATIONS

There were multiple limitations in this study. First, we obtained our patient population by performing a database query. It is possible that patients seen by Amy Sturm, MS, LGC during the specified time frame had not yet been entered into the database at the time we performed the query. If a patient was not in the database at the time of our query, they would not have been included in this study. By missing patients who would have qualified for this retrospective chart review but who were not in the database at the time of query, this study would not have accounted for additional variants that may have been identified in these patients. Therefore, some of the statistical analysis that was not significant due to the limited number of variants in some of the genes may have been strengthened if these patients had been included. Not every genetic testing report listed the gene transcript used for analysis. Therefore, we could not confirm that we were using the correct transcript during my evidence collection. This limitation was particularly important to consider when variants were not found in databases such as ClinVar or HGMD. It is possible that the variants were present in these databases under alternative nomenclature or different gene transcripts. We obtained our in silico predictions from multiple sources for this study. Therefore, we were unable to confirm that all variants were assessed using the same versions of these prediction programs. This lack of consistency could have impacted our variant classification due to potential differences in the predictions we used for classification. We were unable to obtain access
to every published journal article that was referenced while we were collecting evidence for each variant. Therefore, it is possible that the information in these articles was missed and not considered in our variant reclassification. We also used specific search methods in PubMed to obtain functional studies for each variant. The use of these specific methods could have resulted in us missing functional information for some of the variants. Similarly, we used specific search methods to obtain the information about each gene that was necessary for variant classification. Therefore, it is possible that these methods resulted in obtaining certain information for a gene which could have limited our ability to accurately classify variants in some genes. Finally, this study made modifications to the current ACMG guidelines, and these modified guidelines were used to re-evaluate all variants in this study. Therefore, other reviewers may not reach the same variant reclassification as was made in this study due to different interpretations of these guidelines.
REFERENCES


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doi:10.1016/j.hrthm.2013.05.014 [doi]

doi:10.1038/gim.2013.204 [doi]

doi:10.1074/jbc.M110.156646 [doi]

doi:10.1097/GIM.0b013e31816b5cae [doi]


APPENDIX A: SUPPLEMENTARY DATA
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<th>Protein</th>
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Table 8: Supplementary Data

continued
Table 8 continued

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continued
Table 8 continued

| 23 | 99 | NM_00023       | 8.2 | KCNH2 | c.982C>T | p.ARG328CY | p.R328C | VUS | LQTS | unknown | unknown | Likely pathogenic |
| 25 | 134 | NM_00192       | 7.3 | DES   | c.1353C>G | p.Ile451Met | p.I451M | Disease causing | Arrhythmia | European | European | Likely pathogenic |
| 26 | 1   | NM_02442       | 2.3 | DSC2  | c.327A>G  | p.Ile109Met | p.I109M | VUS | DCM | unknown | unknown | Likely benign |
| 26 | 1   | NM_00025       | 7.2 | MYH7  | c.4377G>T | p.Lys1459Asn | p.K1459N | Disease causing | DCM | unknown | unknown | VUS |
| 27 | 157 | NM_00125       | 6850.1 | TTN   | c.71192dupA | p.Asn23731LysfsX5 | p.N23731KfsX5 | Disease causing | DCM | unknown | unknown | Pathogenic |

continued
| 28 | 49 | NM_00575 1.4 | **AKAP9** | c.4342A>G | p.Ile1448Val | p.I1448V | VUS | CPVT | unknown | unknown | VUS |
| 28 | 49 | NM_19805 6.2 | **SCN5A** | c.1338+2T>A, c.IVS10+2T>A | Pathogenic | | | | | | |
| 29 | 122 | NM_00194 3.3 | **DSG2** | c.1003A>G | p.Thr335Ala | p.T335A | VUS | ARVC | European | European | VUS |
| 29 | 123 | NM_00194 3.3 | **DSG2** | c.1003A>G | p.Thr335Ala | p.T335A | VUS | ARVC | unknown | European | VUS |
| 31 | 82 | NM_00194 3.3* | **DSG2** | c.1439C>T | p.Thr480Ile | p.T480I | Likely pathogenic | ARVC | unknown | unknown | VUS |
| 31 | 83 | NM_00194 3.3* | **DSG2** | c.1439C>T | p.Thr480Ile | p.T480I | Likely pathogenic | ARVC | unknown | unknown | VUS |
| 31 | 220 | NM_00441 5.2 | **DSP** | c.1696G>A | p.p.Ala566Thr | p.A566T | VUS | DCM | unknown | unknown | Likely benign |

**Table 8 continued**
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| 80 | 115 | NM_17070 7.2 | LMNA | c.179G>C | p.Arg60Pro | p.R60P | Likely disease causing | DCM | unknown | unknown | Likely pathogenic |
| 81 | 191 | NM_17070 7.2 | LMNA | c.585C>G | p.As195Lys | p.N195K | Disease causing | DCM | unknown | unknown | Pathogenic |
| 81 | 192 | NM_17070 7.2 | LMNA | c.585C>G | p.As195Lys | p.N195K | Disease causing | DCM | unknown | unknown | Pathogenic |
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| 83 | 43 | NM_00025 6.3* | MYBPC3 | c.1624+2T>C, c.IVS17+2T>C | | | Presumed pathogenic | clinical data unavailable | European | European | Likely pathogenic |

Table 8 continued

| 86 | 80 | NM_17070 7.2 | LMNA | c.179G>C | p.Arg60Pro | p.R60P | Likely disease causing | DCM | unknown | unknown | Likely pathogenic |
| 81 | 191 | NM_17070 7.2 | LMNA | c.585C>G | p.As195Lys | p.N195K | Disease causing | DCM | unknown | unknown | Pathogenic |
| 81 | 192 | NM_17070 7.2 | LMNA | c.585C>G | p.As195Lys | p.N195K | Disease causing | DCM | unknown | unknown | Pathogenic |
| 82 | 80 | NM_00025 6* | MYBPC3 | c.459delC | p.Ile154fs | p.I154fs | Presumed pathogenic | HCM | Native American | European | Pathogenic |
| 83 | 43 | NM_00025 6.3* | MYBPC3 | c.1624+2T>C, c.IVS17+2T>C | | | Presumed pathogenic | clinical data unavailable | European | European | Likely pathogenic |
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| 130 | 210 | NM_00103 5.2 | **RYR2** | c.6916G>C | p.Val2306Leu | p.V2306L | Likely disease causing | CPVT | unknown | unknown | Likely pathogenic |
| 131 | 15 | NM_00103 5.2 | **RYR2** | c.4828C>T | p.Arg1610stop | p.R1610X | VUS | DCM | European | European | VUS |
| 131 | 15 | NM_00100 1430.1 | **TNNT2** | c.742T>C | p.Phe248Leu | p.F248L | VUS | DCM | European | European | VUS |
| 132 | 121 | NM_00103 5.2 | **RYR2** | c.EX30_32del | | | VUS | DCM | African | African | VUS |
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| 150 | 86 | NM_001001430.1 | TNNT2 | c.83C>T | p.Ala28Val | p.A28V | VUS | Cardiomyopathy | European | European | VUS |
| 150 | 86 | NM_001256850.1 | TTN | c.30812-1G>A, c.IVS119-1G>A | VUS | Cardiomyopathy | European | European | VUS |
| 151 | 133 | NM_001001430.1 | TNNT2 | c.832C>T | p.Arg278Cys | p.R278C | Disease causing | HCM | European | European | Likely pathogenic |
| 152 | 68 | NM_001018005.1 | TPM1 | c.743A>C | p.Lys248Thr | p.K248T | VUS, likely disease causing | 19y female with normal Echocardiogram and Electrocardiogram | European | unknown | Likely pathogenic |
| 152 | 69 | NM_001018005.1 | TPM1 | c.743A>C | p.Lys248Thr | p.K248T | VUS, likely disease causing | HCM | European | European | Likely pathogenic |

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<td>p.S3373Y</td>
<td>VUS</td>
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continued
| Chromosome | BP   | Gene   | Exon   | cDNA   | Protein   | Effect   | Heart Block/Pacemaker | Age of Onset | Type of Cardiac Event | Classification |
|------------|------|--------|--------|--------|-----------|----------|-----------------------|--------------|------------------------|----------------|----------------------|
| 163        | 153  | NM_17070 | 7.2    | c.1698C>T | p.His566His | p.H566H | Not associated         | unknown     | unknown                | Benign         |                     |
| 163        | 153  | NM_00025 | 6      | c.506-12delC |           |          | Not associated         | unknown     | unknown                | Benign         |                     |
| 163        | 153  | NM_00025 | 6.3    | c.537C>T | p.Ala179Ala | p.A179A | Not associated         | unknown     | unknown                | VUS            |                     |
| 163        | 153  | NM_00025 | 7.2    | c.189C>T | p.Thr63Thr | p.T63T | Not associated         | unknown     | unknown                | Benign         |                     |
| 163        | 153  | TNNT2   | 430.1  | c.318C>T | p.Ile106Ile | p.I106I | not associated          | unknown     | unknown                | Benign         |                     |

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<th>unknown</th>
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<td>unknown</td>
<td>unknown</td>
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<td>164</td>
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<td>BrS</td>
<td>European</td>
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APPENDIX B: REFERENCES USED IN VARIANT RECLASSIFICATION

**ABCC9**


**ACTN2**


**AKAP9**


**ANK2**

123


**ANKRD1**


**APOB**


doi:10.1258/acb.2007.007077 [doi]


**CACNA1C**


**CACNB2**


**CSRP3**


**DES**

125
References


**DMD**


**DSC2**


**DSG2**


dysplasia/cardiomyopathy genotype-phenotype follow-up study. *Circulation*, 123(23), 2690-2700. doi:10.1161/CIRCULATIONAHA.110.988287 [doi]


**DSP**


**DTNA**


**EMD**


**FBN2**


**GPD1L**

**HCN4**


**JPH2**


**KCNE1**


Nakajima, T., Kaneko, Y., Manita, M., Iso, T., & Kurabayashi, M. (2010). Aborted cardiac arrest in a patient carrying KCNE1 D85N variant during the postpartum period. *Internal Medicine (Tokyo, Japan),* 49(17), 1875-1878. doi:JST.JSTAGE/internalmedicine/49.3859 [pii]


**KCNH2**


novel mutations and an amino acid polymorphism with possible phenotypic
effects. Human Mutation, 15(6), 580-581. doi:10.1002/1098-
1004(200006)15:6<580::AID-HUMU16>3.0.CO;2-0 [pii]

015-0038-y [doi]


Novotny, T., Kadlecova, J., Raudenska, M., Bittnerova, A., Andrsova, I., Florianova, A., . . .


Paavonen, K. J., Chapman, H., Laitinen, P. J., Fodstad, H., Piippo, K., Swan, H., . . .


patients with KvLQT1 and HERG potassium channel defects. *Journal of the American College of Cardiology, 34*(3), 823-829. doi:S0735-1097(99)00255-7 [pii]


Zhao, J. T., Hill, A. P., Varghese, A., Cooper, A. A., Swan, H., Laitinen-Forsblom, P. J., . . . Vandenberg, J. I. (2009). Not all hERG pore domain mutations have a severe phenotype: G584S has an inactivation gating defect with mild phenotype compared to G572S, which has a dominant negative trafficking defect and a severe phenotype. *Journal of Cardiovascular Electrophysiology, 20*(8), 923-930. doi:10.1111/j.1540-8167.2009.01468.x [doi]

**KCNJ2**


**KCNQ1**


doi:10.1016/j.ajhg.2013.08.006 [doi]

doi:10.1161/CIRCGENETICS.112.964684 [doi]

doi:10.1161/CIRCGENETICS.112.963785 [doi]


**LAMP2**


**LDB3**


**LDLR**


LDLR mutations and genotype-phenotype correlations. *Atherosclerosis*, 223(2), 401-408. doi:10.1016/j.atherosclerosis.2012.05.014 [doi]


**LMNA**


**MYBPC3**


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TNNT3 from 312 patients with familial or idiopathic dilated cardiomyopathy. *Circulation.Cardiovascular Genetics,* 3(2), 155-161. doi:10.1161/CIRCGENETICS.109.912345 [doi]


Molecular Medicine (Berlin, Germany), 80(7), 412-422. doi:10.1007/s00109-002-0323-9


of the American College of Cardiology, 58(23), 2406-2414.


doi:10.1161/CIRCGENETICS.113.000039 [doi]


doi:10.1161/CIRCGENETICS.111.961805 [doi]


**MYH6**


196


Hershberger, R. E., Norton, N., Morales, A., Li, D., Siegfried, J. D., & Gonzalez-Quintana, J. (2010). Coding sequence rare variants identified in MYBPC3, MYH6, TPM1, TNNC1, and TNNI3 from 312 patients with familial or idiopathic dilated cardiomyopathy. *Circulation Cardiovascular Genetics, 3*(2), 155-161. doi:10.1161/CIRCGENETICS.109.912345 [doi]


**MYH7**


*MYL2*

**MYOZ2**


**MYPN**


**NEBL**


**NEXN**


**PCSK9**

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**PKP2**


**PRKAG2**


**RBM20**


surveyed by clinical DNA sequencing. *Genetics in Medicine: Official Journal of the American College of Medical Genetics, 16*(8), 601-608. doi:10.1038/gim.2013.204 [doi]


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Marks, A. R., Priori, S., Memmi, M., Kontula, K., & Laitinen, P. J. (2002). Involvement of the cardiac ryanodine receptor/calcium release channel in catecholaminergic polymorphic ventricular tachycardia. *Journal of Cellular Physiology, 190*(1), 1-6. doi:10.1002/jcp.10031 [pii]

comprehensive open reading frame mutational analysis. *Journal of the American College of Cardiology, 54*(22), 2065-2074. doi:10.1016/j.jacc.2009.08.022 [doi]

**SCN1B**


**SCN4B**


**SCN5A**


Olesen, M. S. (2013). Mutations in genes encoding cardiac ion channels previously associated with sudden infant death syndrome (SIDS) are present with high frequency in new exome data. The Canadian Journal of Cardiology, 29(9), 1104-1109. doi:10.1016/j.cjca.2012.12.002 [doi]


syndrome. *Proceedings of the National Academy of Sciences of the United States of America, 104*(52), 20990-20995. doi:0710527105 [pii]


Hwang, H. W., Chen, J. J., Lin, Y. J., Shieh, R. C., Lee, M. T., Hung, S. I., ... Hwang, B. T. (2005). R1193Q of SCN5A, a brugada and long QT mutation, is a common polymorphism in han chinese. *Journal of Medical Genetics, 42*(2), e7; author reply e8. doi:42/2/e7 [pii]


doi:10.1126/science.1073569 [doi]

doi:10.1161/CIRCGENETICS.110.958652 [doi]

doi:S0024320503001218 [pii]


**TNNC1**


**TNNI3**


**TNNT2**


Midde, K., Dumka, V., Pinto, J. R., Muthu, P., Marandos, P., Gryczynski, I., . . . Borejdo, J.

Miliou, A., Anastasakis, A., D'Cruz, L. G., Theopistou, A., Rigopoulos, A., Rizos, I., . . .


*TPM1*


TTN


TTR


disease. Cell, 121(1), 73-85. doi:S0092-8674(05)00092-9 [pii]

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Investigation : The Official Journal of the International Society of Amyloidosis, 22(2),

Taylor, P. N., Porcu, E., Chew, S., Campbell, P. J., Traglia, M., Brown, S. J., . . . UK0K
Communications, 6, 5681. doi:10.1038/ncomms6681 [doi]

(2014). Isolated heart transplantation for familial transthyretin (TTR) V122I cardiac
amyloidosis. Amyloid : The International Journal of Experimental and Clinical


**VCL**