

# Genetic advances in sarcomeric cardiomyopathies: state of the art

Carolyn Y. Ho<sup>1\*</sup>, Philippe Charron<sup>2,3</sup>, Pascale Richard<sup>2</sup>, Francesca Girolami<sup>4</sup>, Karin Y. Van Spaendonck-Zwarts<sup>5</sup>, and Yigal Pinto<sup>6</sup>

<sup>1</sup>Cardiovascular Division, Brigham and Women's Hospital, Boston, MA, USA; <sup>2</sup>Centre de référence maladies cardiaques héréditaires, ICAN, Inserm UMR\_1166, Hôpital Pitié-Salpêtrière, AP-HP, Paris, France; <sup>3</sup>Université de Versailles-Saint Quentin, Hôpital Ambroise Paré, AP-HP, Boulogne-Billancourt, France; <sup>4</sup>Genetic Diagnostic Unit, Careggi University Hospital, Florence, Italy; <sup>5</sup>Department of Clinical Genetics, Academic Medical Center Amsterdam, University of Amsterdam, Amsterdam, The Netherlands; and <sup>6</sup>Department of Cardiology, Academic Medical Center Amsterdam, University of Amsterdam, Amsterdam, The Netherlands

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Genetic studies in the 1980s and 1990s led to landmark discoveries that sarcomere mutations cause both hypertrophic and dilated cardiomyopathies. Sarcomere mutations also likely play a role in more complex phenotypes and overlap cardiomyopathies with features of hypertrophy, dilation, diastolic abnormalities, and non-compaction. Identification of the genetic cause of these important conditions provides unique opportunities to interrogate and characterize disease pathogenesis and pathophysiology, starting from the molecular level and expanding from there. With such insights, there is potential for clinical translation that may transform management of patients and families with inherited cardiomyopathies. If key pathways for disease development can be identified, they could potentially serve as targets for novel disease-modifying or disease-preventing therapies. By utilizing gene-based diagnostic testing, we can identify at-risk individuals prior to the onset of clinical disease, allowing for disease-modifying therapy to be initiated early in life, at a time that such treatment may be most successful. In this section, we review the current application of genetics in clinical management, focusing on hypertrophic cardiomyopathy as a paradigm; discuss state-of-the-art genetic testing technology; review emerging knowledge of gene expression in sarcomeric cardiomyopathies; and discuss both the prospects, as well as the challenges, of bringing genetics to medicine.

## Keywords

Genetics • Hypertrophic cardiomyopathy • Dilated cardiomyopathy • Sarcomere • Next Generation Sequencing

This article is part of the Spotlight Issue on Sarcomeric cardiomyopathies: from bedside to bench and back.

## 1. Introduction

The 'genetic era' of cardiomyopathies was ushered in by the pivotal discovery that sarcomere mutations cause hypertrophic cardiomyopathy (HCM),<sup>1</sup> and the more recent recognition that they also cause dilated cardiomyopathy (DCM).<sup>2</sup> Now, >25 years after initial description, genetic studies continue to demonstrate that HCM is predominantly a disease of the sarcomere, although the genetic basis of DCM is more diverse. Additionally, sarcomere mutations have been identified in association with more complex disorders of cardiac structure and function, including restrictive physiology and left ventricular non-compaction.<sup>3,4</sup> These observations illustrate that different variants within the same sarcomere gene can result in both overlapping and divergent clinical manifestations. The considerable variability in penetrance and expressivity displayed by individual mutations, not only across unrelated families, but

even within the same family, indicate that factors other than the sarcomere mutation itself influence clinical course and outcomes. These genetic, epigenetic, and environmental modifiers play an important role but are not yet characterized or understood.

Genetic testing was initially possible only in research laboratories capable of performing meticulous linkage analysis and candidate gene sequencing in large, well-characterized families with obvious inherited disease. However, advances in contemporary DNA-sequencing methodology make gene-based diagnosis increasingly feasible in routine clinical practice. Faster and more affordable genetic testing provides unprecedented opportunities to improve diagnostic certainty when evaluating patients and families with relatively non-specific phenotypes of cardiac hypertrophy, dilation, and contractile dysfunction.

\* Corresponding author. Cardiovascular Division, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA. Tel: +1 617 732 5685; fax: +1 617 264 5265, Email: cho@partners.org

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With molecular-level diagnosis, physicians are able to specifically differentiate primary sarcomeric cardiomyopathies from other entities that may have a similar crude appearance, but different underlying pathophysiology, inheritance patterns, natural history, and treatment strategies. In this manner, genetic cardiomyopathies can be differentiated from phenocopies, such as hypertensive heart disease, athlete's heart, and storage/metabolic disorders. This critical advantage, afforded by genetic testing, has important implications for the care of the individual patient, as well as their family.

Moreover, gene-based diagnosis is independent of clinical manifestations, allowing precise identification of at-risk individuals in the preclinical stage—prior to diagnosis. If a pathogenic mutation can be identified in a family, other relatives can undergo predictive genetic testing to determine whether they have inherited the mutation, well before a clinical diagnosis can be established. In addition to guiding the care of the individual and family, this capability creates unique prospects for developing therapies that can modify or prevent disease; targeting at-risk individuals before irreversible changes to the heart's structure and function have occurred and at a stage where disease-modifying therapy may be more successful. From an investigative perspective, identifying pathogenic sarcomere mutations underlying hypertrophic and dilated cardiomyopathies provides a powerful handle to interrogate fundamental mechanisms governing myocyte biology, structure, and function in health and in disease. Ongoing and future basic science and clinical translational collaborations will continue to dissect the precise pathways driving how these mutations remodel the heart, and identify rational targets for therapies designed to interrupt the emergence of pathology.

In this section, we review the genetic landscape of sarcomeric cardiomyopathies, focusing on the most robustly described conditions of HCM and DCM. We discuss how gene-based diagnosis of HCM can now be applied in the clinical setting; the state-of-the-art regarding genetic testing technology and interpretation; and emerging knowledge regarding modifiers of gene expression that may partly explain the remarkable phenotypic diversity of these important and intriguing conditions. Identifying the genetic basis of disease is a critical first step on a path that will hopefully advance and transform medicine, by allowing rational treatment based on mechanistic insight, early diagnosis, and disease prevention.

## 2. Gene-based diagnosis of HCM in the clinical setting

### 2.1 Historical perspective

Although familial aggregation with disease has been demonstrated since the first modern description of HCM in the late 1950s, the genetic basis was definitely demonstrated only in 1989, when linkage analysis revealed an association between HCM and a locus on chromosome 14q1.<sup>5</sup> The responsible gene, *MYH7*, coding for the beta-myosin heavy chain protein was identified shortly thereafter.<sup>1</sup> In this manner, HCM became the first cardiac disease for which a molecular genetic cause was determined. Since that description, well over a thousand mutations in numerous genes have been described as disease causing, most commonly involving eight major sarcomeric genes (Table 1).

### 2.2 Genetic knowledge: where are we now?

#### 2.2.1 Prevalence and inheritance

HCM is a relatively common monogenic cardiovascular disease. The prevalence of the clinical phenotype of unexplained LVH is ~1:500 in

the general population.<sup>6</sup> If systematic cardiac screening of the family is performed, we roughly estimate that >50% of patients have familial disease, with at least one other affected family member. Autosomal dominant inheritance is most commonly seen. HCM is a global disease, described in >50 countries representing a variety of different ethnic origins.

#### 2.2.2 Sarcomere gene mutations

In 1990, a mutation in  $\beta$ -myosin heavy chain (*MYH7*) was first identified as responsible for causing HCM.<sup>1</sup> During the next decade, numerous genes were reported to be associated with disease (Table 1). Most of these genes encode proteins of the myofilaments or Z-disc of the sarcomeres. As such, HCM has been described as a 'disease of the sarcomere'. Sarcomere genes definitively shown to have a pathogenic role in HCM include three elements of the thick filament: *MYH7*— $\beta$ -myosin heavy chain, *MYL2*—regulatory myosin light chain, and *MYL3*—essential myosin light chain; four elements of the thin filament: *TPM1*— $\alpha$  tropomyosin, *TNNT2*—cardiac troponin T, *TNNI3*—cardiac troponin I, and *ACTC1*—cardiac actin; and an assembly protein: *MYBPC3*—cardiac myosin-binding protein C. Rare variants have been reported in other genes of the sarcomere apparatus, or genes coding for proteins of the adjacent Z-disc, such as *MYH6*— $\alpha$  myosin heavy chain, *CSRP3*—muscle LIM, *TCAP*—telethonin,<sup>7</sup> as well as in genes involved in calcium homeostasis pathways (*VCL*—vinculin and *JPH2*—junctophilin 2). However, for some of these genes, evidence for direct pathogenicity in HCM has not been clearly established.<sup>8</sup>

Over the past two decades, there has been extensive molecular screening of sarcomere genes. More than 1400 mutations have been described in association with HCM. Pathogenic mutations are found in ~50–60% of familial HCM and in 30–40% of apparently sporadic cases with no family history.<sup>9</sup> Almost all mutations associated with HCM are 'private mutations', identified in one or only a few families. However, rare mutational 'hot spots' have been recognized, such as *MYH7* Arg403Gln and Arg453Cys; *TNNT2* Arg92Gln and Arg92Trp; and *MYBPC3* Arg502Trp and c.1928-2A>G.<sup>9,10</sup> These mutations have recurrently arisen in a larger number of unrelated families.

Pathogenic mutations are often missense substitutions, resulting in exchange of a single amino acid for another at the protein level. Alternatively, and particularly in the *MYBPC3* gene, mutations predicted to lead to more radical structural changes or truncation of the protein are identified, such as frameshift mutations (mutations resulting in the insertion or deletion of  $\geq 1$  nucleotide in the coding region of genes), non-sense mutations leading to premature termination of translation, or mutations affecting normal splicing of mRNA (mutations involving the consensus splice sites, or intronic mutations leading to cryptic abnormal splice sites).<sup>11</sup> These putative truncating mutations can result in shortened proteins or the absence of the mutant protein due to mRNA decay mechanisms, leading to haploinsufficiency or disruption of normal sarcomere function. Additionally, mutations in genes encoding sarcomere proteins are associated with highly variable clinical phenotypes, including restrictive cardiomyopathies, and left ventricular non-compaction.<sup>12,13</sup>

#### 2.2.3 Distribution of genes

Using current genetic approaches, roughly 50% of patients with a clinical diagnosis of HCM will not have a specific genetic defect identified after genetic testing. In patients who are found to have a pathogenic or likely pathogenic DNA variant, not all disease genes are equally represented. Mutations in  $\beta$ -myosin heavy chain (*MYH7*) and myosin-binding

**Table 1** Genes associated with hypertrophic cardiomyopathy

	Gene	Location	Frequency (%)
<b>Prevalent sarcomeric genes</b>			
Thick filament			
β-Myosin heavy chain	<i>MYH7</i>	14q11.2	20–30
Regulatory myosin light chain	<i>MYL2</i>	12q23–q24	2–4
Essential myosin light chain	<i>MYL3</i>	3p21.3	1–2
Thin filament			
Cardiac troponin T	<i>TNNT2</i>	1q32.1	10
Cardiac troponin I	<i>TNNI3</i>	19q13.4	7
α-Tropomyosin	<i>TPM1</i>	15q22.1	<1
α-Cardiac actin	<i>ACTC1</i>	15q11q14	<1
Intermediate filament			
Cardiac myosin-binding protein C	<i>MYBPC3</i>	11p11.2	30–40
<b>Rare sarcomeric and Z-disc genes<sup>a</sup></b>			
α-Actinin 2	<i>ACTN2</i>	1q43	<1
α-Myosin heavy chain	<i>MYH6</i>	14q11.2	Rare
Muscle LIM protein	<i>CSRP3</i>	11p15.1	Rare
Telethonin	<i>TCAP</i>	17q12	Rare
<b>Calcium handling or regulation genes<sup>a</sup></b>			
Phospholamban	<i>PLN</i>	6q22.3	Rare
Calsequestrin	<i>CASQ2</i>	1p13.1	Rare
Junctophilin 2	<i>JPH2</i>	20q13.12	Rare
<b>Non-sarcomeric genes associated with cardiac hypertrophy</b>			
Gene		Associated phenotypes	Transmission/Frequency
Protein kinase, AMP-activated, gamma 2 subunit	<i>PRKAG2</i>	Wolff–Parkinson–White syndrome	Dominant/rare
Lysosomal-associated membrane protein 2	<i>LAMP2</i>	Danon disease	Dominant/rare
Galactosidase, alpha	<i>GLA</i>	Fabry	X Linked/1–2% of males
Four and a half LIM domains 1	<i>FHL1</i>	FHL1-related diseases	X Linked/rare
Transthyretin	<i>TTR</i>	Amylose	Dominant
Glucosidase, alpha	<i>GAA</i>	Pompe	Recessive/rare
Protein tyrosine phosphatase, non-receptor type 11	<i>PTPN11</i>	Noonan	Dominant/rare
Frataxin	<i>FXN</i>	Friedreich	Recessive/rare

<sup>a</sup>For these genes, evidence for pathogenicity in HCM has not been clearly established.

protein C (*MYBPC3*) are responsible for the great majority of HCM, accounting for >70% of identified mutations (~25 and 45% of cases, respectively). Other genes including *TNNT2*, *TNNI3*, *TPM1*, *MYL2*, *MYL3*, and *ACTC1* each account for a small proportion of patients (1–5% each).<sup>9,12</sup>

### 2.2.4 Complex genotypes

A patient with HCM typically carries one heterozygous disease-causing mutation in a single allele of a sarcomeric gene. However, an important proportion of HCM patients, 5–7% of patients in our experience (using Sanger Sequencing), has been reported to carry multiple (2 or more rarely 3) sequence variants in sarcomere genes. Higher proportions of complex genotypes have also been recently reported using next-generation sequencing (NGS) technologies; however, these reports often include variants of uncertain pathogenicity. These complex genotypes include compound heterozygous patients (heterozygous mutation in each allele of the same gene), double heterozygous patients (heterozygous mutations in two different genes: digenism), and rare homozygous patients.<sup>9,14</sup>

### 2.2.5 Pathogenic vs. non-pathogenic mutations: pitfalls in the interpretation of variants

As described below in the Section 3, a major challenge facing the molecular diagnosis of genotypically diverse diseases such as HCM lies in determining whether the observed sequence variant should be considered a pathogenic, disease-causing mutation or as a non-pathogenic variant (benign polymorphism). Making the correct variant classification is critical to the appropriate use of genetic testing in the clinical management of the patient and their family.

## 2.3 Penetrance and phenotype–genotype relationships

### 2.3.1 Penetrance

Before the molecular era, it was commonly assumed that HCM would be fully manifested before 20 years of age. We know now that mutation penetrance (the percentage of subjects bearing a mutation who express the phenotype) is highly variable and may be substantially delayed<sup>15–20</sup> or incomplete. Left ventricular hypertrophy (LVH) may not develop until the third decade or beyond. In a French study, penetrance was 55% between 10 and 29 years, 75% between 30 and

49 years, and 95% after 50 years.<sup>15</sup> Furthermore, penetrance may differ according to gender (potentially earlier in males than in females), and in some cases it may be incomplete (no cardiac expression at advanced age),<sup>15–19</sup> especially in the context of founder mutations<sup>21,22</sup> that are expected to have less dramatic impact as they have escaped evolutionary pressure.

Early investigations suggested rudimentary phenotype–genotype correlations since the high degree of phenotypic variability appeared to be partly explained by genetic heterogeneity. Some genes, or some particular mutations, were described as associated with a high degree of sudden death (e.g. *TNNT2* mutations; *MYH7* p.Arg403Gln), modest LVH (*TNNT2*), or delayed cardiac expression (*MYBPC3*).<sup>16–20</sup> Subsequent studies, based primarily on individual probands rather than families, have reported exceptions and more heterogeneous cardiac expression within a given gene or mutation, although recent reports from the UK suggest a higher risk of sudden death in *TNNT2* vs. *MYBPC3* families (0.93 vs. 0.46% per year).<sup>23,24</sup> With currently available phenotyping tools, no clear and consistent correlations are observed for most of mutations. Our ability to draw more accurate genotype–phenotype correlations over the spectrum of disease will improve in the future as more robust and quantitative methods to assess phenotype are identified, and as we are able to follow larger genotyped cohorts over time. At a broader level, patients with complex genotype and multiple simultaneous mutations may have more severe or early disease expression, related to a gene dosage effect. This was clearly described in small families<sup>9,14,25–27</sup> and recently suggested in a larger study.<sup>28</sup> Finally, some studies have observed that patients with a pathogenic sarcomere mutation had an increased risk of cardiovascular events, especially heart failure, compared with patients with negative genetic testing and no sarcomere mutation.<sup>29–31</sup>

## 2.4 Non-sarcomeric genetic aetiologies

Some patients may have conditions that bear superficial similarity to HCM, as defined by the presence of ‘unexplained LVH’, even familial in some cases, but genetic testing reveals that disease is not caused by sarcomeric mutations (Table 1). These phenocopies represent distinct disease entities that differ with respect to inheritance, pathophysiology, natural history, extra-cardiac features, and management. In a US paediatric registry, ~25% of children diagnosed with HCM have non-sarcomeric genetic aetiologies, mainly related to inborn errors of metabolism (e.g. Pompe disease), malformation syndromes (e.g. Noonan syndrome), and neuromuscular disorders (e.g. Friedreich ataxia).<sup>32</sup> The proportion of non-sarcomeric aetiologies in adults is lower but includes metabolic storage diseases such as Fabry disease (*GLA* gene), Danon Disease (*LAMP2* gene), familial amyloidosis (*TTR* gene), LVH associated with Wolff–Parkinson–White syndrome (*PRKAG2* gene), and mitochondrial cardiomyopathies. Genetic testing, guided by a careful diagnostic work-up of the patient and their family,<sup>33</sup> is a crucial tool for early recognition and appropriate management of these diseases.

## 2.5 Current use of genetic testing in clinical management

### 2.5.1 Genetic testing and genetic counselling

Advances in the understanding of the molecular genetics of HCM have created new expectations regarding genetic counselling, DNA testing, and applying this knowledge in clinical practice. Specific clinical objectives include: informing patients and families about the genetic aspects of their disease, including the risk of transmitting the disease within

the family; organization of appropriate cardiac evaluation and follow-up of relatives; and consideration of genetic testing, which may have the potential to improve medical management. Strategies to appropriately achieve these goals have been detailed in various guidelines or expert consensus reports.<sup>34–36</sup> As a general statement, genetic counselling is recommended for all patients with HCM, unless an acquired cause is demonstrated. Counselling should be performed by trained healthcare professionals working within multidisciplinary teams to help patients understand and manage the psychological, social, professional, ethical, and legal implications of genetic disease.

When genetic testing is considered, pretest counselling is of paramount importance to assist decision-making by fully informing patients about the potential benefit, but also about the limitations of testing and the sometimes complex consequences that genetic testing results may have for them (e.g. on sport activity, employment, life/disability insurance), and for their family (e.g. the responsibility in sharing the information with relatives to promote the family screening). Information should be provided on existing legislative protection for discrimination based on genetic testing, including discussion of areas that are not protected. These laws vary from country to country. Psychological support can be provided, especially with post-test counselling, to help individuals cope with anxiety associated with the disease or genetic result. Counselling is also critical to help ensure that patients and families have appropriate expectations about what genetic testing can provide, and correct understanding of the often ambiguous results.

### 2.5.2 Predictive genetic testing in relatives

There is general agreement that predictive genetic testing is indicated in asymptomatic relatives of a patient with HCM when a definitive disease-causing mutation has been previously characterized in the family. In this manner, relatives who are found to not carry the mutation can be reassured and longitudinal cardiac follow-up is no longer required (in the absence of clinical signs or symptoms). Moreover, they can be reassured that there is no risk of transmitting the disease to offspring. If a relative is found to carry the family’s pathogenic mutation, then regular, longitudinal medical follow-up is required to detect cardiac expression early and optimize their care. Predictive testing can also be performed in their offspring. The cost-effectiveness of this cascade approach to family screening has been favourably evaluated in economic decision models.<sup>37,38</sup> A Dutch study suggests that the potential adverse psychological consequences are minimal when individuals are counselled appropriately.<sup>39</sup> In a child, the guiding principle is that a test should be performed in the best interest of the child and should have a clear impact on management. Therefore, predictive diagnosis in children is often considered at the age at which cardiac examinations are initiated (~10 years of age) but may occur earlier in families with early-onset disease or as guided by other concerns.<sup>34</sup> Predictive testing in the family should generally not be pursued if there is any doubt that the results of genetic testing are definitive and that the variant identified may not be truly pathogenic.

### 2.5.3 Other clinical situations

Genetic testing can be discussed in various clinical situations. Genetic testing is recommended to attempt to diagnose potential phenocopies of sarcomeric HCM, especially when atypical phenotypic features are present. Such a diagnosis may importantly modify medical management including therapeutics (e.g. enzyme replacement therapy in Pompe or Fabry disease). Genetic testing may also be useful in selected cases of borderline hypertrophy, such as in differentiating HCM from athlete’s heart,<sup>40</sup> although there is no general consensus in this situation.

Genetic testing has limited impact on prognostic stratification in the context of established sarcomeric HCM, except for very rare, selected mutations that have demonstrated consistently severe outcomes in unrelated families, or in the presence of multiple mutations.

Prenatal genetic diagnosis can be performed at the beginning of pregnancy using chorionic villus sampling or amniocentesis, although this procedure is not legal in some countries and may be controversial in others given the considerable variability in the phenotypic expression,<sup>41</sup> even within the same family. Alternative options to prenatal diagnosis, such as adoption, artificial insemination using donated gametes, and pre-implantation genetic diagnosis (PGD), can be also reviewed during discussions on reproductive options. PGD is an established clinical procedure, more recently applied to inherited cardiac diseases,<sup>42</sup> that allows couples to conceive a child without passing on a pathogenic mutation. After *in vitro* fertilization, a single cell is removed from each embryo at the 3-day stage and analysed for the causative mutation in the family. Only embryos unaffected by the condition are transferred to initiate pregnancy. The success rate of PGD is ~30% live births per cycle started.

### 3. New technologies and bioinformatics: genetic testing and interpretation

#### 3.1 Next-generation sequencing

NGS technologies first arrived in 2005 and have allowed a revolutionary increase in high-throughput sequencing capability. These advances have dramatically decreased costs and timelines; both significant advantages over traditional Sanger sequencing.<sup>43,44</sup> With this technology has come an evolving transformation of our approach to genetic testing. No longer strictly confined by the amount of sequencing that can be feasibly performed, genetic testing is moving towards far more comprehensive strategies, including large multigene testing panels, whole-exome sequencing (WES), and whole-genome sequencing (WGS). These opportunities have the potential to improve both clinical diagnostics, as well as research aimed to discover novel disease-causing genes. However, the bioinformatic processing of data generated by such massive sequencing runs will be a major challenge. DNA variants identified by comprehensive genetic sequencing require meticulous curation and interpretation to determine which are clinically relevant and to reduce the risk of false-positive findings.<sup>45</sup>

The fundamental strategy employed by all NGS platforms is to sequence millions of short-DNA fragments in massively parallel arrays and then realign and map the short reads back to the reference genome (Figure 1). The basic steps include the following: (i) fragmentation of genomic DNA into fragments of a few hundred bp, (ii) library generation by ligating adapter sequences on both ends of genomic fragments, (iii) enrichment of targeted regions of interest by multiplexed PCR-based methods or by in-solution oligonucleotide hybridization-based methods that capture baits with streptavidin beads in solution, and (iv) massively parallel sequencing to generate strings of bases called 'reads' that are then aligned and assembled against the reference genome, and interpreted, as described below.<sup>46–50</sup> In this manner, three types of analysis are typically undertaken using NGS: DNA sequencing of disease-specific gene panels, WES, and WGS.

##### 3.1.1 Target sequencing in custom disease-specific panels

By using disease-targeted gene panels, it is possible to analyse multiple genes simultaneously rather than the traditional 'one gene at a time'

approach.<sup>51</sup> Genetic testing panels are typically designed by including some or all of the genes that have been associated with a given condition. Virtually, all genes with a published association with the disease of interest can be analysed at once using NGS technology. However, recognizing the limitations of NGS technology, Sanger sequencing to 'fill in' missing data from regions with insufficient coverage (e.g. regions where each bp is sequenced <10–20 times) is recommended.<sup>52</sup> This targeted approach, focusing on a limited set of genes with scientific evidence to support a causative role in the disease, produces greater depth of coverage, allows the laboratory to run more patient samples in a single run, and gives rise to results that are relatively straightforward to interpret. Currently, targeted disease-specific panels are the most commonly used approach in clinical genetic testing. More comprehensive genetic analysis may result in a higher diagnostic yield for phenotypically and genotypically heterogeneous conditions such as HCM and DCM,<sup>50</sup> although the precise impact is not yet known, as the prevalence of variants of unknown significance (VUS) will also increase as the number of genes included on testing panels increases.

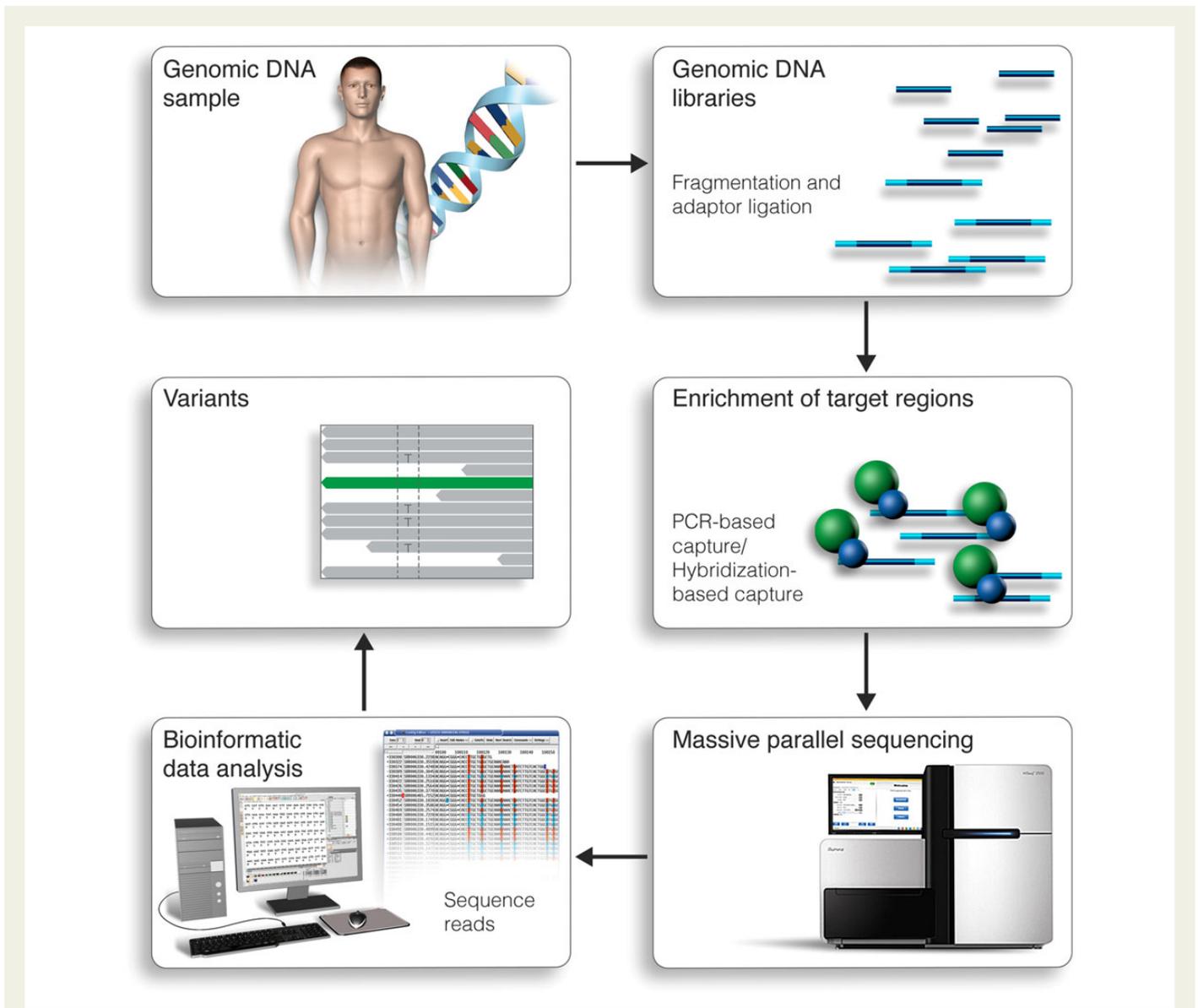
Tables 1 and 2 list the genes previously associated with HCM and DCM, respectively,<sup>50,52–57</sup> and illustrate the important role that sarcomere gene mutations play in both HCM and DCM. Based on these genetic associations for cardiomyopathies, testing panels comprise 10–50 genes have been developed by genetic testing laboratories.

In addition to allowing a large number of genes to be sequenced simultaneously, another advantage offered by more efficient NGS technology is the opportunity to analyse giant genes like titin (*TTN*) and dystrophin (*DMD*) (>400 and 80 exons, respectively). For example, *TTN* has been known to cause DCM for several years; however, the enormous size of this gene precluded systematic analysis and determination of its contribution to disease. Using NGS methodology, Herman *et al.* demonstrated that *TTN* truncations may be responsible for up to ~20% of 'idiopathic' DCM. This finding identifies *TTN* as the single largest genetic contributor to DCM yet identified.<sup>58</sup>

NGS also offers the advantage of being able to identify small insertion or deletions (InDels) of 1–20 nucleotides, as well as larger loss or gain of regions of DNA known as CNV (copy number variations), simultaneously with analysis for missense variants. Currently, the contribution of CNV to cardiomyopathies is largely unknown, because this type of sequence variation is not detected by traditional Sanger sequencing methods. Preliminary studies suggest that CNV could account for a notable proportion of DCM patients.<sup>59</sup> CNV analysis may importantly aid in the differential diagnosis of phenocopies of HCM and DCM and in the confirmation of diagnosis in atypical or overlapping phenotypes.<sup>12</sup>

##### 3.1.2 Whole-exome sequencing

WES involves sequencing all coding regions of the genome. The exome represents ~1% of total genomic DNA and consists of ~20 000 genes, 180 000 exons, and 30 million bp of DNA. WES is increasingly being applied to identify otherwise undetermined genetic causes of cardiomyopathies: individuals with suspected genetic cardiomyopathy in whom no causal variant is identified using targeted gene panels. WES can identify variants in genes known to be associated with specific disease, but it can also provide a powerful investigative tool to discover novel gene–disease associations.<sup>51,60,61</sup> Over recent years, variants in different genes (*BAG3*, *ACSF3*, *AARS2*, *MRPL3*, *GATAD1*) were identified as possible causes of cardiomyopathies by WES.<sup>62–64</sup> One of the major advantages of WES is the ability to detect a putative mutation in small nuclear families and in sporadic cases that are not approachable by traditional linkage analysis. However, there are important considerations when performing



**Figure 1** NGS Strategy. It involved three major steps: library preparation from genomic DNA, enrichment of target regions by hybridization, and massively parallel sequencing of all captured fragments.

whole-exome analysis. Major challenges facing WES currently include the following: appropriately classifying and determining the clinical significance of the high number of variants inevitably discovered, ethical problems related to incidental findings (genetic variants discovered that may have important medical or social implications that are unrelated to the indication for testing), and technical difficulties obtaining adequate sequence quality, particularly reaching sufficient coverage of key disease-related genes, including *MYBPC3*.<sup>45,65–67</sup>

One strategy to address the challenges of interpreting the massive amount of data returned by WES has been to perform WES, but to initially restrict analysis to genes already known to be associated with the disease. The advantage of this strategy over current genetic testing panels lies in the opportunity to re-analyse the sample if no causative mutation is identified.

### 3.1.3 Whole-genome sequencing

WGS involves sequencing all 3 billion bp of the human genome, covering both coding and non-coding regions. It is the most comprehensive and

expensive sequencing approach. WGS has mainly been used in the research realm to discover novel disease-related genes, although there have been examples of successful use in clinical diagnostic odysseys.<sup>52</sup> Even more than WES, the major obstacles facing WGS are data management and interpretation. Thousands of sequence variants will be detected in any individual, the vast majority of no or uncertain clinical relevance. Making sense of the output of WGS and determining which variants are clinically important are major challenges to this approach.

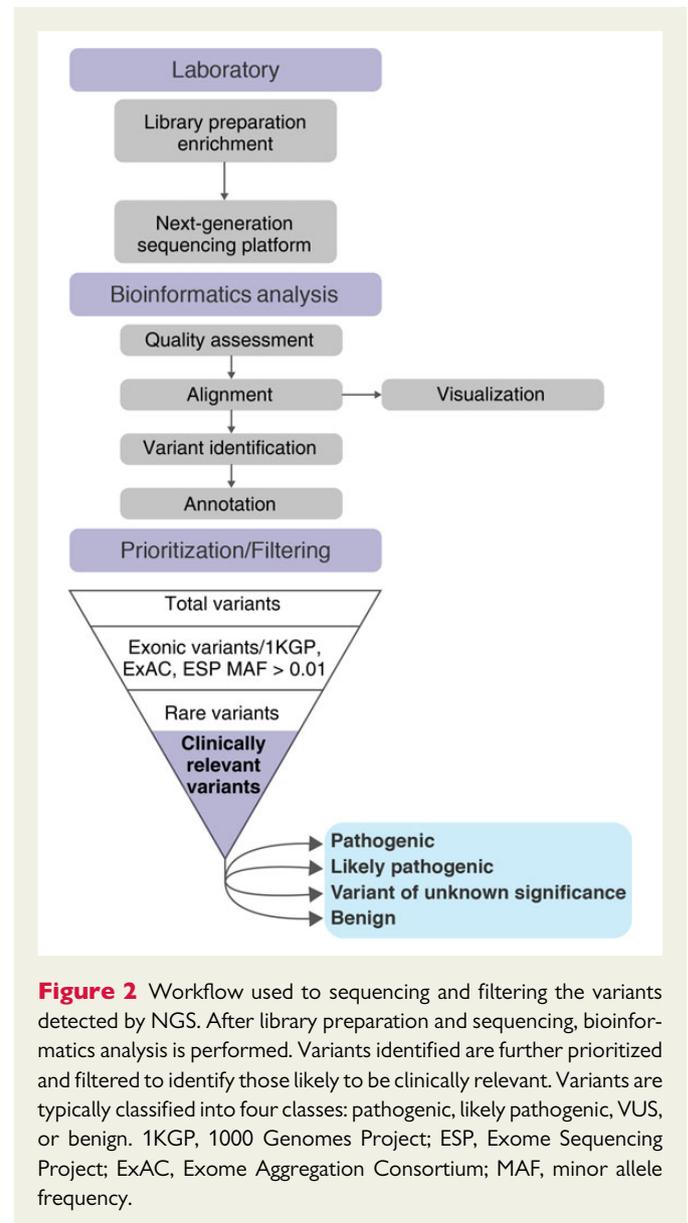
## 3.2 Bioinformatics data analysis, filtering, and determining the pathogenicity of DNA variants

Managing and curating the voluminous sequencing data generated from WES and WGS require considerable experience and expertise. After library preparation and sequencing, data analysis of generated sequences (reads) comprise different steps, including: (i) quality assessment of raw sequence data, (ii) alignment of reads to the correct position relative to

**Table 2** Genes associated with DCMs

Gene	Estimated prevalence (%)
<i>TTN</i>	20
<i>MYH7</i>	4–7
<i>LMNA</i>	2–6
<i>SCN5A</i>	2–3
<i>TNNI3</i>	2–3
<i>LDB3</i>	1–3
<i>PLN</i>	1–3
<i>TNNT2</i>	1–3
<i>TNNC1</i>	Rare
<i>TAZ</i>	Rare
<i>CSRP3</i>	Rare
<i>DES</i>	Rare
<i>ACTN2</i>	Rare
<i>ANKRD1</i>	Rare
<i>ABCC9</i>	Rare
<i>TPM1</i>	Rare
<i>DMD</i>	Rare
<i>VCL</i>	Rare
<i>EMD</i>	Rare
<i>MYOZ1</i>	Rare
<i>MYBPC3</i>	Rare
<i>BAG3</i>	Rare
<i>ABCC9</i>	Rare
<i>LAMP2</i>	Rare
<i>EYA4</i>	Rare
<i>TMPO</i>	Rare
<i>PSEN1</i>	Rare
<i>PSEN2</i>	Rare
<i>SGCD</i>	Rare

Rare is defined as <1% contribution to the disease. For some of these rare genes, evidence for direct pathogenicity in DCM has not been clearly established.



the reference genome, (iii) variant calling, and (iv) functional annotation and filtering (Figure 2).

The final two steps are the most labour intensive as they serve to determine functional and clinical relevance of the thousands of sequence variants identified in a sequencing run. This analysis requires sophisticated manual curation and literature review to contextualize the effect of variants with respect to several biological data including: protein-coding changes, affected amino acids, allele frequency, known single-nucleotide polymorphisms (SNPs), conserved regions, and predicted pathogenicity scores. All of this information can be integrated to filter and prioritize detected variants to identify those that are most likely to be clinically relevant.

Variant interpretation involves several steps (Table 3):<sup>68,69</sup> (i) determining whether the variant in question has previously been reported in the literature as associated with the disease (including assessment of the quality of prior publications as many variants previously published as ‘pathogenic’ have subsequently been shown to be of unknown significance or likely benign); (ii) assessing for appropriate co-segregation with disease if permitted by family size and structure; (iii) determining if the involved amino acid residue is conserved among species and isoforms;

(iv) determining if the nucleotide is located in a strategic functional domain; (v) assessing the variant’s prevalence in ethnically matched controls, based on prior publications and large, publicly-available databases of human genomic variation;<sup>52,68</sup> (vi) using software [e.g. Polyphen2, Mutation Taster, sorting intolerant from tolerant (SIFT)]<sup>45,69</sup> to predict the potential impact of the variant on protein structure/function; and (vii) determining whether the variant was present in the same gene in unrelated individuals with the same phenotype. The most important sources of broad population frequencies that can be used for classification of variants include 1000Genomes Project (1KGP) ([www.1000genomes.org](http://www.1000genomes.org)), the Exome Sequencing Project (ESP) ([evs.gs.washington.edu/EVS/](http://evs.gs.washington.edu/EVS/)), dbSNP ([www.ncbi.nlm.nih.gov/SNP](http://www.ncbi.nlm.nih.gov/SNP)), and the recently released results of the large-scale Exome Aggregation Consortium (ExAc) ([exac.broadinstitute.org](http://exac.broadinstitute.org)). In some circumstances, investigators have taken additional steps to assess mutation pathogenicity using *in vitro* cellular or *in vivo* animal assay, but this approach is not yet scalable to allow real-time interrogation of all variants identified in all patients who undergo NGS.

**Table 3** Commonly used criteria to determine the pathogenicity of DNA sequence variants

Criteria	Description
Previously reported in the literature	Documentation that the variant is associated to the disease in >1 patient in literature. Assessment of the quality of prior publications as many previously published 'pathogenic' variants have subsequently been shown to be of unknown significance or likely benign. For this reason, it is very important to evaluate whether the variant reported is supported by other evidence supporting pathogenicity.
Co-segregation in families	Determine whether variant is present in affected relatives if permitted by family structure. Requires appropriate segregation in at least 3 informative meioses; ideally at least 10 meioses; more distant relationships are more informative.
Conservation (amino acid/species)	Determine whether the impacted amino acid residue is conserved among 10 species and isoforms
Functional domains	Determine whether the variant is likely to cause a disruption of protein structure
Frequency/absence in control populations (1000Genome, ExomeSequencing Project)	Check the prevalence of the variant in public databases; confidence for pathogenicity increases when variant is confirmed to be rare and absent from ethnically matched control population.
Predictive tools (e.g. Polyphen 2, SIFT, Mutation Taster)	Confidence for pathogenicity increases if the variant is predicted to have a 'deleterious' impact on protein structure/function consistently in all tools, rather than just one
Presence in unrelated individuals with the same phenotype	Confidence for pathogenicity increases if the variant is previously found in unrelated probands with the same disease
Functional studies	Study <i>in vitro</i> such as mouse model is very important to classify variants. This approach is not yet scalable to allow interrogation of all identified variants
Type of variant (missense, non-sense, frameshift, splice variant)	Confidence for pathogenicity is higher for radical variants such as nonsense, InDels, or splice site variants affecting canonical acceptor or donor sites, relative missense variants. This criteria is usually true for DCM genes; in HCM, it is true for MYBPC3 gene

By applying the above rules, the variants can usually be classified into four main classes such as known pathogenic, likely (probably) pathogenic, VUS, and benign variants.<sup>52</sup> Pathogenic and likely pathogenic variants identified by NGS methods are typically confirmed to be present in the patient's genomic DNA using standard Sanger sequencing methods. Follow-up segregation studies on affected family members (if available) can play a key role in the interpretation of variants and, in informative families, can provide pivotal information regarding the causal role of a putative mutation.

### 3.3 Separating the signal from the noise: the challenge of genetic testing interpretation

The major challenge facing new sequencing technologies and comprehensive testing relates to the large number of DNA sequence variants present and identifiable in each individual. Distinguishing variants truly capable of causing disease (pathogenic mutation) from the 'background noise' of human genetic variation has increasingly emerged as a dilemma for interpreting cardiomyopathy genetic testing results and has been regarded as the 'Achilles heel' of diagnostic genetic testing.<sup>12,70,71</sup> An important limitation is that the wealth of information derived from NGS is not yet matched by the clinical knowledge needed to accurately gauge the clinical relevance of each identified variant.<sup>58,63</sup> The challenges of distinguishing pathogenic mutation from background genetic noise were described for inherited channelopathies where Ackerman et al.<sup>64</sup> estimated that Long QT genetic testing performed by analysing the three major genes (*KCNQ1*, *KCNQ2*, and *SCN5A*) has background 'signal-to-noise' ratio of ~19:1 in a cohort of Caucasian patients with a robust clinical phenotype. In sarcomeric cardiomyopathies, the signal-to-noise varies within different genes and is generally better in HCM than in DCM. Assessing pathogenicity is particularly difficult in titin, as 3% of the general population may carry a truncation variant in this gene.<sup>71</sup>

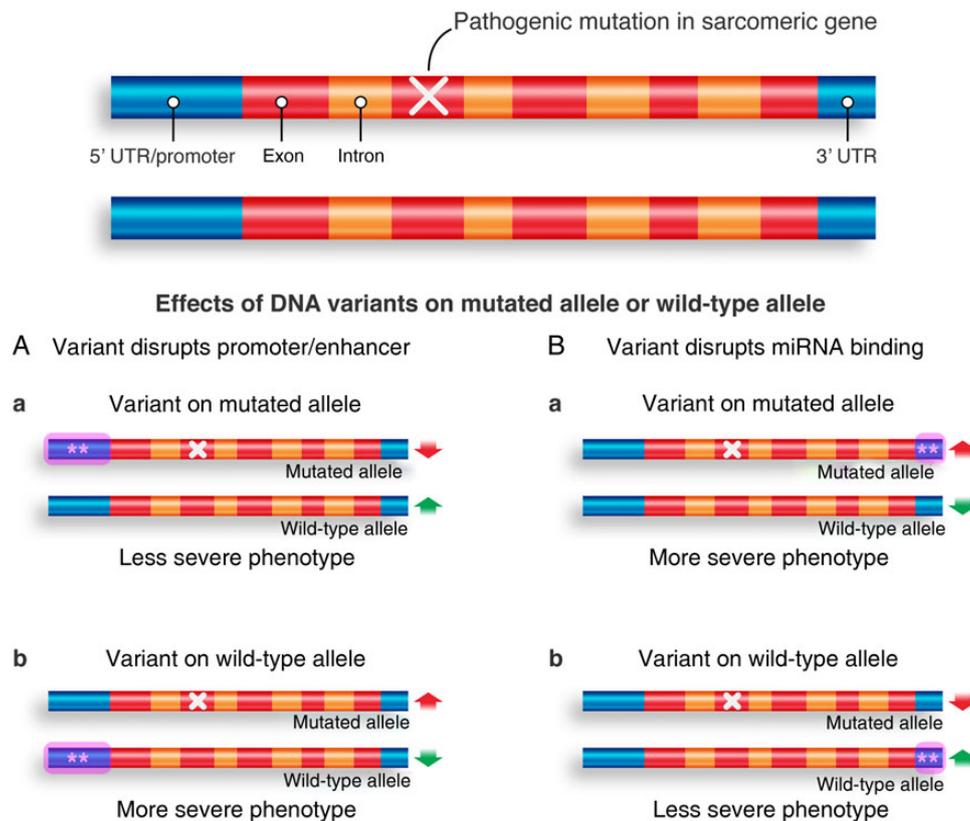
Owing to the complexity involved in correctly interpreting DNA variants, genetic tests are best interpreted in a fully integrated context

encompassing expert clinical evaluation, together with comprehensive family history and assessment. Multidisciplinary teams composed of cardiologists, geneticists, and genetic counsellors are often required to appropriately synthesize clinical and genetic data to provide optimal care to the patient and their family.<sup>52,64,72</sup>

## 4. Epigenetics, genetic modifiers, and phenotypic diversity

Sarcomeric cardiomyopathies are remarkably heterogeneous disorders, like other autosomal dominant inherited adult onset disorders. The clinical presentation ranges from no or minimal symptoms to the most serious complications including heart failure and sudden cardiac death. Even for a given single mutation and even within a single family, the complete phenotypic spectrum from unaffected to severely affected can be seen. This implies that factors beyond just the pathogenic sarcomere mutation influence the phenotype. As a result, the model of sarcomeric cardiomyopathies as a strictly monogenic disease following simple Mendelian patterns of inheritance is increasingly recognized as an oversimplification.<sup>73,74</sup> In theory, a discrete number of reasons can account for phenotypic diversity: genetic, environmental, or mixed. However, the precise mechanisms have not yet been elucidated. It would be interesting to unravel to what extent the diverse phenotypes are genomically defined, for example with the help of twin studies.

Genetic mechanisms can be locally acting variants, where nearby DNA variants directly influence regulation of the mutated allele or the wild-type counterpart allele (Figure 3). Additionally, distant-acting mechanisms may be present, where DNA variants located remotely from the primary mutation, or mutations on separate loci (e.g. additional mutations/polymorphisms in separate genes) influence the biology of the sarcomere. The most well-known locally acting modification occurs when there are two disease-causing variants in the same sarcomeric gene (homozygosity or compound heterozygosity).<sup>9,26,75–79</sup> This has been recently illustrated with a mouse model



**Figure 3** Schematic overview of locally acting variants on different positions (A, B, C, D) with influence on the mutated allele and the wild-type counterpart allele (red arrows), and the subsequent expected effect on the phenotype (less or more severe). UTR, untranslated region.

bearing *MYH7* mutations.<sup>73</sup> Interestingly, apart from these rare double hits, very common variants can have profound locally acting effects if located near to a disease-causing mutation in the same gene. The first example of such local-acting modification was shown in another genetic cardiac disease, long QT syndrome type 1. Here, we showed that SNPs in the 3' untranslated region (UTR) in the *KCNQ1* gene alter the expression of the allele on which they reside, influencing the balance between proteins stemming from either the normal or the mutant *KCNQ1* allele.<sup>80</sup> This showed that common SNPs that are typically silent, suddenly become highly relevant when they are located in the 3' UTR of a mutated gene. It would be interesting to explore such polymorphisms in UTRs of sarcomeric genes, by example the 3'-UTR of the *TTN* gene that contains 20 SNPs (Ensembl).

An example of distant-acting modifiers is the presence of multiple gene mutations in other sarcomeric genes, which can result in a more severe clinical phenotype.<sup>9,26,75,78</sup> As genetic sequencing technology advances and genetic testing becomes more comprehensive, cardiomyopathies attributable to 'double trouble' like digenic/oligogenic heterozygosity (two or more mutations in different genes) will be identified with increasing frequency. Apart from rare variants in sarcomeric genes, distant-acting mechanisms have also been proposed to occur with more common variants. These include polymorphisms influencing the sarcomere,<sup>81</sup> and polymorphisms in genes with functions beyond the contractile apparatus, like the renin-angiotensin-aldosterone system.<sup>82,83</sup>

Loading conditions (related to hypertension or valvular heart disease), exercise, diet, environmental exposures, and other co-morbid

medical conditions are examples of environmental factors that may modify phenotypic expression of sarcomeric genes. Remarkably, there have been almost no studies describing how environmental factors may affect phenotype in sarcomeric cardiomyopathies. Olivotto *et al.*<sup>84</sup> studied a large cohort of HCM patients and showed that obesity and systemic hypertension are associated with increase in left ventricular mass, thus act as modifiers for the HCM phenotype. However, Schmitt *et al.*<sup>85</sup> showed that pressure overload induced by trans-aortic banding did not profoundly exacerbate cardiac hypertrophy in an HCM mouse model with a myosin heavy chain mutation. It is also important to acknowledge that environmental influences on a single cardiovascular condition, like HCM, may be linked to other coexisting factors. For example, obesity is associated with hypertension and diabetes. Therefore, interpretation of clinical impact of each of these factors in isolation is challenging. Moreover, genetic modifiers can coexist with environmental modifiers, all contributing to the phenotypic diversity of sarcomeric cardiomyopathies.

Additionally, epigenetics are likely to be relevant in sarcomeric cardiomyopathies. The term 'epigenetics' is defined as a change in gene expression that cannot be explained by changes directly in the DNA sequence itself, but rather as a result of alterations related to the packaging and/or translation of genetic information.<sup>86,87</sup> Epigenetic mechanisms can be acquired or heritable and constitute a means by which interactions between genes and environment can occur. Epigenetic regulation occurs by three key mechanisms: (i) methylation of CpG islands, mediated by DNA methyltransferases (DNMTs), (ii) modification of histone proteins, and (iii) microRNAs (miRNAs). Such modifications will

lead to differential expression of similar information depending on the surrounding conditions, resulting in gene activation or silencing. No epigenetic modifications have yet been reported in sarcomeric cardiomyopathies. The role of epigenetics has been mainly evaluated in cancer, but recent studies have begun to address the involvement of epigenetics in the development and progression of cardiovascular diseases such as heart failure. In particular, the role of miRNAs in heart failure has been studied intensively in the last years.

MicroRNAs are a class of small non-coding RNA molecules that modify the expression of messenger RNAs (mRNAs) by interfering with their translation, either by repressing the translation and/or by decreasing stability of mRNAs.<sup>88</sup> A miRNA binds often to the 3'UTR mRNA target region,<sup>89</sup> although target sites within the 5'UTR are described.<sup>90</sup> Three types of mutations can affect miRNA function: mutations affecting primarily miRNAs, mutations affecting genes involved in general miRNA processing, and mutations affecting the target region of miRNAs (the 3'UTR in mRNAs). An individual miRNA can regulate hundreds of target genes. Around 1000 miRNA genes have been identified, together regulating at least one-third of all genes.<sup>91</sup> Four miRNAs are highly expressed in the heart, miR-1, miR-133, miR-208, and miR-499.<sup>92</sup> Studies on mice (and limited data derived from human heart tissue) show promising results about miRNAs in relation to hypertrophy (e.g. miR-195) and heart failure (e.g. miR-133).<sup>92,93</sup> Although common variants in the 3'UTR have been described to have clinical relevance in a cardiogenetic disease (see above), mutations in the genes encoding miRNAs have not yet been described for sarcomeric cardiomyopathies.

## 5. Future directions: using genetics to improve care of sarcomeric cardiomyopathies

Identifying the genetic basis of human heart disease creates remarkable opportunities to understand how disease develops, and by extension, how to disrupt disease progression. By identifying at-risk individuals prior to clinical diagnosis, by characterizing early pathways involved in disease progression, and by fostering the development of novel therapies to target these pathways in order to delay or prevent full clinical expression, genetic discoveries can change medicine. Moreover, new strategies are emerging where mutant alleles may be effectively silenced.<sup>94</sup> Rather than trying to target downstream pathways set into motion by the sarcomere mutation, these approaches have the potential to strike at the root cause of disease—inhibiting mutant gene expression itself. And finally, although opportunities may begin in rare Mendelian conditions like sarcomeric cardiomyopathies, the insights gained are likely to also advance understanding of cardiac function relevant to more common forms of heart failure.

The discovery that sarcomere gene mutations cause both HCM and DCM was a landmark event that characterized inherited heart disease at the molecular level. Genotype provides a valuable anchor for detailed basic and clinical translational studies to interrogate how mutations in contractile genes affect the biochemistry, electrophysiology, structure and function of the cardiomyocyte. Gene-based diagnosis can importantly guide family management today by allowing a cost-effective and decisive way to identify at-risk relatives who may transmit and develop disease in the future and who therefore require longitudinal clinical follow-up.

However, much work remains. These initial discoveries are a call to action to realize the promise of genetics to transform medicine. What

is the impact of these mutations at the level of whole heart and the whole individual? How do mutations alter the function of the encoded protein and how do they influence other cellular pathways to produce complex and variable clinical phenotypes? How can we use this information to improve health in a proactive manner, rather than merely identifying those at risk?

Many fundamental questions must be answered to translate genetic findings to enhance the care of patients. With current knowledge, we fail to identify mutations in sarcomere genes in approximately half of HCM patients and the majority of those with DCM. We seek a more complete understanding of the burden of genetic disease in “sarcomere-negative” patients and identification of other disease-causing genes. Although comprehensive genetic testing, such as WGS, will identify new genes implicated in cardiomyopathy, a substantially higher number of VUS's will also be generated, potentially increasing overall ambiguity. A more sophisticated understanding of human genetic variation and more robust approaches to assess the pathogenicity of sequence variants are needed to complement the massive amount of information returned from comprehensive genotyping. High-throughput model systems must be developed to interrogate the functional consequences of DNA variation and aid in the assessment of pathogenicity.

Furthermore, we will need to gain much better understanding about the great phenotypic diversity of sarcomere mutations and the modulation of gene expression throughout an individual's lifetime. How can we fully characterize genetic, epigenetic and environmental modifiers and explain the diverse clinical manifestations and outcomes of these intriguing disorders? Are there dynamic elements that explain the phenotypic variation over time? Larger cohorts that marry genotyping and longitudinal clinical and cellular phenotypes will foster further insights. Given the rarity of these conditions, multicentre collaboration will be essential for success.

Considerable advances must still be made before we fully understand the complex connections between sarcomere mutations and their clinical consequences. Nonetheless, these cardiomyopathies serve as a remarkable model of how insights gained by the molecular characterization of Mendelian disorders can enlighten understanding of key biological processes and ultimately translate into better ways to diagnose, treat, and prevent disease.

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