# Delivering Clinical Grade Sequencing and Genetic Test Interpretation for Cardiovascular Medicine

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Contemporary DNA sequencing approaches are increasingly used as diagnostic tools within clinical medicine, driven by rapid reductions in cost and improvements in speed. In 2014, Illumina, Inc. launched a system that could sequence an entire human genome for under $1000,[1] with sequencing and analysis achievable in under 2 days.[2,3] For inherited disease, next-generation sequencing (NGS) technologies have been applied in 3 broad categories: (1) gene panels, where a collection of predefined genes for a given condition, or a group of closely related conditions, are sequenced; (2) whole-exome sequencing (WES), where the majority of the protein-coding portions of the genome (≈2% of the genome) is sequenced; and (3) whole-genome sequencing (WGS), where the majority of the genome is sequenced, including nonprotein-coding DNA.

In the management of inherited cardiovascular disease, there has been increasing use of genetic testing as major healthcare systems establish centers of excellence. In most cases, these tests now feature NGS approaches. However, as we transition from traditional to NGS approaches, it is important that noninferiority with traditional practices is firmly established. Furthermore, our ability to correctly interpret the clinical impact of variants derived from these sequencing efforts remains suboptimal. The recent analysis and public release of sequence data from tens of thousands of individuals established that rare variation is common in humans, meaning that only a small proportion of rare variants will actually be causal of rare genetic disease. Indeed, even when rare variants emerge that could potentially explain a given presentation, the classification of such variants is often discordant between laboratories.

Here, we will explore the challenge and opportunity of NGS for inherited cardiovascular disease. First, we describe recent advances in sequencing and interpretation facilitated by large-scale population genomics studies. Next, we outline current approaches to clinical genetic testing and describe areas of technical need. Finally, we describe the process of clinical test interpretation and discuss how to improve concordance of reporting among professionals.

## Clinical Sequencing in Inherited Cardiovascular Disease

DNA sequencing has increasing importance in the delivery of clinical care to patients with inherited cardiovascular disease. In some cases, sequencing can help to clarify a diagnosis, such as in cases of concentric left ventricular hypertrophy where a sarcomeric form of hypertrophic cardiomyopathy can be distinguished from other causes of hypertrophy, such as storage diseases or amyloidosis.[4–7] In other settings, a genetic diagnosis can be used to guide therapy, such as in the use of sodium channel blockers for long-QT type 3, enzymatic replacement for Fabry disease, or silencing and tetramer stabilization therapies for transthyretin (*TTR*) cardiac amyloidosis.[8–10] However, in most cases, the major utility for sequencing is in the identification of a causal variant for predictive testing in the patient's family. This can help focus clinical screening on those family members at genetic risk of developing the condition. Where it has been formally studied, in diseases such as hypertrophic cardiomyopathy, this approach, named cascade screening, has been shown to be cost-effective.[11]

With the rapidly increasing clinical availability of genetic testing for cardiovascular disease, several governing bodies have published guidelines for its use across conditions (Table 1).[12–19] These recommendations reflect overall agreement between experts, but vary in the strength of recommendations for genetic testing across different diseases. For example, strong recommendations are made for testing index patients with hypertrophic cardiomyopathy (HCM), long-QT syndrome, and arrhythmogenic right ventricular cardiomyopathy (Table 1). However, in pathogenically diverse diseases with lower diagnostic yield, such as dilated cardiomyopathy, genetic testing in index cases is only strongly recommended in the setting of concomitant conduction system disease, for which genetic testing for specific genes may have higher specificity.

Significant benefits of commercially available genetic testing are realized only in those families for whom a causal genetic variant is identified. The percentage of families for whom this is achieved varies widely according to the condition and presentation. In some diseases, such as long-QT

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**Table 1. Summary of Consensus Recommendations from ESC, EHRA, HRS, AHA/ACC, and HFSA on Genetic Testing in Index Patients for Cardiomyopathies and Inherited Arrhythmias**

| Disease | Recommendations for Genetic Testing in the Index Patient (Class; Level of Evidence) | Assoc (Year) |
|---|---|---|
| HCM | Genetic testing as part of diagnosis and management | |
| | Genetic testing is recommended in patients fulfilling diagnostic criteria for HCM, to confirm the diagnosis (I,B) and that it be performed in certified diagnostic laboratories with expertise in the interpretation of cardiomyopathy-related mutations (I,C) Genetic testing in patients with a borderline diagnosis of HCM should be performed only after detailed assessment by specialist teams (IIa,C) | ESC (2014) |
| | Comprehensive or targeted (*MYBPC3, MYH7, TNNI3, TNNT2, TPM1*) HCM genetic testing is recommended for any patient in whom a cardiologist has established a clinical diagnosis of HCM based on examination of the patient's clinical history, family history, and electrocardiographic/echocardiographic phenotype | EHRA/HRS (2011) |
| | Genetic testing for HCM and other genetic causes of unexplained cardiac hypertrophy is recommended in patients with an atypical clinical presentation of HCM or when another genetic condition is suspected to be the cause (I,B) and is reasonable in the index patient to facilitate the identification of first-degree family members at risk (IIa,B) | ACC/AHA (2011) |
| | Genetic testing to facilitate family screening | |
| | Postmortem genetic analysis of stored DNA should be considered in deceased patients with confirmed HCM, to enable genetic screening of relatives (IIa,C) | ESC (2014) |
| | Genetic testing is reasonable in the index patient to facilitate the identification of first-degree family members at risk for developing HCM (IIa,B) | ACC/AHA (2011) |
| | Genetic testing should be considered for the one most clearly affected person in a family to facilitate family screening and management (A*) | HFSA (2010) |
| DCM | Genetic testing as part of diagnosis and management | |
| | Genetic testing can be useful for patients with familial DCM to confirm the diagnosis, to recognize those who are at highest risk of arrhythmia and syndromic features. (IIa,C) Comprehensive or targeted (LMNA and SCN5A) DCM genetic testing is recommended for patients with DCM and significant cardiac conduction disease (ie, first-, second-, or third-degree heart block) and a family history of premature unexpected sudden death (I,C) | EHRA/HRS (2011) |
| | In familial DCM (defined as 2 closely related family members who meet the criteria for idiopathic DCM) genetic testing may be considered in conjunction with genetic counseling. (Class; Level of Evidence not provided) In idiopathic DCM, the utility of genetic testing remains uncertain. Yield of genetic testing may be higher in patients with significant cardiac conduction disease and a family history of premature sudden cardiac death (Class; Level of Evidence not provided) | ACC/AHA (2013) |
| | Genetic testing to facilitate family screening | |
| | Genetic testing can be useful to facilitate cascade screening within the family, and to help with family planning (IIa,C) | EHRA/HRS (2011) |
| | Genetic testing should be considered for the one most clearly affected patient in a family to facilitate family screening and management (B*) | HFSA (2010) |
| ARVC | Genetic testing as part of diagnosis and management | |
| | Comprehensive or targeted (DSC2, DSG2, DSP, JUP, PKP2, and TMEM43) ARVC genetic testing can be useful for patients satisfying task force diagnostic criteria for ARVC. (IIa,C) Genetic testing may be considered for patients with possible ARVC (1 major or 2 minor criteria) according to the 2010 task force criteria (*European Heart Journal*) (IIb,C)Genetic testing is not recommended for patients with only a single minor criterion according to the 2010 task force criteria (III,C) | EHRA/HRS (2011) |
| | Genetic testing to facilitate family screening | |
| | Genetic testing should be considered for the one most clearly affected person in a family to facilitate family screening and management (A*) | HFSA (2010) |
| LVNC | LVNC genetic testing can be useful for patients in whom a cardiologist has established a clinical diagnosis of LVNC based on examination of the patient's clinical history, family history, and electrocardiographic/echocardiographic phenotype (IIa,C) | EHRA/HRS (2011) |
| RCM | RCM genetic testing may be considered for patients in whom a cardiologist has established a clinical index of suspicion for RCM based on examination of the patient's clinical history, family history, and electrocardiographic/echocardiographic phenotype (IIb,C) | EHRA/HRS (2011) |
| LQTS | Comprehensive or LQT1-3 (KCNQ1, KCNH2, and SCN5A) targeted LQTS genetic testing is recommended for any asymptomatic patient with QT prolongation in the absence of other clinical conditions that might prolong the QT interval (such as electrolyte abnormalities, hypertrophy, bundle branch block, ie, otherwise idiopathic) on serial 12-lead ECGs defined as QTc >480 ms (prepuberty) or >500 ms (adults) (I,C)Comprehensive or LQT1-3 (KCNQ1, KCNH2, and SCN5A) targeted LQTS genetic testing is recommended for any patient in whom a cardiologist has established a strong clinical index of suspicion for LQTS based on examination of the patient's clinical history, family history, and expressed electrocardiographic (resting 12-lead ECGs and provocative stress testing with exercise or catecholamine infusion) phenotype (I,C)Comprehensive or LQT1-3 (KCNQ1, KCNH2, and SCN5A) targeted LQTS genetic testing may be considered for any asymptomatic patient with otherwise idiopathic QTc values >460 ms (prepuberty) or >480 ms (adults) on serial 12-lead ECGs (IIb,C) | EHRA/HRS (2011) |

(*Continued*)

**Table 1. Continued**

| Disease | Recommendations for Genetic Testing in the Index Patient (Class; Level of Evidence) | Assoc (Year) |
|---|---|---|
| SQTS | Comprehensive or SQT1-3 (KCNH2, KCNQ1, and KCNJ2) targeted SQTS genetic testing may be considered for any patient in whom a cardiologist has established a strong clinical index of suspicion for SQTS based on examination of the patient's clinical history, family history, and electrocardiographic phenotype (IIb,C) | EHRA/HRS (2011) |
| CPVT | Comprehensive or CPVT1 and CVPT2 (RYR2 and CASQ2) targeted CPVT genetic testing is recommended for any patient in whom a cardiologist has established a clinical index of suspicion for CPVT based on examination of the patient's clinical history, family history, and expressed electrocardiographic phenotype during provocative stress testing with cycle, treadmill, or catecholamine infusion (I,C) | EHRA/HRS (2011) |
| BrS | Comprehensive or BrS1 (SCN5A) targeted BrS genetic testing can be useful for any patient in whom a cardiologist has established a clinical index of suspicion for BrS based on examination of the patient's clinical history, family history, and expressed electrocardiographic (resting 12-lead ECGs and provocative drug challenge testing) phenotype (IIa,C)Genetic testing is not indicated in the setting of an isolated type 2 or type 3 Brugada ECG pattern (III,C) | EHRA/HRS (2011) |
| CCD | Genetic testing may be considered as part of the diagnostic evaluation for patients with either isolated CCD or CCD with concomitant congenital heart disease, especially when there is documentation of a positive family history of CCD (IIb,C) | EHRA/HRS (2011) |
| Out-of-hospital cardiac arrest survivors | In the survivor of an Unexplained Out-of-Hospital Cardiac Arrest, genetic testing should be guided by the results of medical evaluation and is used for the primary purpose of screening at-risk family members for subclinical disease (I,C)Routine genetic testing, in the absence of a clinical index of suspicion for a specific cardiomyopathy or channelopathy, is not indicated for the survivor of an unexplained out-of-hospital cardiac arrest (III,C) | EHRA/HRS (2011) |
| Thoracic aortic aneurysm | Sequencing of other genes known to cause familial thoracic aortic aneurysms and dissection (TGFBR1, TGFBR2, and MYH11) may be considered in patients with a family history and clinical features associated with mutations in these genes (IIb,B)Sequencing of the ACTA2 gene is reasonable in patients with a family history of thoracic aortic aneurysms and dissections to determine whether ACTA2 mutations are responsible for the inherited predisposition (IIa,B) | ACC/AHA (2010) |

For HCM and DCM, recommendations are organized according to outcome (diagnosis/management versus family screening). Otherwise, consensus guidelines are provided with class of recommendation and level of evidence following. In general, Class I signifies the guideline is recommended, IIa that it can be useful, IIb, that it "may be considered, and III that it "is not recommended." Level of evidence B indicates moderate-quality evidence from RCTs or meta- analyses, and level of evidence C indicates expert opinion. ACC/AHA indicates American College of Cardiology/American Heart Association; ARVC, arrhythmogenic right ventricular cardiomyopathy; BrS, Brugada Syndrome; CCD, Cardiac Conduction Disease; CPVT, catecholaminergic polymorphic ventricular tachycardia; DCM, dilated cardiomyopathy; EHRA/HRS, European Heart Rhythm Association/Heart Rhythm Society; ESC, European Society of Cardiology; HCM, hypertrophic cardiomyopathy; HFSA, Heart Failure Society of America; LMNA, lamin A/C; LQTS, long-QT syndrome; LVNC, left ventricular noncompaction; RCM, restrictive cardiomyopathy; RCT, randomized controlled trial; SCN5A, sodium voltage-gated channel $\alpha$ subunit; and SQTS, short-QT syndrome.

*Strength of Evidence rather than Level of Evidence (defined by the HFSA as Strength A: The specific genetic test or clinical test has a high correlation with the cardiomyopathic disease of interest in reasonably large studies from multiple centers. Strength B: The specific genetic test or clinical test has a high correlation with the cardiomyopathic disease of interest in small or single center studies. Strength C: The specific genetic test or clinical test correlates with the cardiomyopathic disease of interest in case reports.) No international recommendations are available for genetic testing in familial hypercholesterolemia although a scientific position statement has been made by the AHA.[12–18]



syndrome and familial hypercholesterolemia, informative genetic testing has been reported in as many as 60% to 80% of cases, depending on the stringency of diagnostic criteria.[5,20] In others, such as hypertrophic cardiomyopathy, early interpretation of panel testing reported informative genetic testing in 40% to 50% of cases.[21] In dilated cardiomyopathy, where causes are diverse and molecular causes are less well characterized, initial reports of identified variants were as low as 20%.[21]

Importantly, recent population-level genomics data suggest that initial studies may overestimate the diagnostic power of panel-based testing. Walsh et al[22] recently examined the prevalence of variants previously thought to be pathogenic in the healthy and diseased population. Their study found that variants in some genes thought to be pathogenic for inherited cardiomyopathies were in fact over-represented in the healthy population. At the same time, in other genes, they found that current laboratory estimation of pathogenicity may be too conservative. Thus, our understanding of the genetic basis of these diseases remains incomplete. As we discuss in this review, our evolving definitions of genetic disease will rely on continued population-level studies, as well as the clinically and scientifically appropriate implementation of broader sequencing technologies.

## Evolution of Genetic Testing Strategies for Inherited Cardiovascular Disease

Traditional approaches to genetic testing for inherited cardiovascular disease involved amplification of specific exons and untranslated regions of genes of interest, followed by Sanger sequencing. These sequences were then compared with the human reference sequence and variants were called. New variants were viewed in light of a small panel of controls, often anonymous blood donors of unknown ethnicity. If a genotype for every position could be called, a clinical report would be released in which it was not uncommon to presume new variants in known genes were causal of disease.

Cardiovascular genetics was one of the earliest specialties to adopt NGS clinically. Because many inherited cardiovascular diseases are associated with multiple genes, this was a good choice for application of a methodology able to efficiently sequence many genes in parallel. With most NGS approaches, long genomic DNA molecules are first fragmented by ultrasound into billions of smaller molecules. These are then sequenced by methods described elsewhere,[23] and the resulting sequences are matched to the reference sequence creating a consensus sequence with coverage at each position referred to as read depth. From here, a genotype for every position is called (the 2 letters representing

the nucleotide at that position from the maternal and paternal chromosome). The power of this approach is such that most of the genome can be sequenced (WGS) in a fraction of the time compared with traditional sequencing, and at a fraction of the cost. When combined with a capture step (a process that enables DNA fragments from specific regions to be selectively amplified and sequenced[24]), selective regions of the genome can be analyzed, for example, an exome, or a panel of specific genes. A further significant advantage is the possibility of detecting somatic mosaicism, the occurrence, and propagation of variation in specific cell lineages during development. This is a rare but under-recognized cause of negative Sanger sequencing that is relatively easily and cheaply detected using NGS.[25]

## Multigene Panels for Genetic Testing

Regardless of the approach to sequencing, clinical genetic testing in inherited cardiovascular disease most frequently focuses on a small number of genes. Besides logistics and cost, the rationale for this approach is that for many inherited cardiovascular diseases, variants within a small number of genes explain a large proportion of the cases. For example, for hypertrophic cardiomyopathy, data suggest that 50% of identifiable variants are detected within myosin binding protein C (*MYBPC3*) and 33% are located within cardiac β-myosin heavy chain (*MYH7*), with smaller contributions from troponin T (*TNNT2*; 4%) and troponin I (*TNNI3*; 5%).[26] Similarly, 75% of patients with long-QT syndrome harbor pathogenic variants within 1 of the 3 genes: potassium voltage-gated channel subfamily Q member 1 (*KCNQ1)*, potassium voltage-gated channel, subfamily H member 2 (*KCNH2*), and sodium voltage-gated channel α subunit 5 (*SCN5A*).[27] Therefore, by screening a limited selection of genes using a gene panel, a relatively high diagnostic yield can be obtained. In the case where an initial gene panel fails to identify a disease-causing variant, clinicians can opt for a larger panel that includes genes more rarely associated with the phenotype, in addition to genes associated with syndromes that mimic the diagnosis in question, such as transthyretin (*TTR*) and galactosidase α (*GLA*) for hypertrophic cardiomyopathy.[28,29] Alternatively, a broader panel can be used from the outset that includes frequently implicated genes and genes implicated in related conditions. An important caveat to this approach is that broader panels may include genes with spurious disease associations, yielding an increased chance of identifying variants of uncertain significance.

Generating a validated list of genes associated with any inherited cardiovascular disease has proven challenging. This follows the realization that many genes suspected to influence inherited cardiovascular disease pathogenesis were derived from early studies that were underpowered to assign causality. For inherited cardiomyopathies, genes considered causal were recently re-evaluated by leveraging the clinical sequencing results from 7855 cardiomyopathy patients across 2 large clinical genetics services and 60 706 controls from the Exome Aggregation Consortium (ExAC).[22] This approach highlighted a range of hypertrophic cardiomyopathy, dilated cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy genes, previously assumed to be causative for disease, that harbored no greater variant burden than the control cohort.[22] Examples of such genes were troponin C (*TNNC1*), actinin alpha 2 (*ACTN2*), and myozenin 2 (*MYOZ2*) within hypertrophic cardiomyopathy. In dilated cardiomyopathy, genes with equivalent variation to the control population included junction plakoglobin (*JUP*), desmocollin 2 (*DSC2*), and ankyrin repeat domain 1 (*ANKRD1*). Similarly, transmembrane protein 43 (*TMEM43*) and lamin A/C (*LMNA*) failed to demonstrate an excess of protein altering variants within arrhythmogenic right ventricular cardiomyopathy cases compared with ExAC controls. Some of these genes, such as transmembrane protein 43 (*TMEM43*), have very strong evidence supporting their role in disease, but likely their variants are so rare or population-specific that they did not show a signal in this analysis. Other genes, such as myozenin 2 (*MYOZ2*), do not have strong evidence supporting causality.

Such approaches, harnessing data from large population studies, highlight the potential for research using high-throughput sequencing to actually narrow the field of focus for specific inherited conditions, decreasing the likelihood of reporting variants of uncertain significance. Data from such efforts have yet to narrow the breadth of commercial panels and disrupt the common belief that a panel with more genes has greater potential value to provide clinical benefit. Consequently, substantial heterogeneity is observed among panels offered by commercial providers. For example, panels from 14 laboratories offering a test for HCM include a total of 65 different genes, many more than the 8 sarcomeric genes that explain most of positive findings in HCM.[22,26] Although each of these 14 panels include all core sarcomeric HCM genes, a wide range of additional genes are also present, 25 of which feature only once across the 14 laboratories. On average, an HCM gene panel consists of ≈25 genes (median, 23.5; interquartile range, 20–26); however, some laboratories offer far more. For instance, the Illumina, Inc. TruSight Cardio Sequencing Kit is advertised as sequencing 47 genes on an initial inherited cardiac conditions gene panel.[30]

Further hope in consensus building in gene causality comes from projects such as ClinGen.[31,32] ClinGen is a National Institutes of Health–funded resource designed to summarize evidence and provide expert opinion on the clinical validity, pathogenicity, and utility of genes and variants used routinely within clinical genomics. However, disparities between the evidence used by laboratories and contemporary literature persist, and discussion with laboratories on such discrepancies is encouraged. Guidelines for the reporting of genetic testing results have been published by the American College of Medical Genetics and Genomics and the Association of Molecular Pathology, and are briefly summarized in Table 2.[19,31,33,34] A more general policy document was published by the American Heart Association.[35]

## Beyond Panel-Based Genetic Testing: Exome and Genome Sequencing

If a gene panel has returned negative results, more extensive sequencing can be considered in appropriate clinical situations. WES incorporates all exonic and flanking sequences, across ≈20 000 genes, representing ≈2% of the genome. WGS aims to determine the sequence of all ≈6 billion base pairs

**Table 2. Criteria Used to Assess Causality of Variants and Genes in Genetic Cardiovascular Disease**

| |
|---|
| Criteria for ranking variant causality |
| Lies in a gene with strong evidence for causality (see criteria for gene-level causality below) |
| Variants of similar type (eg, missense, frameshifts, or deletions) in this gene have strong evidence for causality |
| Sufficiently rare in reference samples |
| Reported to segregate with disease in multiple families (where other genetic and nongenetic causes have been excluded) |
| Multiple in silico algorithms predict negative impact of the variant on protein function |
| Lies in a domain or region that is enriched for disease-causing variation |
| In vitro or in vivo animal data support pathogenicity |
| Criteria for ranking gene-level causality |
| Segregates with disease in multiple families; at least 1 with LOD score of >3.0 |
| Multiple convincing cases reported |
| Prevalence of rare variation in cases is greater than in reference samples |
| In vitro or in vivo animal data support pathogenicity, preferably replicated by independent groups (including recapitulation of phenotype in animals or induced pluripotent stem cells) |

LOD indicates logarithm of odds.

Detailed information on types of evidence and their incorporation into specific classification of variant causality is available in the American College of Medical Genetics and Genomics guidelines,[33] MacArthur et al[34] and the Clinical Genome Resource (https://clinicalgenome.org/).[19,31]

to provide a comprehensive assessment of variation throughout the human genome. Importantly, in a situation where only a proband is available, candidate variants outside of known genes could be classified, at most, as a suspicious variant of unknown significance. However, in some settings, multiple affected family members are available, and cosegregation of disease with the variant can be demonstrated. In the application of these technologies for undiagnosed diseases (often called diagnostic odysseys), WES and WGS have reported diagnostic yields of 25% to 40%. This yield can be higher if a trio (usually mother–father–child) can be tested, which allows inheritance state modeling,[36–41] and such positive findings have led to the rapid adoption of these technologies for solving these cases both routinely and in international efforts such as the Undiagnosed Diseases Network.[42]

## Technical Considerations for NGS-Based Clinical Tests

For all genetic tests utilizing NGS, clinical decision making is contingent on the quality of the sequencing performed.[43] With a new technology developed primarily for a research market, there is potential for accuracy below clinical standards at each step.

## Reference Materials for Human Genomics

The Human Genome Project sequenced the first human genome.[44] This reference genome was derived from 50 volunteers, DNA from a smaller number contributing variably to allow generation of a haploid (single copy) reference sequence.[45,46] The resulting reference genome then does not represent 1 individual but rather has a disjointed haplotype structure. In addition, it contains many disease-associated alleles. It has benefitted from sustained iterative improvement by the Genome Reference Consortium.[46] The latest version of the reference genome (GRCh38) was released in May 2014.

The current human reference genome provides a standard for identifying and cataloging variants. However, because it was generated from multiple individuals, it is challenging to use for benchmarking the accuracy of new technologies or algorithms. To meet this need, the National Institute of Standards and Technology provided support to a consortium named Genome in a Bottle. This effort generated a set of high confidence genotype calls across the genome for multiple individuals with associated reference materials.[45,47,48] The high confidence genotype calls provide an appropriate standard against which the quality and accuracy of clinical sequencing efforts can be measured.[48–50]

## Target Capture

To selectively sequence a small region of the genome, such as a panel of genes, capture assays can be used. These take advantage of probes complementary to the human reference sequence for the genomic region of interest. This approach has several advantages (eg, cost) and challenges, such as reference bias, coverage trade-offs, and difficulty detecting large structural variants.

Target capture is optimized for pull down of reference sequences as opposed to DNA sequences that include potentially pathogenic variants, a phenomenon known as reference bias. In addition, for exome sequencing, the probe maps were first designed for cohort variant discovery. This meant balancing some degree of genome-wide coverage with an overall median coverage that was commercially viable. Finally, targeted capture approaches have low sensitivity for detecting structural variants in part because of uneven coverage relative to WGS.[51] Emerging solutions to these issues are discussed in detail below.

## Sequencing

Technical descriptions of next-generation sequencing methods are provided elsewhere.[23] The dominant technology in clinical sequencing is sequencing by synthesis from Illumina, Inc. It was developed at Cambridge University, United Kingdom, and first commercialized by Solexa, Inc. The current version generates reads from 100 to 350 bp, termed short-read sequencing, that have a low (eg, 0.1%–1%) per base error rate, but have systematic challenges with areas of high GC or AT content.

Long-read sequencing approaches, such as those emerging from Pacific Biosciences, Inc. and Oxford Nanopore Technologies Ltd, generate reads in the 5- to 10-kb range with a long tail stretching into the tens of thousands of base pairs. Despite the higher per-read error rate (typically 10%–20%), long-read sequencing technologies are particularly useful for genomic regions that short-read sequencing technologies struggle to accurately characterize, for example, highly polymorphic regions (such as the Human Leukocyte Antigen

[HLA] region), tandem repeats, or paralogous sequences. Their error profile tends to be more random although they are more challenged by homopolymer regions (regions with the same nucleotide repeated sequentially). Long-read sequencing technologies have higher sensitivity for structural variants.[52–56] A corollary of this is that current estimations of the prevalence of structural variation in inherited cardiovascular disease are likely underestimations because of the limitations of short-read sequencing technology and targeted capture.[57–59] Recent investigations have demonstrated that structural variation contributes to congenital heart disease[60] and that copy number variants and large deletions explain a non-negligible percentage of long-QT syndrome in patients with negative genetic testing.[61,62] Large deletions have also been reported in the cardiac sarcoplasmic reticulum calcium release channel, ryanodine receptor type 2 (*RYR2*), in catecholaminergic polymorphic ventricular tachycardia.[63,64]

Despite these advantages, long-read sequencing technology remains prohibitively expensive for widespread adoption within clinical genomics at this time, and most laboratories instead attempt to infer structural variation from either read depth analysis performed using short-read exome sequencing data or via the addition of an exon array (using microarray-based comparative genomic hybridization technology).

## Alignment and Variant Calling

After sequencing, bioinformatic tools are used to organize the sequenced reads, a process known as alignment. This involves the accurate mapping of hundreds of millions of sequence reads to corresponding portions of the reference genome. The dominant method of alignment uses a technique borrowed from the science of data compression, known as the Burrows–Wheeler Transform.[65] After alignment, variants from the reference sequence are called. To do this, the first step is to determine whether the nucleotide position has been covered by sufficient reads of adequate quality to allow a genotype call. As noted above, capture approaches lead to highly variable coverage across captured genomic regions. As a result, clinical reporting using median coverage statistics can be misleading. For some time, providers of clinical WES reported a footnote median coverage statistic, for example, ×10 coverage for >90% of the genes (where for Nx, N refers to the number of times a base position has been sequenced). However, recognizing that disease can result from a single base pair change (theoretically anywhere along the length of a gene) and that widely differential coverage along a gene could thus significantly impair the sensitivity of a test, this metric fails to capture the essential data required to judge clinical quality of the test.

In response to this need, we recently proposed a new metric for use in clinical sequencing: the Clinical Quality measure. It describes for a given gene the absolute number of base pairs that are not uniquely covered by 20 high-quality reads (Figure 1).[66] The metric includes 3 distinct components: the number of reads covering a given position, the mapping quality for those reads, and the quality of the bases covering a given position. Base quality in genomics relates to the probability of an accurate base call within a given read. It is expressed as the $-10\times\log_{10}(P)$, where $P$ is the probability of error.[67,68] This is known as the Phred score and denoted with a preceding Q. Typical thresholds are 1% error (Q20) and 0.1% error (Q30). The mapping quality score is a response to the fact that a given short read may map to multiple locations. Indeed, this is an inevitable consequence of the anatomy of the genome, 5% of which originates 100 base pair reads identical to a different part of the genome.[45] These identical regions mostly derive from closely related gene families and pseudogenes. Although no consensus exists on how to treat these reads (discard, map to all locations, and map to random location), the fact that a read is nonuniquely mapped is of importance because each genomic area will be subject to different selection pressure. Our view is that for a metric to represent noninferiority with Sanger sequencing (where a specific region of the genome is selectively and uniquely amplified before sequencing), we should only count uniquely mapped reads (MAPQ>0).[45] Within inherited cardiovascular disease, mapping is particularly relevant for ion channels, which harbor significant repetition and redundancy.[45]

After genome alignment, areas of the genome that do not match the reference are called as variants. However, our current algorithms vary widely in their ability to detect different classes of variation, for example, single-nucleotide variation; small insertions and deletions; and larger structural variants. Algorithms used in clinical environments were developed by academic researchers for the purposes of cohort variant

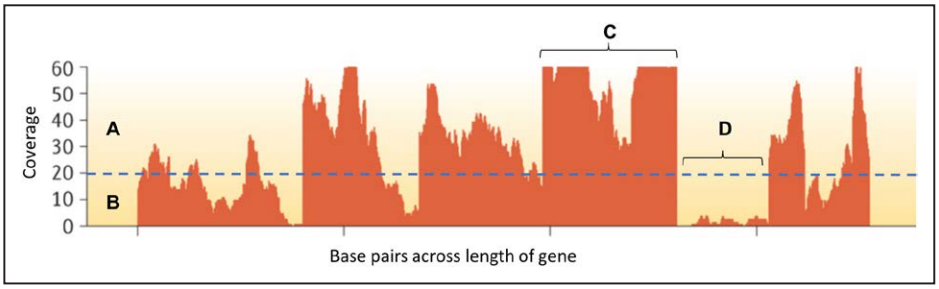


**Figure 1.** Variable sequence coverage within genes makes average coverage an inadequate metric. Not all base pairs within a gene will achieve the necessary ×20 threshold used in the Clinical Quality (CQ) metric. Areas shaded red represent gene base pairs that have been sequenced. Coverage has been represented on the vertical axis and refers to the total number of times a base pair has been sequenced. In this diagram, the label A denotes coverage >×20 and the B represents coverage of <×20. Base pairs sequenced by >20 reads are considered to satisfy the coverage aspect of the CQ metric. The section denoted by the label C represents a region within the gene where all base pairs have achieved at least ×20 coverage. Section D highlights a section of the gene with inadequate coverage and would not satisfy the CQ metric. Adapted from Ashley[66] with permission of the publisher. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.

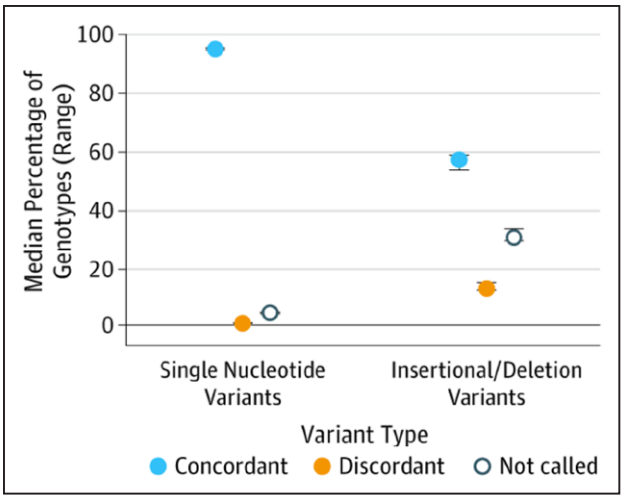


**Figure 2.** Calling algorithms are better suited to the calling of single-nucleotide variation than other forms of variation, such as insertion/deletion variants. Adapted from Dewey et al[69] with permission of the publisher. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.

discovery, shortly after the genome-wide association study era when there was significant focus on single-nucleotide variation. Thus, methods for calling single-nucleotide variation tend to be accurate and reliable, whereas the ability to call other forms of variation is substantially lower (Figure 2).[69] Indeed, in 1 study, although >99% of single-nucleotide variants were concordant with an orthogonal technology, small insertions and deletions exhibited concordance as low as 33%.[45,69] Newer approaches are being developed that improve these metrics, but continued work is required.

## Exome Versus Whole Genome

There remains ongoing debate as to whether exome or genome sequencing is preferable for genome-wide testing. The Clinical Quality metric described above provides a useful framework to investigate this question.[70] We calculated this metric for data sets in each of 3 categories: standard exome capture, exome capture augmented for clinical purposes, and WGS. An augmented exome is enhanced either by post hoc Sanger sequencing or through the addition of specific capture probes along with sequencing parameters optimized to improve overall sequencing coverage. We found that for genes known to be associated with disease, augmented exome approaches were superior to a standard clinical whole genome (sequenced to an average of ×30 coverage). Augmented exome approaches and whole-genome approaches were both markedly better than a standard exome (Figure 3).[43] However, for genes not currently known to play a role in human disease (and therefore not augmented in the clinical exome), the whole-genome approach performed better. We hypothesized that at a certain coverage level, the whole-genome approach could equal the Clinical Quality of the augmented exome even for the medical genome. We found this was established at whole-genome coverage of ≈×45.[43] However, achieving this requires 50-fold more sequence data than the augmented exome approach, with attendant implications for the cost of storage and compute across a much larger data file.[43]

## Filtering Methods for Called Variants

Most human genetic variation is rare. This means that in any given individual's whole-genome sequence, many millions of variants will be reported as different from the reference genome. Some of these variants will be commonly found in the population, some will be rarely found, and many will never have been seen before. This general principle applies to

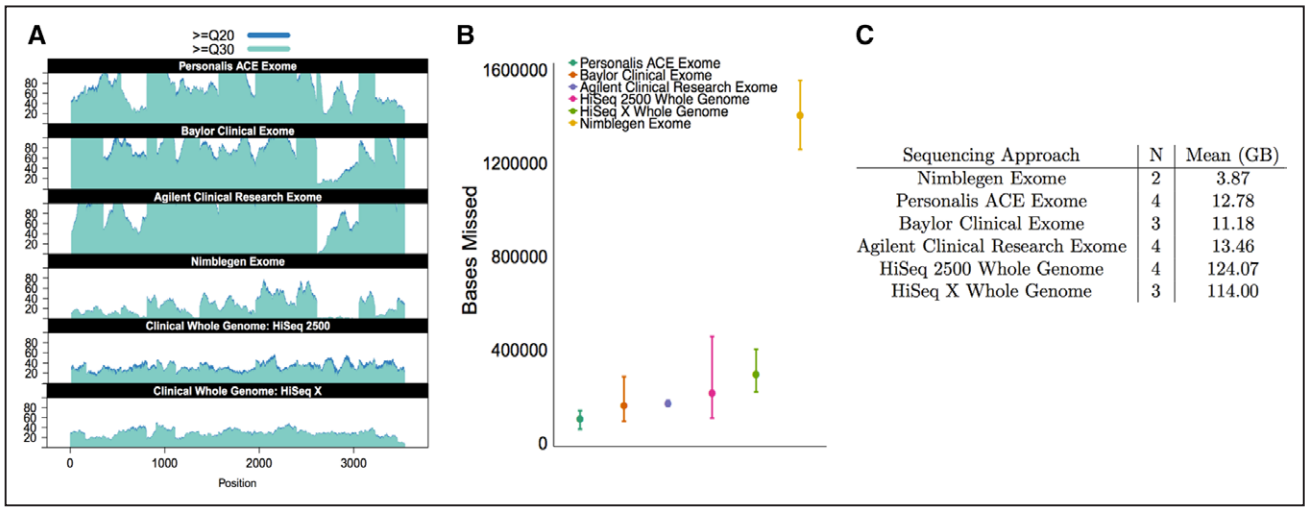


**Figure 3.** A visual demonstration of the variable coverage achieved across a gene. **A**, The varying levels of coverage seen across a gene (in this case *KCNH2)* when utilizing clinical whole-genome sequencing (HiSeq 2500 and HiSeq X), standard exome sequencing (Nimblegen), and a selection of augmented exomes (Personalis ACE exome, Baylor Clinical exome and Agilent Clinical Research Exome). **B**, A graphical representation of the number of base positions that do not meet the Clinical Quality threshold, defined as ×20 Q20 mapQ >0, for all clinically relevant genes catalogued within the ClinVar database (n=3062) using clinical whole-genome sequencing (HiSeq 2500 and HiSeq X), standard exome sequencing (Nimblegen), and a selection of augmented exomes (Personalis ACE exome, Baylor Clinical exome, and Agilent Clinical Research Exome). **C**, Mean gigabases of sequencing for each platform and shows the number of samples included from each platform in **B**. Note that these data are not from polymerase chain reaction–free preparations. Adapted from Goldfeder and Ashley[43] with permission of the publisher. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.

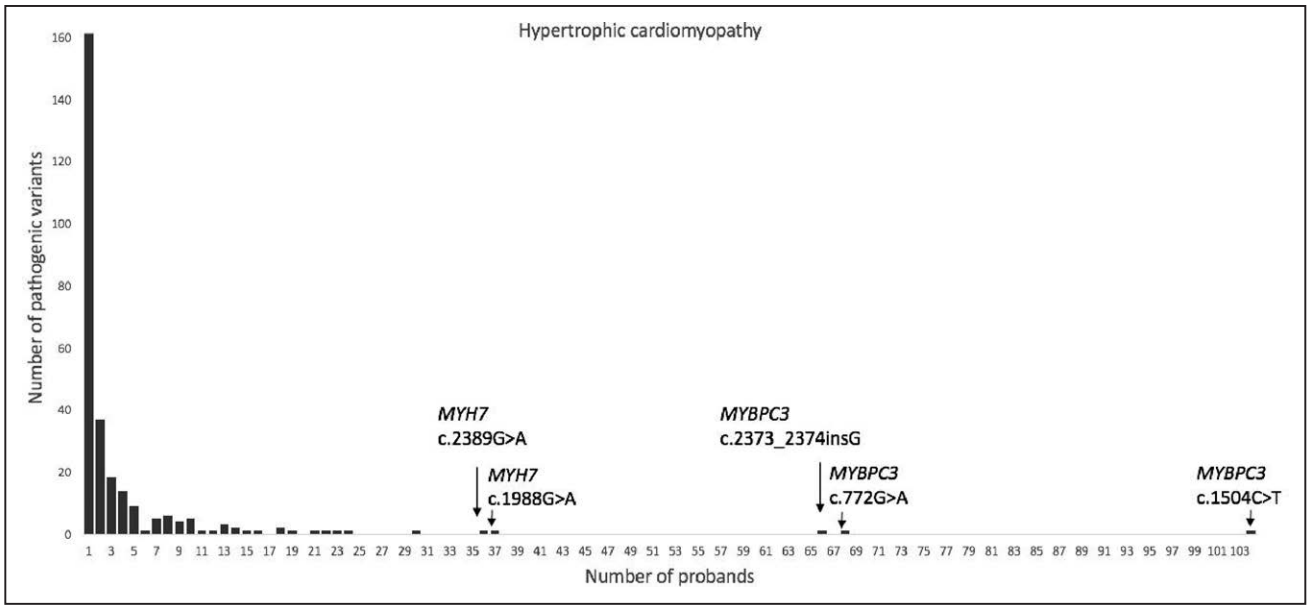


**Figure 4.** Frequency plot demonstrating the marked locus heterogeneity of pathogenic variants observed within eight robustly associated sarcomeric genes (*MYBPC3*, *MYH7*, *TNNI3*, *TNNT2*, *TPM1*, *MYL2*, *MYL3*, and *ACTC1*) for hypertrophic cardiomyopathy. Adapted from Alfares et al[26] with data derived from Walsh et al.[22] with permission of the publisher. The 5 most frequent variants are individually annotated. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.

variants that are benign, pathogenic, or neutral in their effect. A useful example is seen in Figure 4, where the frequency distribution of pathogenic variants for HCM demonstrates that most have only been reported once.[22,26] After variant calling, the first step is filtering to exclude variants that are not likely to be the cause of a Mendelian syndrome. Recent study has provided a statistical framework for a frequency cutoff useful for this purpose.[71] Based on maximum currently observed frequencies of pathogenic variants in genes with known population variant frequencies, this study provides a practical approach to filtering.[72] For inherited cardiovascular diseases, such calculations suggest that the maximum allele frequency of any potentially causative variant could be much lower than traditional threshold of ≈0.1%. For example, in hypertrophic cardiomyopathy, it has been estimated that the maximal allele frequency for a causative variant will be no greater than $4\times10^{-5}$.[71]

## Interpreting Results From Next-Generation Sequencing

Once a shortlist of variants has been generated, variants are annotated and passed to biocurators who provide broad interpretation and classification of the variants.[73] Accurate interpretation of NGS variants requires understanding and evaluation of multiple sources of evidence.[34] These can be divided into evidence that (1) the gene is causal for the disease in the population and (2) the variant is causal for the disease in the individual, as outlined in Table 2. Detailed guidelines for variant and gene interpretation have been published by the American College of Medical Genetics and Genomics and are widely used for reference in clinical practice.[33] Variants can also be characterized by computational predictions, which are based on factors such as nucleotide conservation across phylogeny or predicted effects on protein structures.[74–76] Thorough assessment of gene or variant causality requires extensive literature review and critical reappraisal of the primary data. Variants are then placed in a category based on the level of evidence supporting causality: (1) pathogenic, (2) likely pathogenic, (3) variant of unknown significance, (4) likely benign, or (5) benign. These categories are not intended to represent the probability of causality in a linear proportional manner; labeling a variant with pathogenic or benign is intended to require ≈90% confidence in either direction (Figure 5).[77]

## Standardizing Sequencing and Interpretation Practices

Classifying variants accurately is challenging, and significant discordance in variant classification is observed between

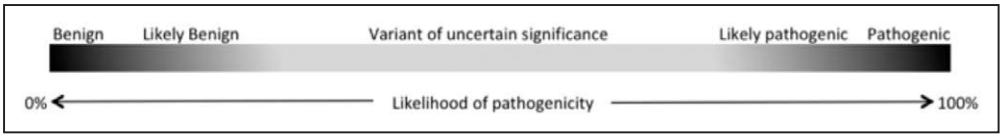


**Figure 5.** Genetic test interpretation is probabilistic. Variants found through genetic testing are classified as benign, likely benign, uncertain significance, likely pathogenic, or pathogenic as per American College of Medical Genetics and Genomics guidelines. These various classifications fall along a spectrum of likelihood that the variant is disease causing, with benign at one end, pathogenic at the other, and a broad range of uncertainty in the middle. Reprinted from Wilson et al[77] with permission of the publisher.

professionals and laboratories.[31,78,79] Certainly, the American College of Medical Genetics has published detailed guidelines for both laboratories and clinicians on the classification and reporting of variants.[33] Despite these efforts, a recent study of the ClinVar database showed discordant classification of 17% of variants with >1 submitter.[31] Another study of interpretation of 99 variants spanning the classification system showed concordance between 9 laboratories as low at 34% based on the American College of Medical Genetics and Genomics guidelines. This rose to 71% after discussion between these laboratories.[79] Systematizing the approach to variant classification and adding refining detail for specific conditions and genes may diminish this discordance. Meanwhile, development of computational and biophysical estimation of variant effects continues. Some recent efforts have focused on determining the tolerance[80] of defined areas of a gene to variation based on structural data including the location of internal promoters and 3-dimensional protein modeling. These emerging tools, in combination with kindred-level segregation data and population-level data, continue to improve concordance in variant classification.[81,82] Furthermore, to assist with the widespread adoption of best practices, the Food and Drug Administration has launched a public, crowd-based platform, termed precisionFDA (https://precision.fda.gov/).[83] One of PrecisionFDA's objectives is to help maximize best practice in NGS application by supporting regulatory science in this area and encouraging free sharing of open source software.[50]

## Conclusion

Clinical genetic testing for inherited cardiovascular disease provides value for the patient, family, and provider. For individuals with an inherited cardiovascular disease, testing can clarify diagnosis and reported pathogenic variants can be used for predictive testing in family members. Focused panels are preferred over broad panels. For patients in whom panel-based testing is unrevealing, WES and WGS may be considered, especially when the cardiovascular abnormality is part of a syndromic presentation or where multiple family members are affected and can be tested for segregation of suspicious variants. With appropriate augmentation, exome approaches can be used with interpretation limited to a focused number of genes.

As the power and availability of our clinical genetic technologies increase, several challenges remain. Applying a noninferiority standard for NGS with respect to Sanger sequencing is critical. This will maximize our confidence in calls made at every position for every gene of interest, the reproducibility of each call (particularly for complex variation), and the concordance with respect to assessment of each gene or variant's pathogenicity. New long-read sequencing approaches and ongoing improvement in algorithms for calling and interpretation are the next steps to ensuring the clinical accuracy and validity of these technologies. We are confident that the promise of NGS, so richly realized in genetic discovery to date, can continue to pay dividends for the clinical management of patients with inherited cardiovascular disease.

## Disclosures

C. Caleshu is a consultant/advisor for Recombine and advisor for Invitae and a consultant for GeneDx. Euan Ashley has ownership interest and is an advisor for Personalis Inc. He is an advisor for SequenceBio, Myokardia, Heart Metabolics, Genome Medical, and Avive. The other authors report no conflicts.

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