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SUMMARY

Arrhythmogenic cardiomyopathy is a progressive, heritable myocardial disorder resulting in ventricular arrhythmias and sudden cardiac death, but also in end-stage heart failure.¹ Initially, it was believed that the disease resulted from abnormal development of the right ventricle, resulting in the term “right ventricular dysplasia”.² Early publications described involvement of the right ventricle, which was considered the classic subtype of the disease. More recently however, involvement of both ventricles and left ventricular predominance have been described.³⁻⁵ The terms “right ventricular” and “dysplasia” seem to be outdated and the preferred term for this type of inherited cardiomyopathy is now “arrhythmogenic cardiomyopathy” (ACM). The disease usually presents in young adolescence or early adulthood, but a diagnosis in children under the age of 10 years is rare.^{6,7} Genetic causes, mainly mutations in genes encoding for desmosomal proteins, can be identified in about 50% of cases.

The first part of this thesis describes genetic studies and phenotypic characterisation in ACM. Chapter 2 describes the application of the haplotype-sharing test to identify disease-causing mutations. Many families affected with a subtype of cardiomyopathy are too small to perform classical linkage analysis. We therefore performed the haplotype-sharing test to search for large shared segments in the individuals’ genomes that are most likely to contain the causative mutation in a family. Affected individuals will have inherited the haplotype with this mutation from a common ancestor. The common ancestor could be from several generations ago, so that the affected individuals might be unaware of the fact that they are related to each other. DNA from proven affected family members was genotyped using single nucleotide polymorphism (SNP) arrays and the data from these analyses were combined to search for shared haplotypes. Both a family with ACM and a family with dilated cardiomyopathy (DCM) were analysed using the haplotype-sharing test. In both pedigrees, we searched for mutations in the genes located in the largest shared haplotypes and we were able to identify mutations in *PKP2* and *MYH7*, respectively. Furthermore, we calculated that with a pedigree containing at least seven meioses, the chances are high that the largest shared haplotype will be the one to contain the causative mutation. This proof-of-principle study showed that the haplotype-sharing test can assist in identifying causative genes in families with low penetrance Mendelian diseases, in which standard tools cannot be used because of insufficient pedigree information. The haplotype-sharing test can also be used if a common ancestor is suspected based on the place of birth of the individuals, even if they are not known to be related.

Chapter 3 describes molecular studies on the *PKP2* mutation identified in chapter 2, together with the clinical data of the mutation carriers. The identified *PKP2* splice site mutation (c.2489+4A>C) is predicted to lead to the expression of a dysfunctional *PKP2* protein, rather than a loss-of-function protein. The clinical manifestations in mutation carriers ranged from severe disease to non-penetrance in elderly mutation carriers. This study suggests that this *PKP2* mutation alone is not sufficient to cause disease and that other genetic and/or environmental factors also contribute to the development of the disease. This exemplifies the variable expression and incomplete penetrance characteristic of ACM.

Founder mutations are frequently identified in Dutch patients with inherited cardiac diseases. Chapter 4 describes the largest series of patients with the same founder mutation in the *PKP2* gene (p.Arg79X). Twelve index patients and 41 family members were evaluated. Haplotype analysis revealed a shared haplotype among all mutation carriers, indicating a common founder. Sudden cardiac death (SCD) in individuals younger than 40 years of age had occurred in 50% of these families, while only 60% of the mutation carriers had experienced any symptoms by age 60, again illustrating the clinical variability and reduced penetrance in ACM.

The pathogenicity of identified genetic variants in ACM patients is not always clear. In Chapter 5, the genetic variants database for ACM (available from www.arvcdatabase.info) is described in detail. This freely available, online database lists the genetic variants in genes associated with ACM and is a digital repository for both molecular data and all the publications containing additional information on clinical and/or genetic data of identified variants. In June 2012, the database contained 856 variants and data from 111 publications. The database has been proven to be useful: since its launch in October 2008, it has been visited 125,000 times by 11,500 returning visitors from more than 50 countries.

Chapter 6 reports a large cohort of Dutch ACM families, collected in all the university medical centres in the Netherlands. To gain more insight into the natural variability of the disease expression and phenotypic consequences of genetic findings in ACM, all five desmosomal genes were sequenced in 149 Dutch ACM index patients. In total, 302 family members were screened for carriership of the identified pathogenic mutations in their family. Pathogenic mutations were found in 58% of index patients; these were mainly truncating *PKP2* mutations but included three cases with *PKP2* exon deletions. A *PKP2* mutation could be identified in 90% of families with more than one affected individual. The discovery of a pathogenic mutation in index patients enabled the identification of those relatives who have a 6-fold increased risk of developing ACM, justifying regularly monitoring for early symptoms.

The second part of this thesis focuses on the identification of a founder mutation in the phospholamban gene (PLN p.Arg14del) in a substantial number of Dutch patients with ACM and/or DCM. The clinical characteristics of index patients carrying this mutation are outlined in Chapter 7. A cohort of 97 unrelated ACM index patients and 257 unrelated DCM index patients was screened for *PLN* mutations. The p.Arg14del mutation was identified in 12% of ACM patients and in 15% of DCM patients in the Netherlands. This is the highest single mutation yield reported in Dutch cardiomyopathy patients. Patients carrying the PLN p.Arg14del mutation presented with a low voltage ECG in 46% of cases and demonstrated an arrhythmogenic phenotype, with high rates of appropriate implantable cardioverter defibrillator (ICD) discharges, cardiac transplantation, and a family history of sudden cardiac death. The average age of 26 family members who died of SCD was 37.7 years. Surprisingly, immunohistochemical analysis in myocardial samples, considered a diagnostic test for ACM, revealed absent or depressed plakoglobin levels at intercalated disks in five of seven (71%) p.Arg14del ACM samples, but in only one of nine (11%) p.Arg14del DCM samples. This chapter illustrates how one mutation can result in different clinical diagnoses, in this case both ACM and DCM, and that there is an overlap between these inherited cardiomyopathy subtypes.

The geographical distribution of carriers of this PLN founder mutation in the Netherlands is described in Chapter 8. Haplotype analysis revealed a common founder, estimated to be between 575 and 825 years old. Over 450 carriers have been identified and the majority live in the northern part of the Netherlands, including the province of Noord-Holland. Analysis of their grandparents' places of birth indicated that the mutation likely originated in the eastern part of the province Friesland. Screening of a large population-based cohort (PREVEND) for the presence of this mutation identified six PLN p.Arg14del mutation carriers out of 8,267 subjects (0.07%). These findings illustrated that the PLN p.Arg14del mutation is highly prevalent in the general population in the northern part of the Netherlands and that this mutation is also one of the most prevalent cardiomyopathy related genes in other parts of the Netherlands.

In Chapter 9, a large pedigree with ACM was studied. A previously identified mutation in *PKP2* (p.Ser140Phe) was found not to co-segregate with the disease in this family. After meticulous phenotypic characterisation, we could find no evidence that the *PKP2* variant contributed to the disease. However, the PLN p.Arg14del mutation was present in all family members with ACM and all of them had left ventricular involvement. Out of nine identified mutation carriers, four also had a low voltage ECG.

In patients with inherited cardiomyopathy the identification of a pathogenic mutation can confirm the diagnosis and subsequently asymptomatic relatives can be identified after cascade family screening. Clinical geneticists are at the heart of multidisciplinary teams consisting of genetic counsellors, molecular geneticists, cardiologists, pathologists, neurologists, and researchers, who together care for cardiomyopathy patients and their families. Following the recent advances in genetic research, as addressed in the General discussion, many novel mutations will be identified and counselling these families will become increasingly difficult. A challenging future lies ahead for clinical geneticists and their colleagues, but their patients and families will benefit from the progress that will be made by dealing with these challenges.

GENERAL DISCUSSION

In the last two decades, genetic research in families with cardiomyopathy has rapidly shifted from basic science towards routine clinical application, thereby changing the clinical practice. For instance, it has resulted in the fact that nowadays genetic counselling is recommended for all patients and their relatives with cardiomyopathy.⁸ The yield of genetic testing for cardiomyopathies ranges from <20% for restrictive cardiomyopathy to around 60% for hypertrophic cardiomyopathy.⁸ This means that currently no mutation can be identified in 40-80% of index patients with a suspected inherited form of cardiomyopathy. It is important to realise that not all forms of idiopathic cardiomyopathy are genetic. Unknown environmental factors could cause isolated or even familial cases of cardiomyopathy in the absence of a pathogenic mutation, so a yield of 100% is highly unlikely. Another factor that hampers genetic analyses in cardiomyopathy patients is the genetic heterogeneity. This is especially the case in dilated cardiomyopathy (DCM) where nearly 50 genes have now been identified, but most of these genes are found to hold mutations in only a small percentage of cases or even in single families. The recent identification of truncating titin gene (*TTN*) mutations in 25% of familial DCM cases is the exception to this rule.⁹ Due to the high costs and laborious tests, many cardiomyopathy patients will usually be screened for mutations in only a few genes. As a result, only the genes that supposedly account for the highest percentage of cases are being analysed. Sequence analysis of the remaining genes is hardly ever performed, and a definite diagnosis cannot be made in many cases.¹⁰ In general, it is rare that we have any clinical clues to guide the genetic screening in inherited cardiomyopathies. Examples of phenotypical information that could guide specific genetic testing are the presence of cardiac conduction disorders in ACM or DCM, which are frequently observed in patients with mutations in the *LMNA*, *DES*, and *SCN5A* genes,¹¹⁻¹³ or relatively less hypertrophy in patients with HCM caused by mutations in the *TNNT2* gene.¹⁴ Moreover, mutations can be present in more than one gene and the number of mutations can sometimes be related to the clinical impact; the involvement of two or three genes is usually associated with a more severe clinical picture.¹⁵⁻¹⁹

The identification of multiple mutations that can be associated with the phenotype poses a great challenge for clinical and molecular geneticists. Cardiomyopathies have previously been considered to be monogenic disorders, but the presence of multiple mutations indicates a more complex genetic background. A pathogenic mutation could be potentiated by other variants present in the same gene, in other genes, or even in the intergenic region. On the other hand, additional mutations could also reduce the strength of a pathogenic mutation. The presence of such additional mutations could explain the large clinical variability seen in patients and

within families with inherited cardiomyopathies. At the same time, this illustrates the importance of correctly interpreting the identified variants. Although *in silico* prediction programs and co-segregation analysis can assist, a lack of functional data and animal models hampers our interpretation of the mutations identified. Frequency data on identified mutations can be helpful, but large series of well-characterised patients are needed to establish enhancing or reducing effects between mutations.

The gathering of genetic data is rapidly becoming easier but, as a result, the classification and interpretation is becoming more and more challenging. To establish the effects of variants of unknown significance, we need to perform large-scale functional studies, but these are expensive and laborious. Gehmlich et al. studied *DSC2* mutations identified in ACM patients by expressing mutant proteins in multiple cellular systems.²⁰ A Western blot of the p.Thr275Met mutant protein, expressed in COS-1 cells, showed an impaired maturation process from the uncleaved precursor form to the cleaved mature form. The p.Arg203Cys protein was only detected in the precursor form. Localisation studies of the *DSC2* mutants in the cardiac cell line HL-1 showed that the p.Thr275Met protein was able to localise in the desmosomes, but a shift towards vesicular and Golgi apparatus localisation was observed. The p.Arg203Cys protein failed to localise at the cell borders and was absent from desmosomal structures.²⁰ These results show how functional assays could aid in the interpretation of (missense) mutations.

The study of mutations in zebrafish (*Danio rerio*) could be another approach for systematic mutation screens. Zebrafish that carried human Nexilin (*NEXN*) mutations, introduced by PCR mutagenesis, showed a similar cardiac pathology as human *NEXN* mutation carriers, including induced Z-disk damage and heart failure, confirming the disease-causing nature of these mutations.²¹

These approaches are not yet available for large-scale use, due to the needed time and resources. A model that classifies identified mutations without the need of additional experiments would be very helpful in this era of rapid emerging genetic data. A classification system combining *in silico* prediction methods and a structural model with phenotypic and segregation data to predict *CHD7* missense variants in CHARGE syndrome has been published.²² The model led to a more confident prediction of pathogenicity. Such a model could also be useful for the classification of missense variants in cardiomyopathy related genes. The incorporation of segregation studies, however, is difficult in inherited cardiomyopathies, since carriers of pathogenic mutations associated with cardiomyopathy could be mildly affected or even unaffected, given the variable expression and incomplete penetrance. The confirmation of model-based predictions by functional assays would be a powerful method to prove the strength and reliability of a model.

Given the challenges in the interpretation of variants, counselling patients and their families will also become increasingly difficult. Multiple variants are being identified in cardiomyopathy patients and the effects of these variants, both private and in combination with others, are often not yet understood. However, the identification of a pathogenic mutation can be of great clinical significance for the index patient under evaluation, because some genes are associated with important phenotypes such as early onset and/or sudden cardiac death, which could be prevented by placing an implantable cardioverter defibrillator.^{23,24} Much effort and resources are needed to correctly interpret identified variants. When a variant is classified as disease-causing or disease-contributing, cascade genetic screening of family members can be initiated. Those family members who are found not to be a carrier of the identified mutation(s) can be dismissed from frequent follow-up, further illustrating the importance of genetic testing in patients with cardiomyopathy.²⁵

The study of induced pluripotent stem cells (iPSCs), derived from patients with inherited cardiomyopathy, offers an opportunity for creating disease-specific cellular models to investigate underlying mechanisms. Generated cardiomyocytes from iPSCs derived from DCM patients carrying a mutation (p.Arg173Trp) in the *TNNT2* gene recapitulated the morphological and functional phenotypes of DCM to some extent.²⁶ Compared to controls, these cardiomyocytes exhibited altered calcium regulation and decreased contractility. Treatment with β -adrenergic blockers improved the function of iPSC-derived cardiomyocytes from DCM patients, illustrating that these cells may serve as a useful platform for exploring the effects of identified mutations and disease mechanisms.²⁶ In another study, generated cardiomyocytes from iPSCs from a patient with ACM, carrying a p.Leu614Pro mutation in the *PKP2* gene, showed reduced cell surface localisation of desmosomal proteins and a more adipogenic phenotype.²⁷ These patient-specific cells may therefore be a useful tool to study these cardiomyopathies and although iPSC-studies cannot discriminate between multiple mutations identified in one patient, these studies could prove to be useful in selecting specific therapeutic options.

Mutation detection arrays

After years of serial testing of one or a few genes at a time, several customised resequencing arrays have recently been developed to sequence large numbers of genes in parallel, in a cost-efficient manner. Analysis of all coding exons, splice site junctions and 5' untranslated regions of 12 genes in 38 unrelated HCM patients led to the identification of a pathogenic mutation in 60% of familial cases and in 10% of sporadic cases.²⁸ A similar approach was applied in 73 previously tested DCM patients, who were screened for mutations in 19 genes using the 'DCM CardioChip'. Combined with previously published data, the authors calculated a clinical sensitiv-

ity for this chip of 26-29% (notably, the chip did not include the *TTN* gene).²⁹ Since not many clear genotype-phenotype relationships have been identified that can guide the genetic screening in inherited cardiomyopathies, sequencing of multiple genes in the same experiment is a reasonable approach to adopt and several arrays/chips are now commercially available.

Next-generation sequencing

Rapid technological advances have led to the application of novel platforms which supersede the array/chip techniques. The introduction of so-called next-generation sequencing (NGS) has led to spectacular developments in genetic research. Sanger sequencing is currently being replaced by different sequencing technologies able to rapidly sequence millions of nucleotides in multiple samples in parallel. Next-generation techniques are based on library preparation: Genomic DNA is randomly broken into small fragments. These fragments are hybridised using a library containing the sequences of the genes of interest. In the next step, these fragments are amplified and sequenced. Imaging techniques and bioinformatics tools enable the generation of large volumes of sequence data and the identification of mutations in the patient's DNA. The risk of false-positive results (i.e. erroneously reported mutations which are not actually present) due to technical and bioinformatical limitations of these methods means the reported mutations must be confirmed, which is still done by Sanger sequencing.³⁰ These targeted platforms can generate a large coverage (the number of reads covering a specific DNA sequence), thereby lowering the risk of false-positives. In our first series of 96 cardiomyopathy patients, we found no false-positives after confirming 40 identified variants. In addition, we identified all the 91 variants previously detected using Sanger sequencing (unpublished data).

Targeted enrichment of DNA fragments encoding known (or suspected) disease genes is a promising application of NGS in the field of inherited cardiomyopathies.¹⁰ A proof-of-principle study analysed 47 cardiomyopathy-related genes in five HCM and five DCM patients. The targeted approach was applied to increase the coverage of the genomic regions of interest, i.e. the 47 genes, which was increased more than 2,000-fold compared to the whole genome. A pathogenic mutation was identified in six of these ten patients.³¹ The authors stated that the results can be made available within two weeks and the testing costs less than \$1,800 (€1,500). In our laboratory, using a targeted approach containing 50 cardiomyopathy genes, the results can be available within three weeks and cost around €400 for the enrichment kit and sequencing chemicals, but excluding salaries and overhead.

Sequencing of dozens of genes in a single experiment will lead to the identification of several previously unknown variants, of which only one or a few will be probably pathogenic. On average, we identified three novel variants (range 0-11)

in our series of 96 cardiomyopathy patients (unpublished data). Multiple affected family members could be studied to gain more insight in the pathogenicity of the identified variants. A variant present in all affected individuals but absent in healthy family members and controls is more likely to be pathogenic, but variants that are only identified in some or even only one of the affected family members could still contribute to the disease in that individual. The interpretation of identified variants is, as mentioned above, a major challenge and will likely be the bottleneck in genetic studies of inherited cardiomyopathies. Much more data will become available with the increased speed and lower costs of next-generation sequencing, but the interpretation of many novel variants requires expertise, time and additional studies, which are often not readily available.

A drawback of any custom-made approach is that they will unavoidably miss the genes that are identified after the panel has been designed. In 2011 three novel cardiomyopathy genes were discovered (Figure 1 in Chapter 1),³²⁻³⁵ illustrating the rapid developments being made in inherited cardiomyopathies.

Exome sequencing

The disadvantage of the non-incorporated genes in custom panels can be overcome by targeting the entire protein-coding sequence of the genome, called the 'exome'. The exome encompasses ~1% of the genome and includes 180,000 exons from more than 20,000 genes. After enrichment of the exome, similar NGS techniques can be applied as described above. Since all coding regions are targeted in exome sequencing, a large number of variants are identified for each sample. Typically, each exome contains between 20,000 and 50,000 variants.³⁶ Multiple bioinformatical filtering steps have to be applied to limit the number of potential disease-causing variants. Quality control steps will exclude those variants that have insufficient coverage, i.e. only a few sequence reads are aligned at a certain position, or are of low confidence, e.g. because only a low percentage of the reads contain a supposedly heterozygous variant. Non-coding variants and synonymous variants are usually filtered out, although non-coding variants in the promoter or untranslated region of a gene could be pathogenic and synonymous variants could influence mRNA splicing. Together with the exclusion of known variants from databases such as dbSNP, the 1000 Genomes Project, the Genome of the Netherlands project and/or in-house databases, the initial number of variants will be reduced by ~99%. However, filtering merely based on the presence in a database carries the risk that truly pathogenic variants may be excluded, since these pathogenic variants can be listed in the databases (known for dbSNP) and because recessive variants with relatively large carrier frequencies could also be listed. After these filtering steps, the remaining 150-500

private and potentially pathogenic variants will need further (*in silico*) analysis to single out the causative mutation or mutations.³⁶

Exome sequencing has been shown to be a very powerful tool in identifying disease-causing genes in a variety of Mendelian disorders.³⁷ In particular, mutations in autosomal recessive diseases and *de novo* mutations in autosomal dominant diseases have been identified using this approach in recent years. In cases of recessively inherited disorders and suspected consanguinity, the pathogenic mutations are most likely homozygous. When there is no evidence of consanguinity, the pathogenic variants could be compound heterozygous, i.e. two different mutations in both alleles of the same gene. Notably, homozygous mutations can also be identified in the absence of consanguinity. Prioritising for genes with two mutations can reduce the number of candidate genes to as few as seven or less, using a sample from only a single patient.³⁸⁻⁴¹

As with recessive disorders, the identification of *de novo* mutations has been shown to be feasible with exome sequencing, using a trio design with sequencing of the parents and the affected offspring. Sporadic diseases with a reduced reproductive capacity could be recessive, but could also be caused by *de novo* autosomal dominant mutations. These mutations can be selected by filtering out all variants that are present in the parents' exomes. This strategy has been used to identify mutations in mental retardation, autism, and schizophrenia.⁴²⁻⁴⁴ The reported rates of *de novo* coding mutations ranged from 0.75 to 0.90 per trio in these studies, which is in agreement with the calculation that, on average, a newborn has 50 to 100 *de novo* mutations in his or her genome, resulting in approximately 0.86 *de novo* amino acid-altering mutations.⁴⁵

The identification of a mutation in an autosomal dominant pedigree is more difficult, since all shared heterozygous variants could be disease-causing. However, successful cases in which the causative mutation was found have recently been reported: In a family with autosomal dominantly inherited amyotrophic lateral sclerosis, exome sequencing was combined with linkage analysis to identify the causative mutation.⁴⁶ In a large family with Charcot-Marie-Tooth disease, exome sequencing in three affected individuals, separated by eight meioses, identified only one variant shared by each individual.⁴⁷

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Exome sequencing for cardiomyopathies

Unfortunately, the powerful strategies mentioned in the previous section are not very suitable for the genetically heterogeneous inherited cardiomyopathies, since they are mostly autosomal dominantly inherited and the affected families are often small. *De novo* mutations have been described in cases of cardiomyopathy,⁴⁸⁻⁵² but they occur infrequently although they seem to be more prevalent in children with

restricted cardiomyopathy.^{53,54} However, assuming a *de novo* mutation in a cardiomyopathy patient is dangerous, since incomplete penetrance could well explain the absence of a phenotype in a parent who is a carrier of the causative mutation.

To apply exome sequencing in families with autosomal dominant diseases such as inherited cardiomyopathies, additional steps need to be taken. Combining exome sequencing with linkage strategies is a possible solution to this problem. In a family with recessively inherited cardiomyopathy, linkage analysis and homozygosity mapping identified the disease locus on chromosome 7q21. Exome sequencing of two affected sisters revealed a single shared homozygous missense mutation in the *GATAD1* gene, located on the 7q21 locus.³⁴ We have shown that a haplotype-sharing test (HST) using SNP-arrays can help in identifying shared loci which are most likely to contain the causative mutation. In the reported families, cardiomyopathy associated genes were located in these regions and mutations in *PKP2* and *MYH7* were identified.⁵⁵ In another family with dilated cardiomyopathy, by applying the HST and exome sequencing, we identified a *TTN* mutation located in the second-largest shared haplotype.⁵⁶ A similar approach identified a *TTN* mutation in four patients with hereditary myopathy with early respiratory failure, who were later found to share a haplotype containing the *TTN* gene, indicating a common ancestor.⁵⁷

Since classic linkage analysis is often not feasible in cardiomyopathy families, due to the small family sizes and the incomplete penetrance, a combined approach of the HST and exome sequencing, preferably with sequencing of at least two affected family members, could be successful. Even in small families, the HST could be useful, since each informative meiosis excludes 50% of the genome. Although the studies mentioned above used SNP arrays for the haplotyping or linkage studies, it is possible to infer haplotypes from the available exome data from multiple family members. This has been shown for the autosomal recessive hyperphosphatasia-mental retardation syndrome.⁵⁸

Exome data could also be used for a targeted approach. In this case there is no targeted capturing of genes associated with cardiomyopathy, but a targeted bioinformatics filtering for variants in any of these cardiomyopathy-related genes. At the moment, this may be more expensive and laborious, and more important, current exome enrichment strategies still result in incomplete representation and coverage of exons of interest, which would result in clinically relevant mutations being missed (see also below). However, when these strategies are improved, an advantage of exome sequencing will be that newly reported disease genes can be studied using the already available data without the need to perform new experiments. With falling costs, exome sequencing will eventually be replaced by whole-genome sequencing, enabling the study of the non-coding part of the genome, in addition to the exome.

Drawbacks of exome sequencing

Although exome sequencing will be successfully applied in many more Mendelian diseases in the coming years, there are a number of drawbacks that need to be recognised. The definition of the exome is not a strict or universal one. Different commercial exome platforms target different exonic segments; the comparison of Nimblegen, Agilent, and Illumina platforms showed that the targeted regions ranged from 44 Mb to 62 Mb and from 188,000 exons to 300,000 exons.^{59,60} So depending on the selected platform, the gene(s) and/or exon(s) containing the disease-causing mutation(s) could not be targeted at all and therefore these mutations will not be identified using that platform. When taking a 10-fold coverage as a minimum, the overall targeting efficiency for each platform (at 80 million reads), ranged from 96.8% for Nimblegen, to 90.0% for Illumina, and 89.6% for Agilent.⁶⁰ This shows that up to 10% of the exome would not pass the quality control filters if set at ≥ 10 fold coverage for a large (80 million reads) dataset, so in one out of ten cases, the causative mutation might be missed due to a lack of coverage. To overcome this issue, more reads could be sequenced but this will mean higher costs.

The filtering steps used could also filter out the mutation due to bioinformatic problems, e.g. the presence of the mutation in a control cohort such as dbSNP. If the mutation is a synonymous variant which results in cryptic RNA splicing, this variant is also likely to be excluded during filtering. Large insertions or deletions might also be missed, although these can be detected by using array-comparative genomic hybridisation (array-CGH) or might be detected by still to be developed bioinformatical tools that will be able to deduce copy number information from exome data. In addition, when the cause of the disease is located outside the exome, i.e. in the non-coding region of the genome, this will not be targeted with an exome platform. To search for variants outside the coding regions, whole-genome sequencing could be performed. As for exome sequencing, performing whole-genome sequencing will result in very large amounts of data that cannot be easily interpreted at the moment, although whole-genome sequencing will become more informative in the future and could also be applied for genome-wide association studies and linkage analysis. With falling costs, a transition from exome sequencing to whole-genome sequencing can be anticipated. The non-targeted whole-genome sequencing approach will result in a more proportionate distribution of sequence reads and therefore in higher coverage.

Genetic strategy for cardiomyopathies

A scheme for the genetic analysis of patients diagnosed with cardiomyopathy is shown in Figure 1. Depending on the phenotype and the costs, one or a few candidate genes with a known high yield in the population could be screened first. In

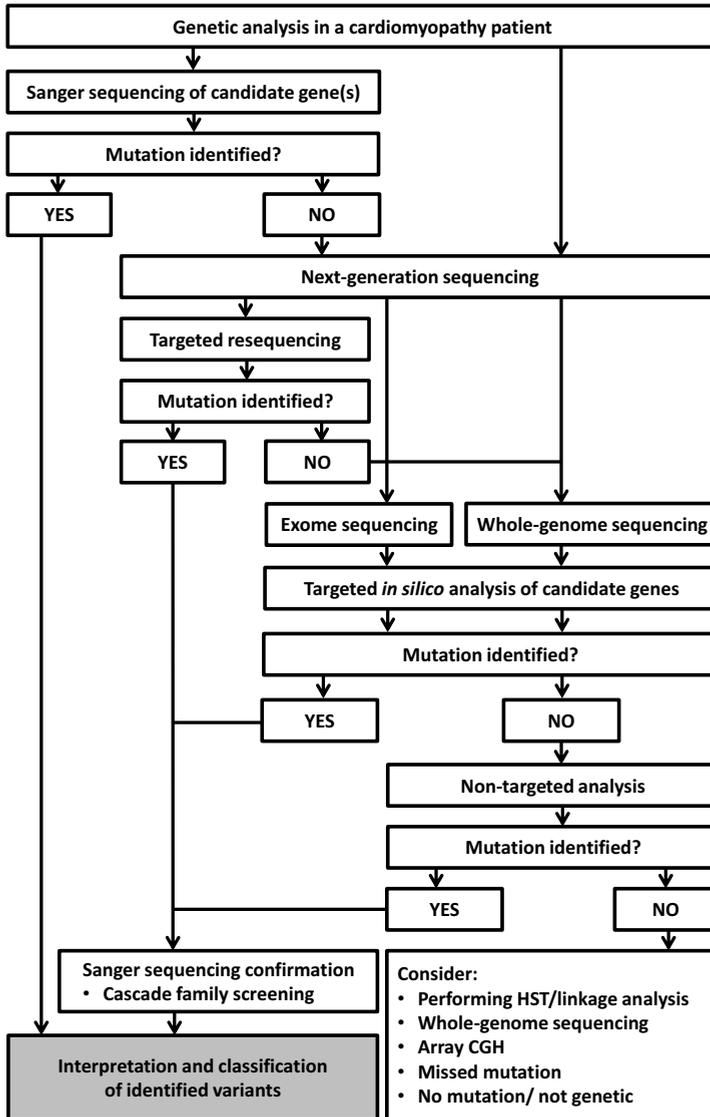


Figure 1 Schematic overview for the genetic analysis in inherited cardiomyopathies. Sanger sequencing of one or a few candidate genes should be considered first. If not performed or no mutations are identified, different next-generation sequencing strategies could be used, depending on the available resources. Targeted analysis could be performed using targeted enrichment or after targeted filtering of exome or whole-genome data. If no mutation is identified, several options could be considered, e.g. missing a mutation due to erroneous filtering. The interpretation and classification of the identified variants is the ultimate and most challenging step in the identification of pathogenic mutations in inherited cardiomyopathies.

patients with ACM, this would mean that sequencing of the *PKP2* gene, and the *PLN* gene in the case of Dutch ancestors, should be performed first, especially in familial Dutch cases, which are in 90% of cases caused by *PKP2* mutations,⁶¹ and the remaining 10% is almost completely caused by *PLN* mutations (unpublished data). If no causative mutation can be identified or multiple mutations are expected, as is the case in about 6% of ACM patients (see Introduction; Table 3), or as could be indicated by the severity of the disease, a targeted analysis could be performed. A targeted approach as a first step is justified for DCM patients, since so many genes are associated with the disease. It depends on the available resources whether this could be done by targeted enrichment of the known cardiomyopathy genes or by exome sequencing with targeted filtering. Although we have not identified any false-positive variants using our targeted approach, Sanger sequencing could be used to confirm true-positive calls and can subsequently be used for co-segregation analysis and cascade screening in family members. If no mutation is identified, the haplotype-sharing test or another type of linkage analysis should preferably be combined with exome sequencing. This requires sampling of additional affected family members. If no mutation can be identified using these strategies, several other options can be considered. First, the option that the disease is not genetic, which should of course be considered from the start, but it becomes more likely after extensive genetic research has not pinpointed a genetic cause. Second, the genetic cause could have been missed due to one of the reasons mentioned in the previous section and alternative techniques like array-CGH should be considered. Third, future developments could help identify a mutation in a patient. These could be: (a) genetic findings, e.g. non-coding variants identified using whole-genome sequencing and/or epigenetic changes (i.e. heritable changes in gene expression without changes in their coding sequence). Examples of such changes are histone modification and DNA methylation.⁶² (b) clinical developments in the family of a patient could also provide new insight; if more family members go on to develop the disease the likely success of haplotype sharing and filtering for shared variants will increase. So, after a certain period, a family could be re-evaluated, using new information.

Risk stratification and personalised medicine

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The implementation of exome or whole-genome sequencing will have a large impact on patient care. The identification of a genetic cause for the disease is only the starting point of the influence of genetic information. It can aid in making a more accurate diagnosis and prognosis, in choosing treatment options, and further enables other family members at risk for the disease to be identified. Genetic information can also be important in the risk stratification of inherited cardiomyopathies. In a

study on risk factors for malignant ventricular arrhythmias in carriers of mutations in the *LMNA* gene, the presence of a truncating or splice mutation was found to be an independent risk factor, when compared to missense mutations.⁶³ Another example is the long QT syndrome type 1 (LQTS1), a disease characterised by a prolonged QT interval and life-threatening arrhythmias, caused by mutations in the *KCNQ1* gene. SNPs in the 3' untranslated region of the *KCNQ1* gene were found to greatly modify disease severity in LQTS1 patients.⁶⁴ Compared with LQTS type 2 and type 3, beta-blocker therapy is more efficient in patients with LQTS1, illustrating a genotype-specific response and the importance of knowing the mutated gene.⁶⁵ Although these are illustrative examples, risk-stratification and treatment strategies based on the genetic background will remain unavailable for the majority of patients with inherited cardiomyopathies until large-scale collaborative studies have been performed. Given the genetic heterogeneity of the cardiomyopathies, this will be a great challenge.

In pharmacogenomics, genetic variation is being used to tailor drug therapies. These variations could modulate the pharmacokinetics of a drug, e.g. by slowing down its metabolism, resulting in increased availability of the compound and thus requiring a lower maintenance dose. A well-known cardiovascular example is the anti-coagulant drug warfarin: polymorphisms in three genes have been identified as influencing the appropriate dose of warfarin and dosage regimens based on the genotype data have been shown to reduce the risk of hospitalisation.^{66,67} Over 2,000 genes have been suggested to be involved in drug response, but the pharmacogenomics of cardiovascular disease is not yet established in clinical practice.⁶⁸ With the increasing emergence of genetic information, the use of pharmacogenomics will progress, but we should be cautious about being too optimistic, because it may prove to be like gene-therapy, which seemed very promising at first, but turned out to be much more difficult than anticipated. For instance, in pharmacogenomics we cannot exclude that proven effects of certain polymorphisms are enhanced or undone by other genetic variants that remain to be elucidated.

A nice example on the incorporation of whole-genome sequencing in risk assessment was published recently.⁶⁹ The authors reported the presence of 69 pharmacogenomic variants, of which 63 had previously been reported and six were novel but located in genes related to drug response. Furthermore, they used the genotype status for SNPs that have been associated in numerous genome-wide association studies to estimate disease probabilities for 55 diseases. For 15 of these diseases, the genetic risk was increased or decreased, defined by a likelihood ratio of >2 or <0.5 , based on the genotype data.⁶⁹

Several companies are nowadays offering SNP genotyping arrays to calculate personalised genetic risks directly to consumers. With the decreasing costs, such

companies will be offering exome or whole-genome sequencing in the near future. Patients will bring their own genetic data to their appointment with a clinical geneticist or other medical doctor, who will be expected to be able to judge these data in an appropriate way. Even though the significance of many variants will remain unknown for a long time, well-known pathogenic mutations will also be revealed and these will need appropriate interpretation and management. Clinical geneticists and genetic counsellors are trained to counsel patients and families about their genetic risks. Ideally, individuals are being counselled about their risks and the consequences of test results before genetic testing is started. With increasing uptake of commercially available genetic tests, however, more and more individuals will not seek genetic counselling until after genetic testing. These companies could hire geneticists to deal with questions from their consumers, but clinical geneticists and other health care professionals need to be aware that the impact of genetic information will rapidly increase. A boom could be expected, but even likely this is followed by decreased enthusiasm of consumers when they realise that at the moment much of their genetic data is non-informative or not interpretable with the current knowledge.

Taken together, the rapid progress in genetics in recent years will revolutionise the field of medicine, offering great opportunities to improve the care of patients but at the same time introducing many challenges. The available exome or whole-genome data of patients will contain many variants of unknown significance. Correct interpretation of these variants will need reliable prediction models and preferably large-scale functional assays, which will form the new bottleneck in the genetic assessment of patients. In the case of arrhythmogenic cardiomyopathy, in the five desmosomal genes alone, multiple variants are expected to be present in 6% of patients. In theory, these additional variants could explain the clinical variability seen between patients and within families, but in practice the weight of each variant and their possible interactions are very hard to determine. The presence of a pathogenic mutation in a patient with arrhythmogenic cardiomyopathy indicates a risk for a malignant course of the disease, but genotype-specific treatment is, as yet, unavailable.

It is important to note is that non-targeted approaches such as exome and whole-genome sequencing could identify mutations in genes not related (or not yet known to be related) to the disease, but which could be clinically relevant. An example of such an accidental finding would be the identification of a mutation in the *BRCA1* gene, which conveys an increased risk of breast cancer and ovarian cancer. Genetic counselling is therefore of the utmost importance. Not all patients want to know their risk of other diseases than the reason they sought medical help

for, even if surveillance and treatment options are available, illustrating the need for proper counselling and informed consent. Whether or not there is a duty for the physician to recontact the patient on discovering something which may, or certainly, impact their (later) health is part of an unresolved legal and ethical discussion.⁷⁰

In theory, insurance companies could use genetic data to assess the risks of individuals and increase their premium, or even exclude individuals at higher risk. To protect individuals from discrimination by life or health insurance companies on the basis of their genetic information, genetic non-discrimination legislation has now been introduced in most European countries, beginning in 1990 in Belgium. Since 2008, the United States has a federal law called the Genetic Information Non-discrimination Act to protect against genetic discrimination for employment and health insurance.⁷¹ In the Netherlands, insurance companies are also restricted in the use of genetic information of their clients. A survey among hypertrophic cardiomyopathy (HCM) mutation carriers showed that they frequently encounter problems when applying for insurances, often in the case of manifest disease, but the risk assessment of insurance companies is largely justified. However, 5% of mutations carriers encountered potentially unjustified problems, indicating the necessity to monitor the application of the existing laws and regulations by insurance companies.⁷² Furthermore, updates of current legislation could be needed to accommodate emerging genetic data.

To keep up with the pace of genetic progress, we need to further combine clinical care with genomic and basic research in order to unravel new molecular pathways underlying diseases and new treatment options. The functional characterisation of novel variants is laborious work but it is proving indispensable. A challenging future lies ahead for clinical geneticists and genetic counsellors who will be confronted with increasing amounts of genetic data, often lacking a well-argued interpretation. We need to be aware of the risks of genetic discrimination and the challenges posed by accidental findings. With these challenges in mind, the sharing of data in large, freely accessible databases or biobanks and large-scale international collaborations are essential. These steps will prevent that the ongoing progress in genetic research remains limited to the scientific community and should guarantee that patients will also benefit from our shared efforts.

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