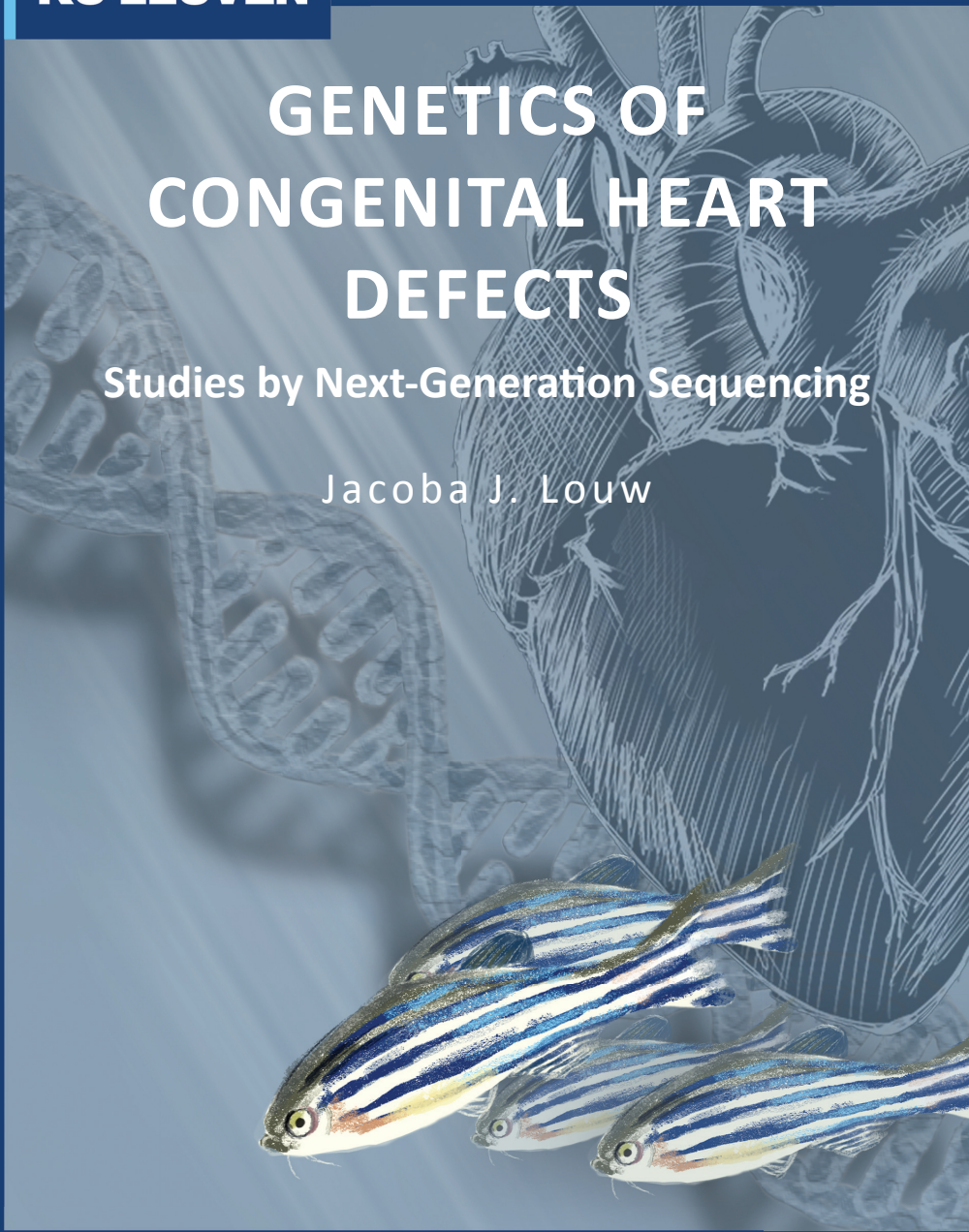
KU LEUVEN
Doctoral School of Biomedical Sciences
Faculty of Medicine
Center for Human Genetics

June 2017

GENETICS OF CONGENITAL HEART DEFECTS

Studies by Next-Generation Sequencing

Jacoba J. Louw



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Co-promotors: Prof. Marc Gewillig, Prof. Werner Budts

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GENETICS OF CONGENITAL HEART DEFECTS:

STUDIES BY

NEXT-GENERATION SEQUENCING

Jacoba J. Louw

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Vir my liefste Pappa

Ds. J.M. Louw

26 Oktober 1946 - 11 Julie 2016

1 Korinthiërs 13:2 en 13

2 En al sou ek die gawe van profesie hê en al die geheimenisse weet en al die kennis, en al sou ek al die geloof hê, sodat ek berge kon versit, en ek het nie die liefde nie, dan sou ek niks wees nie.

13 En nou bly geloof, hoop, liefde — hierdie drie; maar die grootste hiervan is die liefde.

DE STROOMVERSNELLING

In het matrix van genetica zweven ze rond

De oplossingen tot het raadsel, wat een vondst!

Op zoek naar de druppel

Die een zee van antwoorden geven

Tot nu nog door meerdere onbegrepen

Als Sherlock van ouds, gewapend met meer dan een vergrootglas

met data, pipet en microscoop; ook geduld komt handig te pas

juist of fout, herhaaldelijk bewezen

blijven denken en betwijfelen, blijven vragen en lezen

Ineens wordt het duidelijk, nog eentje opgelost!

Euforie! …en dan terug naar de volgende post

Student en leermeester, een gans team erbij

Wij zullen het vinden, zij-aan-zij

Stroomop of in stroomversnelling, blijven gaan, wij zijn er bijna!

En nu, plots, stopt mijn 'officiële' zoektocht…

'Neen', klinkt de chorus in mijn hoofd: 'Het is maar juist begonnen!'

Jacoba J. Louw, 2017

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SUMMARY

The main aim of this thesis was to evaluate the application of a powerful genetic tool, Next Generation Sequencing, to contribute to the understanding of genetics in congenital heart defects (CHD) and congenital cardiomyopathies. We specifically used Whole Exome Sequencing (WES), which analyses the protein coding parts of the human genome, in two distinct clinical groups: sporadic syndromic cases and small families with two affected siblings.

In the sporadic syndromic cases a *de novo* mutation was most likely and analysis was done by trio analysis (WES in the patient and both parents) and compared to index-only analysis (WES only in the patient). Here we could conclude that trio analysis outperforms index-only analysis as filtering was more efficient and resulted in a higher percentage of cases being solved (**Chapter 3**). We reported the first intragenic mutation in *MEIS2* which further confirmed the important role of this gene in normal development (**Chapter 3.1**).

In the families with two siblings sharing a similar phenotype, autosomal recessive inheritance was most likely. (**Chapter 4**). Linkage analysis was combined with WES and resulted in solving two families with a rare, neonatally lethal, cardiomyopathy. In the first family a homozygous mutation in a known gene (*ALMS1*) for Alström syndrome was found. The cardiomyopathy in this family represents the extreme end of the cardiac spectrum in this syndrome (**Chapter 4.1**). In the second family a compound heterozygous mutation was found in a gene (*KIF20A*) which has not been associated previously with human pathology. To prove the pathogenicity we performed additional functional tests, including animal studies in zebrafish which resulted in a progressive and lethal cardiomyopathy phenotype (**Chapter 4.2**). Discovery of these two genes open a new mechanism for future research in linking cardiomyopathies to genes involved in cytokinesis.

Based on our experience as well as current available literature we formulated guidelines for genetic diagnosis in CHD for cardiologists and geneticists. Classifying CHD into four clinical groups based on familial and syndromic characteristics necessitates a different strategic approach. In the last chapter we also addressed the crucial economic aspects and limitations of WES (**Chapter 5**).

SAMENVATTING

Het belangrijkste doel van dit proefschrift was om inzicht te verkrijgen in het genetisch landschap van aangeboren hartafwijkingen en congenitale cardiomyopathieën door de toepassing van een krachtige genetische tool, Next Generation Sequencing. Wij verrichtten analyse door Whole Exoom Sequencing (WES) - het eiwit coderende deel van het menselijk genoom - in twee afzonderlijke klinische groepen: sporadische syndromale patiënten en kleine families met minstens twee siblings met hetzelfde fenotype.

In de sporadische syndromale patiënten was een *de novo* mutatie het meest waarschijnlijk en werd de analyse uitgevoerd door trio analyse (WES in de patiënt en beide ouders) en vergeleken met index-only analyse (WES alleen bij de patiënt). Hieruit concludeerden we dat trio analyse beter functioneert gezien de filtering efficiënter was en frequenter leidde tot het vinden van een causaal genotype (**Hoofdstuk 3**). We publiceerden de eerste intragene mutatie in *MEIS2* die de cruciale rol van dit gen in normale ontwikkeling verder bevestigd (**Hoofdstuk 3.1**).

In families met siblings met een vergelijkbaar fenotype, was een autosomaal recessieve overerving het meest waarschijnlijk (**Hoofdstuk 4**). Koppelingsanalyse werd met WES gecombineerd en leidde tot het vinden van een causaal genotype in twee families met een zeldzame, neonataal lethale, cardiomyopathie. In de eerste familie werd een homozygote mutatie in een bekend gen (*ALMS1*) voor Alström syndroom gevonden. De cardiomyopathie in deze familie past bij het uiterste spectrum van het cardiale fenotype van dit syndroom (**Hoofdstuk 4.1**). In de tweede familie werd een samengesteld heterozygote mutatie in een gen (*KIF20A*) gerapporteerd dat nooit eerder werd geassocieerd met humane pathologie. Om het causale aspect verder te verifiëren werden bijkomende functionele studies uitgevoerd, inclusief een dierenmodel in zebravisjes die resulteerde in een progressieve lethale cardiomyopathie (**Hoofdstuk 4.2**). De ontdekking van deze twee genen opent een nieuw mechanisme voor toekomstig onderzoek door het koppeling van cardiomyopathieën aan mutaties in genen die betrokken zijn bij celdeling of mitose.

Wij hebben, gebaseerd op onze ervaring en de beschikbare literatuur, richtlijnen voor genetische diagnose bij aangeboren hartafwijkingen geformuleerd voor cardiologen en genetici. Op basis van syndromale en familiale gegevens onderscheiden we vier klinische groepen, die een andere strategie in genetisch diagnostiek voor CHD vereisen. Ten slotte werden de economische aspecten en beperkingen van WES die cruciaal zijn in het diagnostisch proces samengevat (**Hoofdstuk 5**).

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

INTRODUCTION

Congenital heart defects (CHD) are structural anomalies of the heart arising from abnormal formation of the heart or major blood vessels. At least 18 distinct types of congenital heart defects are recognized, with many additional anatomic variations. The majority of CHD are structural defects and have an incidence of 7,5/100 live births, if trivial lesions such as small muscular ventricle septal defects are included, making it the most common birth defect [1]. Moderate to severe CHD with functional consequences occur in 6-8 out of 1000 live births (19/1,000 live births if the potentially serious bicuspid aortic valve is included) [1, 2]. These structural defects are the result of abnormal embryonic heart development and can be anatomically classified into abnormalities of the septa, the heart valves, and inflow or outflow tract of the heart. These structural defects most often have functional consequences, but of varying degree and significance. Some structural defects do not have important functional significance in early life such as the aortic valve with two leaflets, the prolapsing mitral valve, a small persistent patency of the arterial duct, small septal defects and patency of the oval foramen. This group might also include structural defects which resolve without ever becoming clinically manifest, such as small muscular ventricular septal defects and atrial septal defects or peripheral pulmonary stenosis. Consideration of these lesions is important because they are common, and might inflate a prevalence estimate. This is also influenced by the surveillance and likelihood of diagnosis which differs greatly in developed and developing countries.

Cardiomyopathies (CM) are a different entity which are defined as primary myocardial disorders in which the heart muscle is structurally and functionally abnormal, in the absence of other causes such as CHD [3]. The annual incidence of pediatric cardiomyopathy is low, 3-6 per 1 million children, with the highest incidence in the first year of life [4, 5]. Four major types of CM are distinguished, i.e., hypertrophic, dilated, restrictive, and arrhythmogenic right ventricular cardiomyopathy (ARVC) [6], but there is considerable etiologic and phenotypic overlap. Previously, hypertrophic CM was described as a sarcomeric disease, with mutations encoding contractile proteins of the cardiac sarcomere, and pathogenic mutations have been detected in 11 genes encoding for sarcomeric proteins. Most mutations, around 80% are detected in *MYH7*

(β-myosin heavy chain) and *MYBPC3* (myosin-binding protein C) [7]. The other 9 genes account for far fewer cases of HCM and include troponin T and I, regulatory and essential myosin light chains, titin, α-tropomyosin, α-actin, α-myosin heavy chain, and muscle LIM protein. There is considerable intragenic heterogeneity, with >400 individual mutations which have been identified. There is also considerable variable expressivity, even within families, which is most probably to the influence of modifier genes and environmental factors [7]. Pathogenic mutations have also been detected in HCM in genes encoding for Z-disk, sarcoplasmic reticulum and plasma membrane proteins. Interestingly, mutations in sarcomeric and Z-disk genes have also been identified in patients with DCM and RCM and desmosomal protein genes in DCM, RCM and ARVC [7].

Reaching an etiological diagnosis is important for counselling on recurrence risks for future siblings or offspring. When no exact cause can be identified, predictions on recurrence risk for additional children in the family or future offspring is mostly based on empirical risks, without knowledge of the true underlying genetic mechanism [8]. Whereas recurrence risks for congenital heart disease usually are low, a large number of individually rare genetic disorders exist that may carry a significant recurrence risk e.g. Noonan syndrome, Barth syndrome or 22q11.2 microdeletion syndrome.

Despite diagnostic and surgical advances, CHD remains a major cause of infant morbidity and mortality. Improved prenatal and neonatal diagnostics, as well as pediatric bypass surgery and percutaneous interventions prolong life expectancy. As more patients survive into adulthood, the burden of disease, and the requirements for resources, have also achieved greater importance. In addition, this has resulted in increased demand for genetic counselling of adults who have been treated for a CHD themselves, and reassessment of recurrence risk for CHD in offspring [9]. In infants and children, one of the major challenges is to predict the long-term prognosis, concomitant morbidities and societal impact. For instance, the prospects of patients diagnosed with a Tetralogy of Fallot (ToF) as an isolated cardiopathy or as part of a 22q11.2 microdeletion syndrome differ significantly. Not only do patients with a 22q11.2 microdeletion syndrome have a different life expectancy but they have a high incidence of intellectual disability (ID), psychiatric

manifestations and medical comorbidity, necessitating life-long surveillance [10-14]. Long-term prognosis is compromised especially in CHD with additional manifestations including ID. Detailed phenotyping and long-term follow-up of many different syndromes have led to clinical guidelines for optimized guidance and follow-up. For instance patients with Down syndrome need to be followed for concomitant hearing loss and thyroid disease [15, 16], children with Noonan syndrome often have short stature and Williams syndrome is associated with a characteristic behavioral phenotype.

Over the past decades, a genetic revolution has taken place, which has largely been driven by technological advances. This has led to new insights in the function and structure of the human genome, and the identification of genes implicated a large number of genetic disorders. This knowledge and technology has rapidly been translated into the clinic, to advance genetic diagnosis in individuals with congenital disorders, including certain categories of CHD.

GENETIC TOOLS

The genetic tools available to study the structure and sequence of the human genome have changed considerably in the last 20 years. Traditionally, one distinguished cytogenetic versus molecular analyses, depending on whether one was studying the number and structure of chromosomes or individual genes, typically at the sequence level. However, with current technology, this distinction is often blurred.

Cytogenetic techniques

1. Karyotype

This classical technique was introduced in 1955 to study the number and structure of chromosomes of a human eukaryotic cell. Over the years, the resolution has increased by chromosome banding techniques and prometaphase chromosome analysis. However, on average, the resolution of karyotyping is limited to fragments of 5-10 Megabases (Mb), containing on average 35-70 genes (Figure 2). A unique feature of karyotyping compared to other cytogenetic tools is that it permits the detection of balanced chromosomal anomalies, without associated loss of genetic material. Such balanced translocations may disrupt a gene and cause a congenital defect. As such, they present unique but rare opportunities to identify genes for specific disorders. Examples in the field of cardiogenetics include *ELN* and Supravalvular aortic stenosis [17], *PROSIT240* and transposition of the great arteries [18] and the *TAB2* gene and cardiac valve disorders [19].

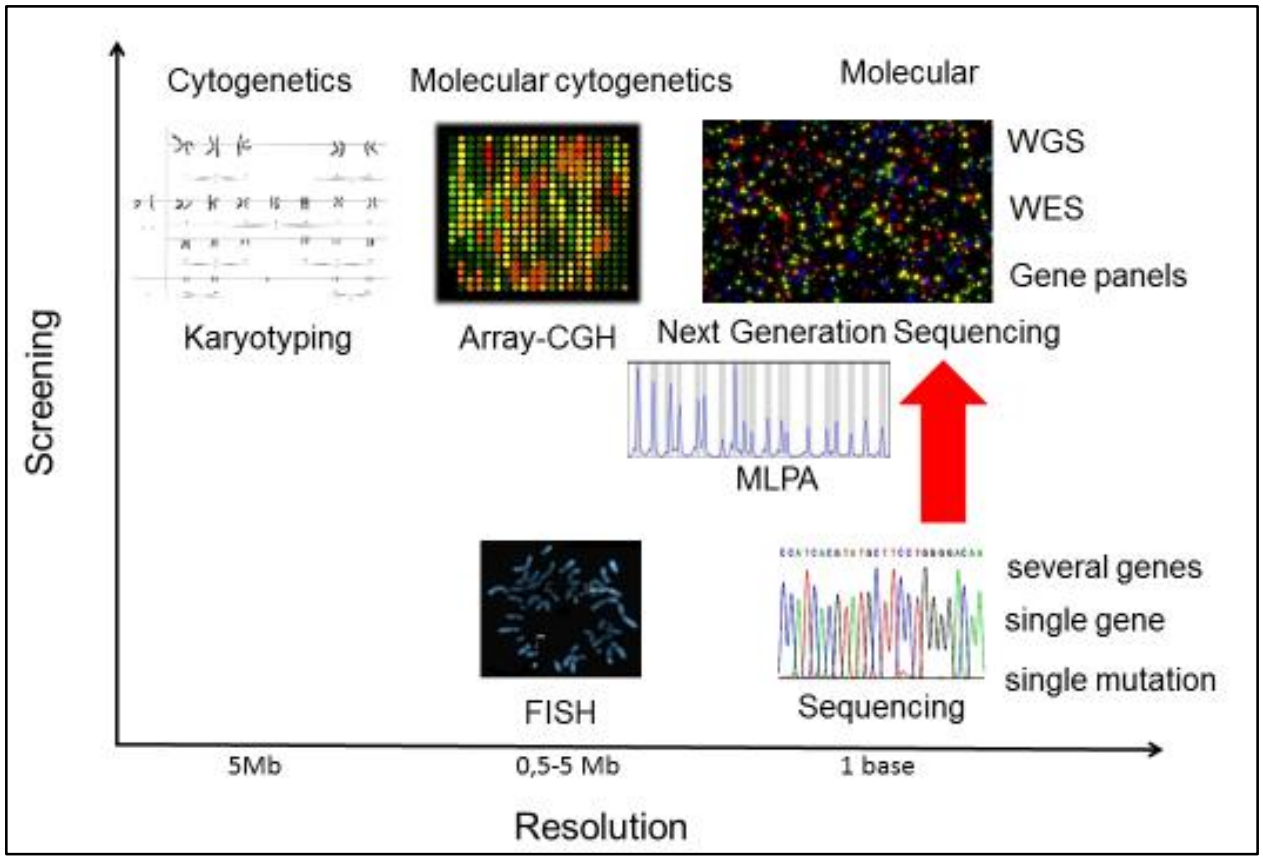

Figure 2. Advances in genetic technology showing the differences in resolution and screening capacity for different tests in research and diagnostics.

2. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) is a cytogenetic technique developed in the early 1980s and used to detect and localize the presence or absence of specific DNA sequences. Fluorescent probes are used which bind complementary sequences on the chromosome with a high degree of sequence complementarity. It is typically used to detect microdeletions, e.g22q11.2 microdeletion syndrome or del7q11.2 (Williams syndrome). Moreover, an important advantage is the possibility to rapidly detect imbalances or aneuploidies in interphase nuclei, e.g. to confirm a suspected diagnosis of Down syndrome. Whereas FISH has a resolution at the single gene level, its application is typically limited to a few loci in one experiment. Therefore, FISH remains targeted at the detection of a suspected imbalance, based on clinical suspicion.

3. Microarray-based copy number variation (CNV) detection

At the beginning of the 21st century, microarray analysis was introduced. It is a molecular technique used for detecting chromosomal copy number variations (CNV), a term used for small deletions (microdeletions) or duplications (microduplications). Two types of microarrays are used: oligonucleotide arrays for array comparative genomic hybridization (also called microarray-CGH, aCGH) and SNP-based microarrays. Microarrays combine the genome-wide screening capacity of karyotyping with the high resolution of FISH. It is now possible to detect small CNV's including a single gene or even a few exons. This technology is currently used as a routine diagnostic test for patients with a developmental disorder including congenital malformations, dysmorphism and intellectual disability. Using array-CGH 15-20% of unknown syndromic cases are currently solved [20]. This technology has also been introduced in prenatal diagnosis of syndromic CHD's in Belgium [21]. Microarray analysis additionally played a crucial role in the hunt for genes involved in CHD's. Numerous genes have thus been identified, often through the identification of the smaller region of overlap in patients with similar phenotype, e.g. *GATA4* on chromosome 8p23.1 [22], *CHD7* in CHARGE syndrome [23] and *NKX2.5* on chromosome *5q35.1* [24].

4. MLPA

MLPA (Multiplex Ligation-dependent Probe Amplification) is a multiplex PCR method that allows the quantitative detection of abnormal copy numbers for small regions (50-70 nt, typically exon level) of up to 50 different loci. It is known that for most monogenic disorders caused by loss-of-function mutations, a small percentage of such mutations are small deletions or duplications (a single or a few exons) which typically escape detection by current sequencing techniques or by microarrays. Therefore MLPA is mostly used to complete gene sequencing to detect such small imbalances [25-27]. An example is the vast variety of mutations in the dystrophin gene (DMD) which lead to Duchenne and Becker muscular dystrophies. The dystrophin gene is the third largest human gene and spans 2.2 Mb and has 79 exons. Intragenic deletions account for 65% of mutations and duplications in 10%. The remaining 25% circa of *DMD* mutations are represented by small mutations, including point mutations (missense, nonsense), frameshifting, indels, and

other rare types (small inversion, complex small rearrangements, atypical deep intronic mutations) [26].

5. Sequencing technology

Specific genes can be sequenced through Sanger sequencing, but this is laborious and costly to perform for multiple genes. More recently, next generation sequencing technology (NGS) was developed and allowed massive parallel sequencing. This made it possible to screen the coding parts (exons) of a large number of target genes (targeted panel) or of all genes (whole exome sequencing) in search of possible causative mutations.

NGS has shifted the challenge from variant detection to variant interpretation: the new "bottle-neck" is data analysis and interpretation. Sifting through the huge amount of data to find a pathogenic mutation in a known or novel gene is time consuming and strongly depends on existing knowledge. In addition, proof that a certain gene is a novel cause of a CHD may require lengthy functional studies, including animal models. This is not trivial, since the ultimate goal is translating this knowledge to the clinic, e.g. offering prenatal diagnosis. This has further led to indistinct borders between diagnostics and research.

5.1. Next-Generation Sequencing Technologies

Currently, we are witnessing a technological revolution of high-throughput massively parallel sequencing methods which allows us to determine the sequence of a large number of genes in one single analysis [28-30]. NGS technologies enable large-scale DNA sequencing, and have dramatically accelerated the genetic research and diagnostics, leading to a rapid increase in the delineation of novel genetic entities, as is shown in Figure 1 [31, 32].

Many of the steps in NGS technology have been evolving rapidly, including sample preparation methods [33], sequencing machines and bioinformatics tools for NGS data processing, but this is beyond the scope of this introduction.

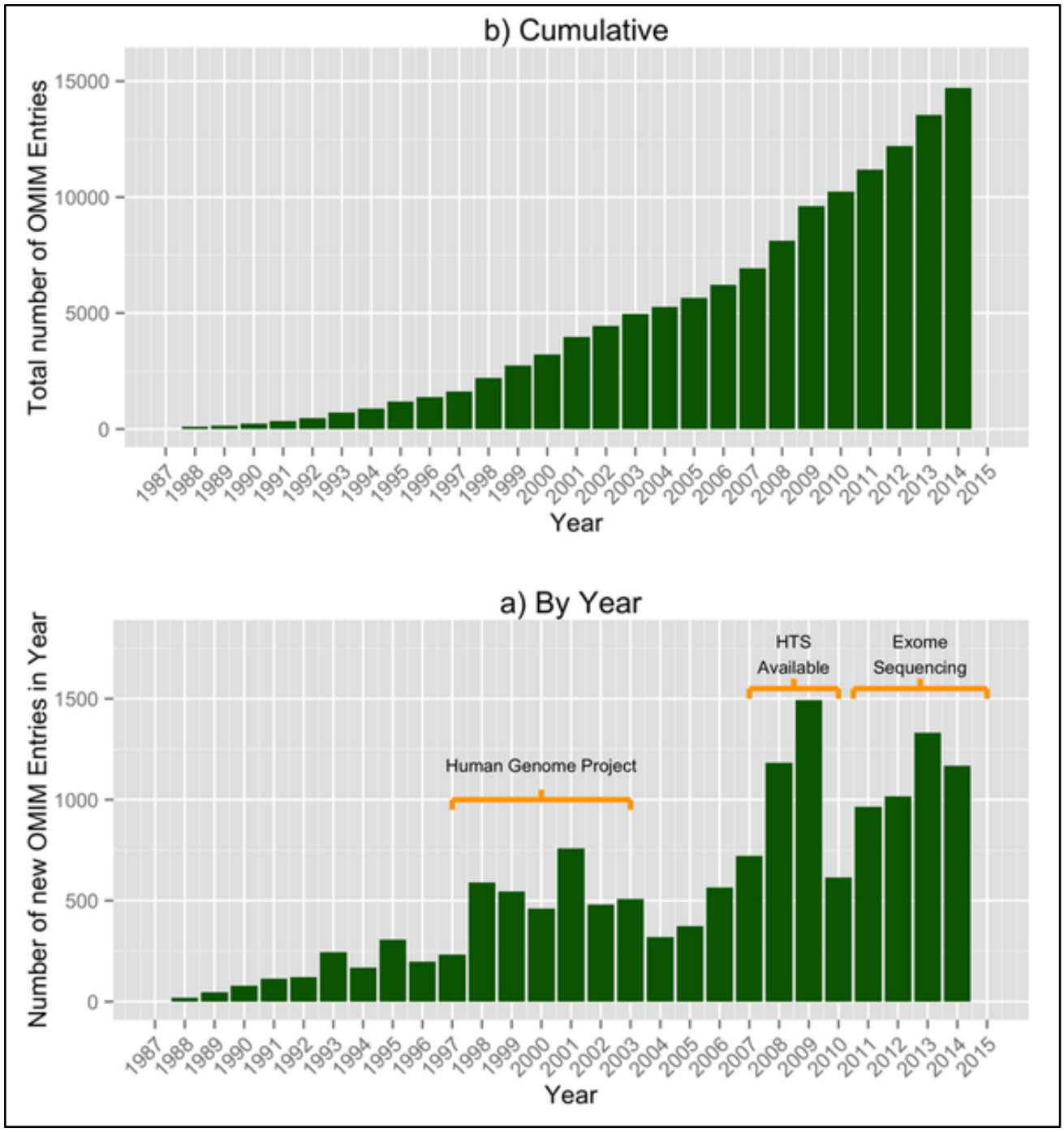

Figure 1. Growth in Number of Online Mendelian Inheritance in Man (OMIM) entries by Year. In the last two decades there has been a tremendous growth in the identification of genes for Mendelian disorders and identification of novel genetic disorders due to advances in genetic technology. Used with permission of Simon Sadedin (December 2015).

Depending on the number of genes resequenced, one can distinguish three different applications:

(1) Sequence analysis of a predetermined set of candidate genes for a specific condition. For instance, panels of known genes causing CHD, CM, or arrhythmias. This is typically used as a diagnostic tool. In the Center for Human Genetics in Leuven, using targeted capture for 57 genes implicated in congenital heart defects, we have thus far identified potential causative mutations in 46% of analysed families with three or more affected individuals (n = 13 families) [24]. For more complex phenotypes (e.g. CHD associated with intellectual disability or multiple malformations) one can use a Mendeliome approach, a panel consisting of the approximately 6000 genes known to be involved in a genetic disorder.

(2) Sequencing the coding sequences (exons) of all 20.000 human genes by Whole Exome Sequencing (WES). The exome encompasses around 1% of the human genome. WES have been used to identify point mutations, as well as small (<50 base pairs) insertions or deletions (indels) in exonic regions. WES is still essentially a research tool, but as mentioned previously, it is also being used in specific diagnostic settings.

As a research tool, WES has achieved a major breakthrough in the identification of genes involved in developmental disorders, including CHD. Since the majority of severe sporadic CHD is caused by a *de novo* mutation, several researchers used WES in a trio (CHD proband-parent) analysis. This has resulted in the identification of several novel CHD genes [34-38]. Of interest, the genetic mechanism for many of these genes is not a loss-of-function but dominant negative or gain-of-function mutation which typically result from missense mutations, and affects crucial functional amino acid residues. Examples include Rasopathies [39-41] where simple dosage alterations as in the deletion or duplication of these genes do not result in the same phenotype, and therefore, previous genome-wide screening studies using CNV analysis failed to identify these genes as candidate genes for CHD.

(3) In Whole Genome Sequencing (WGS) the entire human genome (including the 99% not included by WES) is analysed, thus all coding and non-coding regions. WGS is still an emerging technology, partly due to the relatively high cost of sequencing, but mainly due to the

requirements of more sophisticated bioinformatics tools and computing infrastructure.

GENETICS AND CLASSIFICATION OF CHD

Gene identification for CHD still represents a major challenge, especially since the genetics of CHD is heterogeneous [42]. Not only can different genes be involved, also different inheritance patterns exist. From a clinical point of view, we can distinguish syndromic from non-syndromic forms depending on the presence of dysmorphism, additional major malformations and/or intellectual disability. A second characteristic is the familial history: sporadic or familial occurrence, and the pattern of inheritance. These two characteristics allow us to define four different clinical categories of congenital cardiopathies (Figure 3): (A) sporadic syndromic CHD (22%) [43-45], (B) familial syndromic CHD (exceptional), (C) sporadic non-syndromic CHD (74%) and (D) familial non-syndromic CHD (4%). This distinction with regard to cardiopathies is a clinically useful lead to diagnosis, because each category requires a specific diagnostic approach but also with regard to further follow-up and guidance. Moreover, this distinction may aid directing the set-up of research towards the identification of novel genes.

(A) Sporadic syndromic CHD

This category mostly occurs as the result of a *de novo* mutation, either chromosomal (e.g. Down syndrome and deletion 1p36), or genomic disorders e.g. DiGeorge syndrome (22q11.2 microdeletion syndrome) and Williams syndrome (del7q11.23) or monogenic such as Holt-Oram, Kabuki, Alagille and Noonan syndromes, chromosomal imbalances.

(B) Familial syndromic CHD

Autosomal dominant inheritance of a syndrome is only expected when the expression is compatible with survival and reproduction, which is rare. Affected siblings with normal parents is typically observed in autosomal recessive (e.g. Friedreich ataxia) or X-linked recessive inheritance (e.g. Barth syndrome).

(C) Sporadic, non-syndromic CHD

This is the most commonly observed group, and is thought to have a multifactorial cause, i.e. the interaction of multiple genetic and environmental factors, each with a low risk. However, more recently, exome and copy number variation studies indicate that rare variants with a moderate effect are involved, at least in a small proportion of cases [46, 47].

(D) Familial non-syndromic CHD

Rarely, multiple affected individuals exist with recurrence of a same type of non-syndromic CHD. In most families, inheritance is autosomal dominant, and known genes include *ELN*, *NKX2.5*, *GATA4*, and *NOTCH1*.

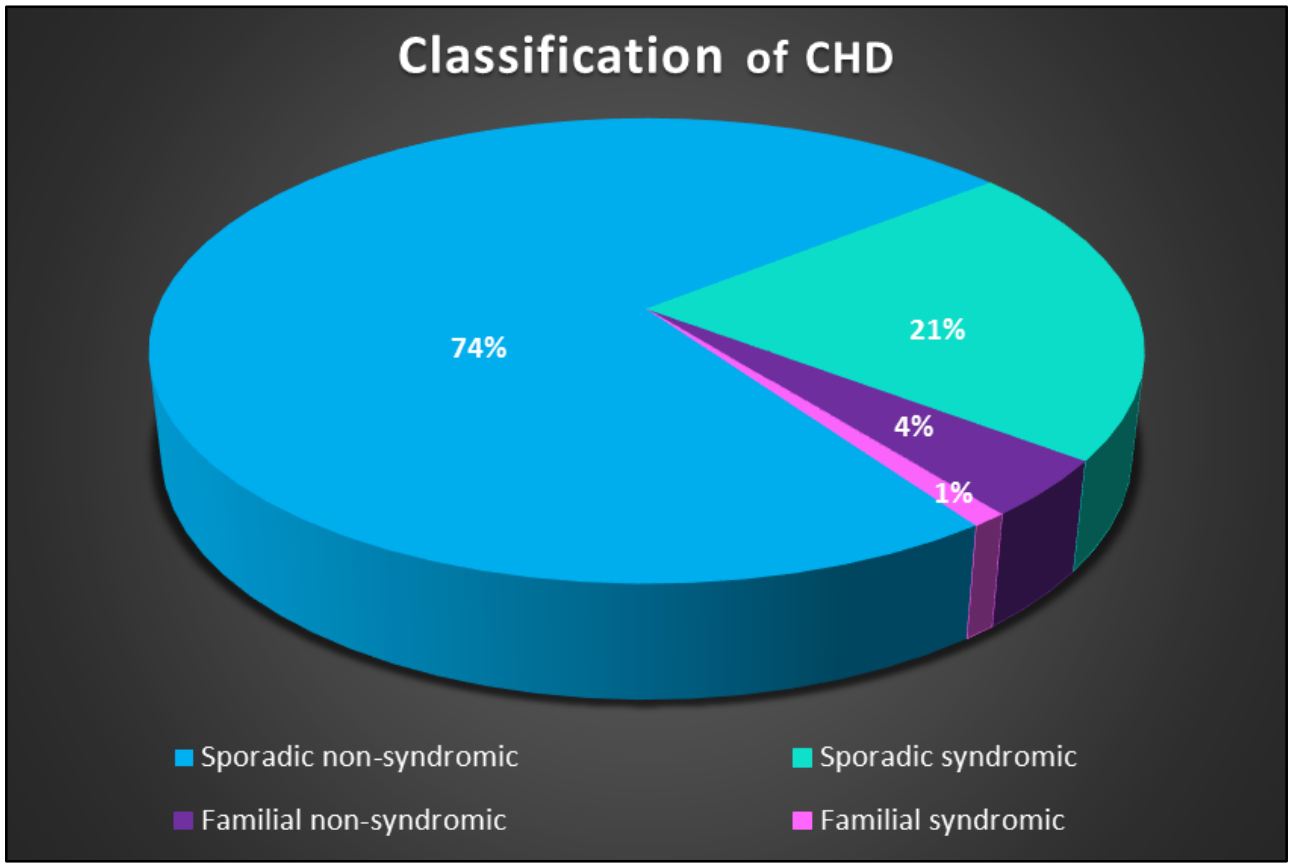

Figure 3. The genetics of CHD results in four different clinical categories: sporadic non-syndromic (74%), sporadic syndromic (21%), familial non-syndromic (4%) and familial syndromic (exceptional <1%).

GENETIC STUDIES OF SYNDROMIC CHD AND CARDIOMYOPATHIES BY NGS

In this study, we evaluated the use of NGS in the identification of the genetic cause in syndromic forms of CHD, both sporadic and familial.

Sporadic syndromic cases with unknown cause

Since the majority of known sporadic syndromes featuring a major malformation have a *de novo* AD cause, we hypothesized that also in those with an unknown cause, a *de novo* dominant mutation is present in a significant proportion. To identify causal mutations, exome sequencing using a trio approach (parents and child) was likely to be the most efficient way. Given the cost, we compared this to an index-only analysis.

Familial syndromic congenital heart defects

When two or more siblings in the same family have the same CHD, the most likely explanation is a multifactorial inheritance. However, certain very rare and specific (syndromic) phenotypes may have a monogenic cause, but no obvious candidate genes exist. This occurs when the combination of distinct anomalies observed in the siblings does not fit a known entity, and thus constitute a new syndrome. Alternatively, the cardiac phenotype is unique, e.g. an unclassified type of CM. In these families, the inheritance pattern is most likely autosomal recessive (AR) and sometimes X-linked. Germline mosaicism in one parent for an AD inherited disorder should also be considered.

We evaluated to what extent exome sequencing in combination with linkage analysis allowed us to identify the causative genes in such small families with unknown cause.

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CHAPTER 2

RESEARCH OBJECTIVES

OBJECTIVES OF THE RESEARCH

The general objective is to contribute to understanding the genetics of congenital heart defects (CHD) and congenital cardiomyopathies.

To this aim, we propose to

1. evaluate the use of Next Generation Sequencing (NGS) in the detection of causal mutations of syndromic CHD in sporadic and familial cases.

2. identify and characterize novel genes in individuals and families.

Based on our findings and current literature, to provide updated guidance for the introduction of NGS into the congenital cardiology clinic.

CHAPTER 3

THE USE OF NEXT GENERATION SEQUENCING (NGS) IN THE DETECTION OF CAUSAL MUTATIONS OF SYNDROMIC CHD IN SPORADIC CASES

INTRODUCTION

Congenital heart defects (CHD) , including trivial lesions such as small muscular ventricle septal defects, have an incidence of 7,5/100 live births making it the most common birth defect [1]. Of these, 22% have a sporadic CHD in the context of a broader syndrome, i.e. the presence of additional major malformations and or dysmorphism [2-4]. This is associated with an increased morbidity and mortality and carries a high risk of developmental delay and intellectual disability. Thus, syndromic CHD has an important impact on the individual, his family and on society. There is an indispensable need for etiological diagnosis, since it is in this group that genetic counselling is frequently requested with regard to recurrence risks. In addition, a diagnosis may aid in establishing the prognosis with regard to intellectual disability (ID) and additional medical complications which are often not yet visible in new-borns.

Identifying the underlying genetic defect in these cases still presents a major challenge due to the vast etiological heterogeneity of syndromic CHD. Not only can different genes be involved, but also different types of mutations (chromosomal or at the gene level) and different inheritance patterns exist [5, 6]. Using a clinical approach, an exact etiological diagnosis can be reached in about 55% of cases, which have a clinically recognisable syndrome [4]. The diagnosis is straightforward for common syndromes (e.g. Down syndrome and 22q11.2 microdeletion syndrome), or for those with a characteristic pattern of malformations (e.g. septal defects and pre-axial hand defects in Holt-Oram syndrome) or with a highly characteristic type of CHD (e.g. supravalvular aortic stenosis in Williams syndrome). The suspected diagnosis can then be confirmed by a targeted genetics test, e.g. FISH, array-CGH or gene sequencing. However, reaching a clinical diagnosis can be challenging is certain circumstances. Special expertise is required to establish a diagnosis in the large number of rare disorders that feature a CHD (e.g. Kabuki syndrome), especially when manifestations can be variable (e.g. Alagille syndrome or Noonan syndrome). Additionally, in the prenatal or early neonatal setting, the full clinical phenotype is not yet apparent, further complicating clinical diagnostics.

In cases where no clinical diagnosis can be reached, genetic testing has an important role. Genome-wide screening for the presence of mutations is therefore an important next step after

clinical evaluation. Since the early 2000's, microarray-CGH was introduced as a tool to screen for small chromosomal imbalances. On average, this has led to a diagnosis in 15-20%, based on the inclusion criteria and expertise of clinical pre-screening [7]. More recently, massive parallel sequencing allowed genome-wide screening of all genes for the presence of sequence alterations.

Previous genetic studies of individuals with a clinically unexplained developmental disorder have shown that de *novo* (dominant) mutations play a major causative role [8-10]. Most syndromic CHDs are also caused by *de novo* dominant mutations, whereas recessive or X-linked inheritance is rare, at least in a society where consanguinity is exceptional [11-13]. We here evaluated, in a pilot study, exome sequencing as a tool to identify the causal mutations in syndromic non-familial CHD. By selecting sporadic cases with a severe and syndromic phenotype, we expected that they are enriched for a *de novo* genetic origin [7, 14]. The most efficient way to identify such de novo mutations is a trio approach, where the whole exome sequence (WES) of both parents and the affected child are compared. Due to the current high cost of WES analysis in a trio setting, we compared this to an index-only approach. Finally, we participated in an international collaboration to study a large cohort of syndromic and non-syndromic CHD patients in a trio setting.

PATIENTS AND METHODS

Patient selection and description

Since it is known that the majority of severe sporadic syndromes have a *de novo* autosomal dominant cause, we hypothesized that also in those with an unknown cause, a *de novo* dominant mutation is present in a significant proportion. For this reason, we analysed nine trios, consisting of both parents and the affected child. In addition, we analysed four index-only cases to compare both approaches.

Inclusion criteria

1. **Syndromic CHD** with syndromic defined as the presence of 1 additional major abnormality apart from the CHD, or intellectual disability not otherwise explained and/or dysmorphism, defined as 3 or more minor anomalies.

2. **An unknown cause** of the syndrome after extensive evaluation:

* All patients were examined by an experienced clinical geneticist and clinical pictures were available for discussion with other clinical geneticists and for review of the phenotype in case a mutation was found. Clinical information on the parents were available.

* The patients were old enough to be clinically (re-)evaluated with regard to psychomotor development i.e. > 12 months.

* High resolution array-CGH was normal in the patient and showed no unclassified variants.

3. **DNA was available** from the patient and both parents in trio analysis.

4. **Sporadic patients**: no familial occurrence i.e. no other family members with the same or a similar condition; no familial occurrence of a CHD in a first or second degree relatives. This was to exclude the possibility that the heart defect and additional features have a separate cause.

5. **No external causes identified**: no teratogens during pregnancy, no neurological damage (e.g. during surgery).

The study was approved by the Ethical Committee University Hospital Leuven (S52853 – B322201010111). Informed consent was obtained from all parents.

The clinical data are presented in Table 1.

TABLE 1

Clinical description of cases

Patient	Gender	Gene	Age	ID	HC	Weight	Height	CHD	Other major malformations	Minor malformations	Other characteristics
1	M	*DYRK1A*	4,23	S	-5	-3,3	-2,8	CoA, HAA, a. lusoria	microcephaly, small cerebellum pachygyria,	sparse hair, deep-set eyes, small scrotum, simple ears	hypertonia, epilepsy, IgG2-deficiency
2	F	*MEIS2*	4,00	M	0,1	-0,6	-1,3	ASD II, VSD, CoA	cleft palate; congenital lobar emphysema, achalasia	bitemporal narrowing, broad 1st ray, gap toe 1-2, syndactyly toe II-III	severe feeding problems, GERD, autism
3	F	*SALL1*	9,69	M	-2,7	-2,8	-1,4	ToF	microcephaly, congenital renal hypoplasia	facial dysmorphism, excessive pubic fat, deep palmar grooves, long slender fingers, short 5th toe	severe feeding problems, dry skin
4	F	*EFTUD2*	35,77	S	-3,1	-1,8	-0,9	Truncus arteriosus	microcephaly, cleft palate, hearing impairment	facial dysmorphism	epilepsy
5	M		4,6	S	-0,4	-1,6	-1,9	AVSD	unilateral renal agenesis, hypospadias, cryptorchidism	facial dysmorphism, branchial appendage, presacral dimple, shawl scrotum, short preputium	
6	M		4,95	NL	-0,1	-3	-4,4	ToF	esophageal atresia, microcolon, omphalocele, pyloric hypertrophy, subependymal cyst	facial dysmorphism, clinodactyly finger 2 & 5 hypoplasia of terminal phalanges, syndactyly toes II-III, sacral hairy dimple	fine motor and social development normal, gross motor and verbal developmental delay
7	M		15,98	S	-1,9	-0,5	-0,2	AP window, IAA	Hirschsprung's disease, microcephaly	none	
8	F		8,21	M	-0,9	-3,1	-2,9	AVSD	agenesis corpus callosum, bilateral focal cataract, unilateral dilated pyelon	facial dysmorphism, posterior rotated ears, transverse palmar grooves, pedal edema	
9	M		18,46	M	NA	-0,3	0,9	ToF	kyphosis and scoliosis	facial dysmorphism, high palate, webbed neck, slender fingers and hands, diffuse striae, tight hamstrings	pes planus, bilateral hallux valgus, pectus carinatum, strabismus

Patient	Gender	Gene	Age	ID	HC	Weight	Height	CHD	Other major malformations	Minor malformations	Other characteristics
10	M	*ANKRD11*	13,61	Mi	-2,2	-4,8	-3,8	ASD II, VSD	hearing loss	facial dysmorphism	autism, microcephaly
11	F		4,52	NL	0,2	0,4	0,1	Right isomerism, UVH, AVSD	none	ectopic thyroid, congenital hypothyroidism	
12	F		19,07	NL	NA	-1,6	-0,3	PDA, CoA, PAPVR	renal hypoplasia, cleft lip and palate	facial dysmorphism	GH deficiency, cholestatic liver disease, retinopathy
13	F		15,2	S	-5,3	-7,28	-9,0	PDA	microcephaly	facial dysmorphism	feeding problems

Cases 1-9 were analysed by means of trio WES, cases 10-14 by index-only WES. Columns show gender, Gene involved in the phenotype, age at examination, level of intellectual disability, biometric values, type of CHD, description of other major malformations, description of minor malformations and other characteristics. Level of intellectual disability is classified into normal (NL), mild (Mi), moderate (M) and severe (S). Congenital Heart Defects are described as coarctation of the Aorta (CoA), Hypoplastic aortic arch (HAA), atrial septal defect type secundum (ASD II), ventricle septal defect (VSD), tetralogy of Fallot (ToF), atrioventricular septal defect (AVSD), aortopulmonary window (AP window), interrupted aortic arch (IAA), univentricular heart (UVH), patent ductus arteriosus (PDA) and partial anomalous pulmonary venous return (PAPVU). Head Circumference (HC), weight and height is given as standard deviations. Some data were not available (NA).

Exome sequencing

Trio analysis

WES was done by the Genomics Core KU Leuven/UZ Leuven as follows: Library construction for all samples were prepared using TruSeq DNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA) in which platform-specific adaptors and unique DNA indexes were ligated. For each sample, 1 µg genomic DNA was sheared by sonication to approximately 300bp fragments, followed by end-repair, adenylation and adapter ligation steps. DNA sequencing libraries were subsequently enriched with the SeqCap EZ Human Exome Library v3.0 (Roche, NimbleGen), reads were generated on the Illumina HiSeq2000 or HiSeq2500 machine using a paired-end 2x100 bps protocol with 3-4 exome-seq samples pooled per lane of a sequencing flow-cell. Sheared DNA, whole genome libraries and enriched exome-seq libraries were validated using DNA-1000 chips on the BioAnalyser (Agilent), and library concentrations were determined using the dsDNA Broad Range Assay using the Qubit (Invitrogen). Post-capture LM-PCR amplification was performed using the Library Amplification Readymix containing KAPA HiFi DNA Polymerase with 14 cycles of amplification.

Sequence reads were aligned to the human genome reference sequence (Genome Assembly GRCh37/hg19) with the Burrows-Wheeler Aligner (BWA v. 0.6.2 or v. 0.7.8). SAMtools (v. 0.1.18 or v. 0.1.19) were used for SAM to BAM files conversion, sorting and indexing alignments. Picard tools (v. 1.78 or v. 1.118) were used to compute quality metrics and mark PCR-generated duplicates. The Genome Analysis Toolkit (GATK v. 2.4.9 or v. 3.2.2) software package was used to perform local realignment around indels and base quality score recalibration. SNPs and small indels were called using GATK HaplotypeCaller (v. 2.4.9 or v.3.2.2). Variants annotation was performed with ANNOVAR (v. 11-0882013 or v. 11-02-2013), including data sets from dbSNP137, the NHLBI 6500 Exome and 1000 Genomes projects for variant frequencies, amino acid change, functional predictions from SIFT, Polyphen2, LRT, MutationTaster and PhyloP.

The total target region was 63 mega base pairs (Mb). Quality assurance was aimed at having 20X coverage in 80% of the targeted exome bases.

Index-only analysis

Due to the cost of WES in Trio analysis, we collaborated with the Human Genome Sequencing Center (HGSC) at Baylor College of Medicine through the Baylor-Hopkins Center for Mendelian Genomics initiative in an index-only analysis. Whole exome sequencing was performed at the HGSC as described previously [15]. Libraries were constructed into Illumina paired-end pre-capture libraries according to the manufacturer's protocol (Illumina Multiplexing_SamplePrep_Guide_1005361_D) with modifications as described in the BCM-HGSC protocol (https://www.hgsc.bcm.edu/content/protocols-sequencing-library-construction). For each sample, 0.5 µg of genomic DNA was sheared by sonication to 200-300bp fragments, followed by end-repair, adenylation and adapter ligation steps. DNA sequencing libraries were subsequently enriched with the SeqCap EZ Human Exome Library v3.0 (Roche, NimbleGen), and paired-end reads were generated on the Illumina HiSeq2000 platform with 6 samples pooled per lane of a sequencing flow-cell. After the final AMPure XP bead purification, quantity and size of the capture library was analyzed using the LabChip GX electrophoresis system. Post-capture LM-PCR amplification was performed using the Library Amplification Readymix containing KAPA HiFi DNA Polymerase with 12 cycles of amplification. The total target region was 37 Mb. The samples achieved 89% of the targeted exome bases covered to a depth of 20X or greater. Initial sequence analysis was performed using the HGSC Mercury analysis pipeline [16, 17] (https://www.hgsc.bcm.edu/software/mercury) which moves data through various analysis tools from the initial sequence generation on the instrument to annotated variant calls (SNPs and intra-read insertions/deletions). Next, the Atlas2 suite (Atlas-SNP and Atlas-indel) was used to call variants and produce a variant call file (VCF). Finally, annotation data was added to the VCF using a suite of annotation tools "Cassandra" that brings together frequency, function, and other relevant information using AnnoVar with UCSC and RefSeq gene models, as well as other internal and external data resources.

Variant filtering

1. Trio analysis

Exome sequences were obtained from both parents and the patient. VCF files were converted into Excel files and all further filtering was done manually. Initially, when developing the filtering process, we compared different annotation tools (Cassava and GATK), different genome browsers (RefGene, Ensembl and knownGene), various chronological orders of filtering steps and stringent versus less stringent filtering. We analyzed variants with two classes of quality. Class 1 calls, the best quality calls, were all calls (reference or mutant) emitted with a genotype quality bigger than 70. The genotype quality is the smallest non-zero phred likelihood (PL), with the zero PL value being the genotype given to the call. The PL values were always given in the following order: homozygous reference, heterozygous, homozygous variant. Class 1 calls were thus calls for which both non-zero PL values were bigger than 70, e.g. a homozygous variant has a PL = 185,96,0. Class 2 calls were less confident calls for which one non-zero PL value is bigger than 70, e.g. a heterozygous variant has a PL = 185,0,45. Class 3 calls were not analyzed as these calls were calls for which both non-zero PL values were smaller than 70, and thus were classified as “no call”.

1.1. De novo dominant or X-linked hypothesis

To exclude local rare variants, we first filtered all variants in the patient against variants in 72 other in-house exomes from patients with various other, distinct phenotypes. All heterozygous variants occurring in one other patient were excluded. Next, we filtered for variants that were reference in the parents and mutant in the patient, thus retaining possible *de novo* variants. We then retained exonic nonsynonymous variants, exonic/splicing and intronic splicing variants (< 5 positions from the splicing site). Next, we only retained variants with a frequency of <1%, or with an unknown frequency, in the 1000 Genomes Project. In-silico predictions by Polyphen, Mutation Taster and SIFT were used as an additional filter when necessary. Only variants predicted as damaging or disease causing by 2 or more in-silico tools were then further analysed. The remaining list was manually curated with literature using UCSC, OMIM, Pubmed and GeneCards.

1.2. Autosomal recessive inheritance

1.2.1. Homozygous inheritance

Firstly, the patient was filtered against 72 other in-house exomes, excluding any variant for which another patient was homozygous. Next, we retained only variants for which the parents were heterozygous. Only exonic, exonic/splicing and potential splicing variants were retained. Only variants occurring with a frequency of <5% or with an unknown frequency in the 1000 Genomes Project were included. Remaining variants were manually filtered according to inheritance pattern: both parents needed to be heterozygote. In-silico predictions and manual curation of the remaining variants were then done as described above.

1.2.2. Compound heterozygous inheritance

Firstly, the patient was filtered against 72 other in-house exomes, excluding any variant for which an individual was homozygous. Next, we retained only variants for which the parents were heterozygous. Only exonic, exonic/splicing and splicing variants were included as described previously. Variants occurring with a frequency of <5% or with an unknown frequency in the 1000 Genomes Project were included. Remaining variants were manually filtered according to the rule: one paternal and one maternal inherited variant occurring in the same gene. In-silico predictions and manual curation of the remaining variants were done as described above.

1.3. X-linked recessive inheritance

Only variants on the X-chromosome were selected in the patient, and reference calls were excluded. Only maternal inherited variants were included as the father would have to be affected if he was carrying the variant. Only exonic, exonic/splicing and splicing as described above were included. Variants occurring with a frequency of <5% or with an unknown frequency in the 1000 Genomes Project were included. In-silico predictions and manual curation of the remaining variants were done as described above.

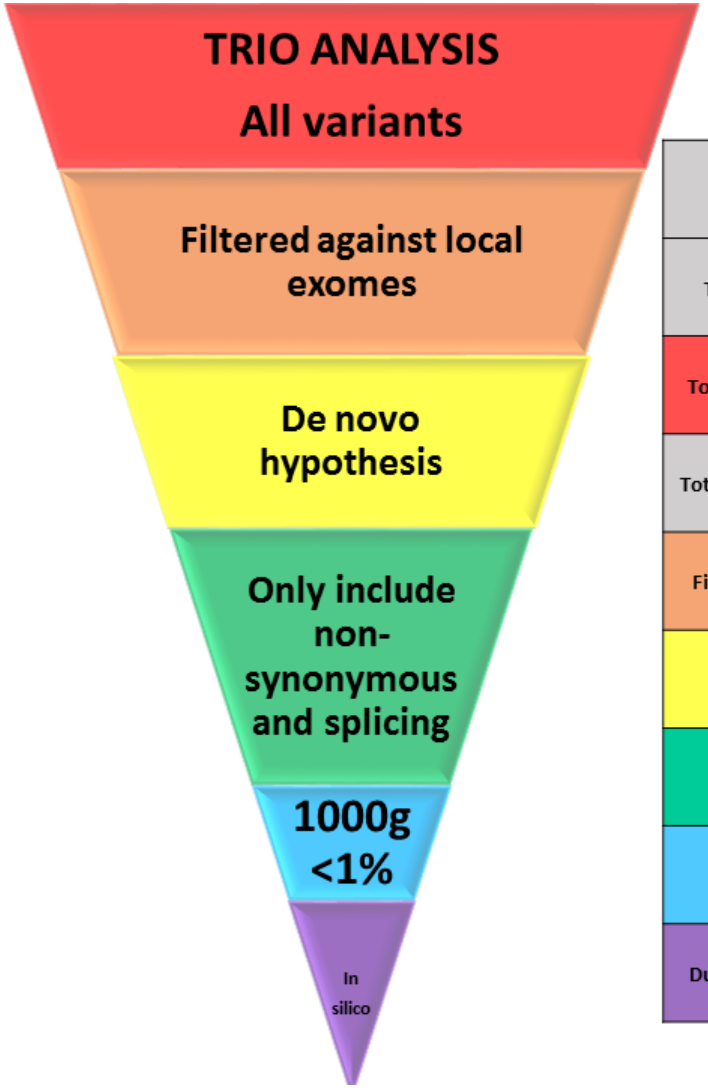
2. Index-only analysis

To identify autosomal dominant or X-linked candidate mutations, the patient was first filtered against 79 local exomes, i.e. where only the patient is heterozygous for a specific variant. Only exonic, exonic/splicing and splicing as described above were included. Variants occurring with a frequency of <1% or with an unknown frequency in the 1000 Genomes Project were included. In-silico predictions by Polyphen, Mutation Taster and SIFT were used with discretion as an additional filter; only variants predicted as damaging or disease causing by 2 or more in-silico tools were further analyzed. The remaining variant list was then manually curated with literature using UCSC, OMIM, Pubmed and GeneCards.

For the autosomal recessive hypothesis, homozygous calls were kept in the patient where no other patient was homozygous. Heterozygous filtering was also done by keeping all heterozygous calls and only retaining the genes where two variants occurred in the same gene. Further filtering was not possible for compound heterozygosity (one maternal and one paternal variant in the same gene) as parental WES data was not available.

RESULTS

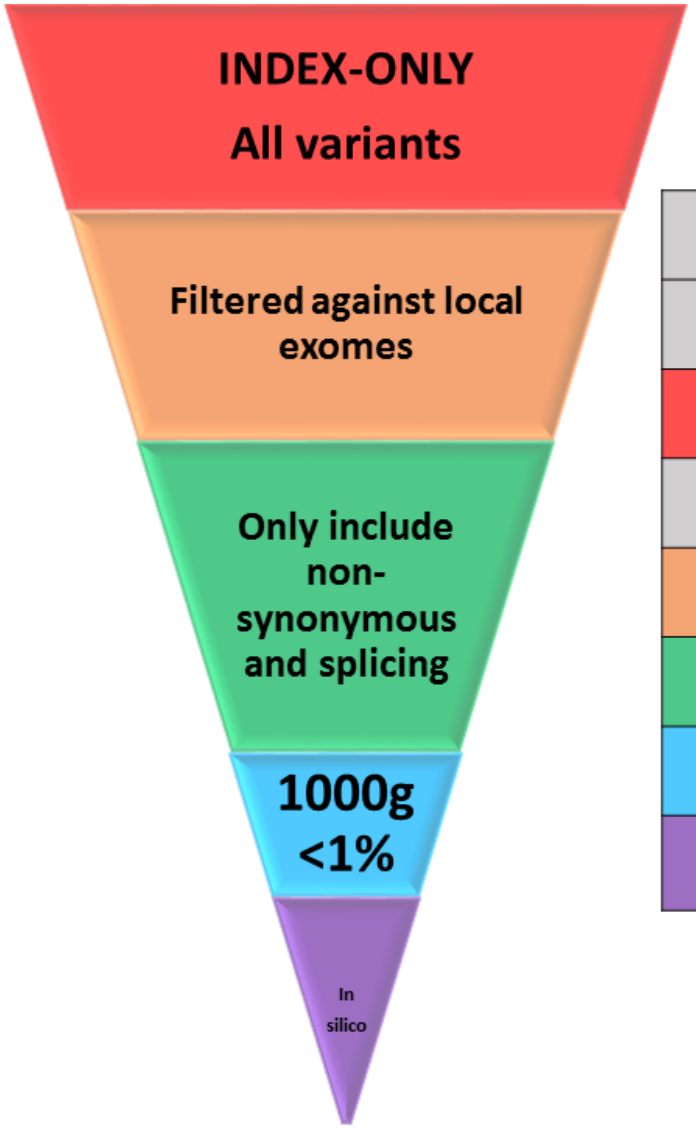
The variants filtering in the different approaches is schematically shown in figure 1 and 2.



Patient	1	2	3	4	5	6	7	8	9
Total local exomes	73	73	73	73	73	49	49	49	49
Total variants in index	429 652	222 012	286 768	153 213	250 923	201 996	195 591	248 683	273 963
Total CS1 reads in index	161 923	100 426	132 214	54 351	137 987	108 508	110 190	140 846	119 552
Filtered local exomes	55 120	45 758	49721	31 300	51 135	53 829	21 576	39 933	30 071
De novo	457	201	381	268	589	282	317	180	210
NS and splicing	237	99	190	147	226	157	31	80	100
1000g < 1%	132	95	171	121	173	129	22	57	84
Duplicates +/- In silico	77	48	97	83	109	45	22	47	59

Figure 1

Variant filtering steps for *de novo* inheritance using trio analysis with the remaining variants after each filtering step shown in the accompanying table.



Patient	10	11	12	13
Total local exomes	80	80	80	80
Total variants in index	171 754	175 362	158 847	154 816
Total CS1 reads	95 882	99 071	85 150	81 213
Filtered in-house	896	1041	876	852
NS and splicing	606	705	576	551
1000g < 1%	590	289	567	539
Duplicates and/or In silico	308	51	339	339

Figure 2

Variant filtering steps for dominant or X-linked inheritance using index-only analysis with the remaining variants after each filtering step shown in the accompanying table.

Identification of pathogenic mutations

Trio-analysis

We analysed 9 patients with a syndromic CHD by using a trio approach. A pathogenic mutation was identified in 4 of them, 3 in known genes and one in *MEIS2* [18] which had previously been suggested as a candidate gene for CHD, ID and cleft palate. Below, we describe clinical and genetic data in these four cases in more detail.

Patient 1 (*DYRK1A*)

This boy was the only child of healthy, unrelated, Caucasian parents. During pregnancy, intra-uterine growth retardation (IUGR) was noted at 34 weeks. He was delivered by caesarean section because of transverse position, at a gestational age of 38 weeks. Weight at birth was 2,7 kg (SD -1,2), length 46 cm (SD -1,64) and head circumference 32,7 cm (SD -1,64). On day 8 he was diagnosed with necrotizing enterocolitis, necessitating abdominal surgery and resulting in short bowel. Due to persistent feeding problems, nasogastric feeding was necessary in infancy. Cardiac evaluation on day 6 showed a bicuspid aortic valve with borderline left ventricle and mitral valve as well as an important preductal coarctation of the aorta. There was a right arteria lusoria. The coarctation was initially treated with percutaneous stenting, at the age of 5 months a coarctectomy was performed. He was hypertonic and had hyperreflexia. MRI at the age of 1 month showed enlarged, symmetrical sulci, cisterns and ventricles with global hypoplasia of the cerebellum - most prominent in the parietal region. Hypoplasia of the corpus callosum was also noted.

Ophthalmologic evaluation revealed small and pale optic discs. At the age of 3 years he was diagnosed with epilepsy. His development was severely delayed, and he followed special education. Facial features included decreased facial expression, sparse hair and deep-set eyes, prominent nasal bridge, bitemporal narrowing, protrusion of the upper lip and mild retrognathia (Figure 3). He had a small scrotum, widely spaced nipples and pectus excavatum. At the age of 6 years 7 months, weight was 16,3 kg (SD -2,8), height 110 cm (SD -2,2) and head circumference 44,5cm (SD -4,7).

After variant filtering as outlined in the methods section, we identified 77 remaining variants. This gene list was manually curated using functional data and genotype-phenotype

correlations for the implicated genes. We thus identified a *de novo* stopgain mutation in the *DYRK1A*-gene resulting in a premature stopcodon NM_001396.3 c.C1309T:p.R437X. Results were confirmed by Sanger sequencing.

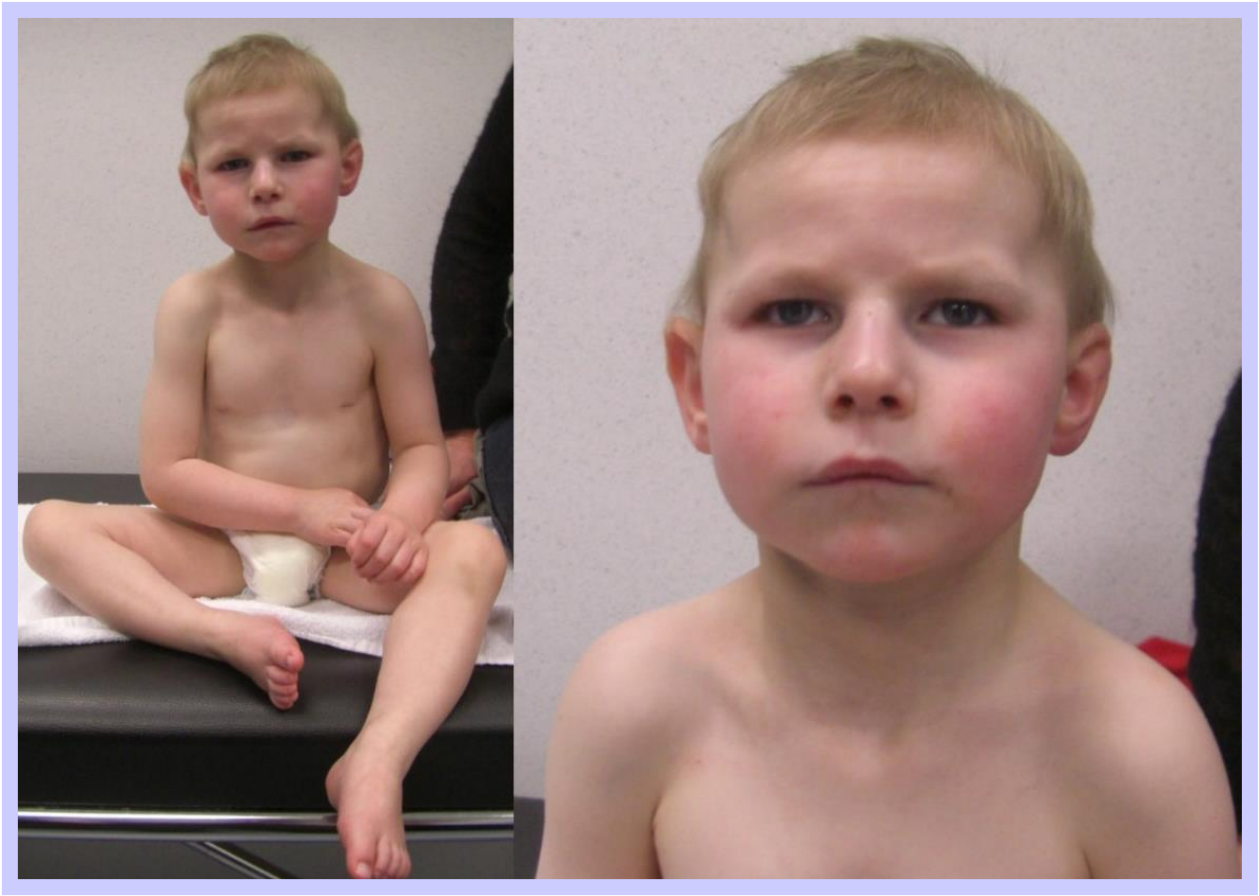


Figure 3

Clinical pictures of the patient with a de novo *DYRK1A* loss-of-function mutation at age 3 years 7 months. He has decreased facial expression, sparse hair, deep-set eyes, prominent nasal bridge, bitemporal narrowing, protrusion of the upper lip and mild retrognathia. He has widely spaced nipples and pectus excavatum.

Patient 2 (*MEIS2*)

This female patient presented with multiple congenital malformations including cleft palate and a CHD (septal defects and aortic coarctation). She had severe feeding problems, severely delayed gross motor and verbal development. She was diagnosed with moderate ID and autism spectrum disorder. Facial dysmorphism consisted of bitemporal narrowing, arched and laterally extended eyebrows, mild upslanting palpebral fissures, deep set eyes, a tented upper

lip, thin upper vermilion, full lower vermilion, broad first ray of hands and feet, a gap between the first and second toes and syndactyly of toe II-III.

After variant filtering we identified variants in 48 candidate genes. This list was manually curated using functional data and genotype-phenotype correlations for the implicated genes. We thus identified a *de novo* non-frameshift deletion of three base pairs in the *MEIS2* gene NM_170674.2 c.998_1000del:p.Arg333del as the most likely cause. This caused a deletion of three nucleotides GAA and thus led to deletion of the amino acid Arginine (Arg). Results were confirmed by Sanger sequencing.

Since this was the first case with an intragenic sequence alteration, we reported this in a separate manuscript (see annex Chapter 3.1 [18]).

Patient 3 (*SALL1*)

This female patient was an only child of unrelated parents. No other family members were known with intellectual disability, congenital heart defects or renal abnormalities. She had IUGR with a birth weight of 1,4 kg (SD -4,2) at the PMA of 38 weeks. She was diagnosed with Tetralogy of Fallot with agenesis of the pulmonary valve and an aneurysmatic pulmonary artery as well as trifurcation of the brachiocephalic truncus for which she underwent surgical correction. She had congenital renal hypoplasia with chronic renal insufficiency. At the age of 9 years her head circumference was 48cm (SD -2.7), her weight 20.2kg (SD -2.8) and her height 127.3 cm (SD -1.4), and she had moderate ID. There was facial dysmorphism with an asymmetric crying face, dry skin, deep palmar grooves, long slender fingers, excessive pubic adipose tissue, a short fifth toe and a relatively long first toe.

After variant filtering as outlined in the methods section, we identified 97 remaining variants. This gene list was manually curated using functional data and genotype-phenotype correlations for the implicated genes. We thus identified a *de novo* frameshift mutation in the *SALL1*-gene c.1998_1999del:p.666_667del. Results were confirmed by Sanger sequencing.

Patient 4 (*EFTUD2*)

This was a female patient with a truncus arteriosus and right aortic arch. The diagnosis was made late in childhood when there was already irreversible obstructive pulmonary

hypertension and Eisenmenger syndrome as well as moderate truncal valve insufficiency grade 2-3/4. She had severe intellectual disability. There was microcephaly with a head circumference of 47 cm (SD -3,1) at the age of 8 years and 5 months. Her weight and height were normal. She had a cleft palate, hearing impairment, epilepsy which started in infancy. She had facial dysmorphism with micrognathia and upslant of the eyes.

After variant filtering, we identified 83 remaining variants. This gene list was manually curated using functional data and genotype-phenotype correlations for the implicated genes. We thus identified a *de novo* frameshift mutation of one base pair in the *EFTUD2*-gene NM_004247:exon9:c.671delG:p.G224fs. Results were confirmed by Sanger sequencing.

Unsolved Trios

In the unsolved trios the same filtering methods were used as mentioned before and resulted in between 22 and 109 variants in *de novo* calls. In the hypothesis of autosomal recessive inheritance between 4 and 26 variants were left.

Index-only analysis

Of the four cases studied, a pathogenic mutation could be identified in one. The detailed clinical description and genetic data are given below.

Patient 10 (*ANKRD11*)

The boy was the only child of unrelated parents. No other family members were known with developmental delay or intellectual disability. He was born at term with a birth weight of 3000 gram. He was edematous. The diagnosis of a congenital cardiopathy was made; i.e. an ASD II and VSD. No cardiac interventions or surgery was necessary. He had a nasal speech and was hearing impaired. He had microcephaly with a head circumference of 48,9cm (SD -2.2), weight 15,7kg (SD-4.8) and height 109cm (SD -3.8) at the age of 8 years. He received growth hormone therapy for his short stature. He had mild intellectual disability and followed special education for children with special needs and autism spectrum disorder. He had camptodactyly of several fingers with a broad first ray and bilateral short fourth metatarsal. The space between the first and second toe was increased. His maxillary incisors were broad. There was endorotation of the femur and mild bowing of the lower legs. He had a rigid back with normal curvature. Previous genetic testing, including microarray-CGH and mutation analysis of *HDAC4*-gene were normal. After variant filtering, 308 variants were left. Further manual curation showed a nonsense mutation in the *ANKRD11* gene (c.7189C>T, p.Gln2397*). This mutation occurred *de novo*, and confirmed the diagnosis of KBG syndrome [19].

DISCUSSION

The introduction of NGS has revolutionized the identification of gene mutations. However, the bottleneck has shifted from variant identification to variant interpretation. In this study, we have applied whole exome sequencing to identify causative mutations in individuals with a sporadic, syndromic CHD.

A *de novo* filter in a trio approach resulted in finding a genetic etiology in 4 out of 9 cases. In the index-only analysis, a causal mutation was identified in only one case out of 4. Whereas the figures in this pilot study were small we found, not unexpectedly, that filtering for *de novo* mutations using a trio approach increased efficiency in filtering. However, even in a trio analysis, on average still 65 candidate variants remained (range 22-109), compared to an average of 259 (range 51-339) in the index-only analysis. This was partly due to the initial poorer quality of the sequencing at the start of this project. At the initial phase of exome introduction in our laboratory with the first variant calling algorithm, calling was less reliable and thus we also included the less reliable calls, resulting in an inflation of the number of remaining potentially *de novo* variants. Currently, with improved sequencing quality and bioinformatics filtering tools, a much lower number of *de novo* variants is expected after filtering.

In a diagnostic setting, additional filtering could be done using a panel of known genes associated to the phenotype under study. One approach is targeted capture and resequencing of a panel of known CHD genes or of all known genes associated to a genetic disorder (Mendeliome). The coverage is greatly improved with this method and the frequency of variants with unknown significance is reduced. The drawback is that no new genes will be discovered to broaden the genetic landscape of unsolved syndromic cardiopathies.

Establishing a genotype-based filter will require an exhaustive list of gene-phenotype associations. However, for CHD, such a gene list is almost certainly incomplete. First, CHD is a feature of many syndromes, and in some, CHD only rarely occurs. In a recent study, syndromic CHD patients were found to carry an excess of *de novo* protein truncating variants in Developmental disorder (DD) genes not known to be associated to CHD [20]. In our study, we identified the first *DYRK1A* mutation associated to a CHD, but since then, *DYRK1A* has been found to be an important ID gene, associated to CHD in a significant proportion of cases [20,

21]. Since all cases we studied were known with ID, we could use a filter for all known genes associated with ID. This list of ID-genes is extensive [10], and still incomplete, since novel genes for ID are being identified continuously. This was illustrated by our finding of a *MEIS2* mutation in one of the cases [18] where it has been a candidate gene for developmental disorders, but no prior intragenic sequence variants have been described. Additionally, it would be difficult to use in a neonatal setting as ID only becomes apparent at a later age.

An additional requirement for optimal functioning of such a phenotype-based filtering is a correct phenotypic description. Standardized nomenclature exists, for instance the Human Phenotype Ontology (HPO) [22], which is being adopted as the standard in the field of developmental disorders. Whereas the standardized description of major malformations (e.g. CHD, cleft lip, polydactyly etc.) is reliable, the description of dysmorphism is much more challenging. Dysmorphism is the combination of several minor anomalies, and a standardized nomenclature for minor anomalies exists [23]. However, many features remain subjective (e.g. deep set eyes, prominent nasal bridge, bitemporal narrowing). HPO terms to classify patients according to phenotypic similarities have been successfully used in computational methods [24]. In this computational study variants in the 2741 established Mendelian disease genes were grouped in a disease-associated genome (DAG) to develop a targeted enrichment panel (7.1 Mb) which had a high coverage of more than 20X in 98%. Data generated was analyzed by a computational method called Phenotypic Interpretation of eXomes (PhenIX) that evaluated and ranked variants based on their predicted pathogenicity (pathogenicity score defined as the single most pathogenic according to three in-silico tools) and semantic similarity of the patient phenotype to known Mendelian diseases. A variant score was calculated (Variant score = population frequency x pathogenicity score) and genes were ranked accordingly. The causal gene was ranked in the first place in computer simulations in 86% of the time and this tool enabled genetic diagnosis in 28% of unknown cases (n= 11) in a prospective study.

A promising future development is the use of automated recognition of facial features or facial gestalt on 2D pictures of a patient, instead of a (subjective) clinical description [25].

We anticipate that improved bioinformatics and the inclusion of standardized phenotypic information will greatly improve the currently slow and labour-intensive process of variant filtering in diagnostic whole exome sequencing.

After initiating this study, we had the opportunity to participate in a large multicenter study, which was led by the Wellcome Trust Sanger Institute in Hinxton UK, with the aim to study the genetics of CHD using WES, in 610 syndromic and 1281 non-syndromic patients [20]. We contributed 60 trios and 30 singletons. In this study, *de novo* mutations (DNM) and rare inherited variants were compared to a null mutation model in genes from 3 groups: autosomal dominant (AD) CHD genes, AD-developmental disorder (DD) associated genes and all remaining protein-coding genes. One of the main conclusions was a larger excess of *de novo* mutations with functional effects (protein truncating (PTV) or missense) in syndromic (S-CHD) compared to non-syndromic (NS) CHD cases (Table 2). This confirms the previous observation by Homsy et al [26] who reported an excess of *de novo* PTV in S-CHD (20% of cases) compared to only 2% in NS-CHD cases. Of interest, when the genes implicated were stratified according to their known gene-phenotype associations, a significant excess of PTV and missense was also detected in genes not previously associated with CDH, known DD-genes but also in the remaining genes. This confirms that the current genotype-phenotype associations are incomplete, and that many CHD genes still remain to be identified. Several novel CHD genes were also reported, and the results of our small study indicate that the *MEIS2* gene can be added to this list.

In conclusion, our study shows that trio exome analysis is an efficient way to identify pathogenic mutations in sporadic cases with a syndromic CHD. The diagnostic yield is high, which has a clear benefit to the patient and his family. Moreover, novel genotype-phenotype correlations emerge, and new genes involved in CHD can thus be identified.

Type of *de novo* mutation	Gene set	S-CHD (n=518)	Excess	P-value	NS-CHD (n=847)	Excess	P-value
Protein Truncating	CHD	27	80,56	$1,21x10^{-43}$	4	7,3	$2,62x10^{-04}$
	DD	12	18,36	$3,49x10^{-13}$	1	0,94	NS
	All	67	1,75	$8,92x10^{-06}$	67	1,07	NS
Missense	CHD	22	8,64	$7,35x10^{-15}$	12	2,88	$3,97x10^{-04}$
	DD	14	2,75	$2,68x10^{-04}$	11	1,32	NS
	All	371	1,34	$2,85x10^{-08}$	593	1,31	$1,15x10^{-10}$

Table 2 Excess of DNMs in S-CHD and NS-CHD showing the types of DNMs with their functional consequences leading to either a protein truncating or missense mutation. Gene sets of congenital heart defects (CHD), developmental disorders (DD) and all remaining protein coding genes were compared. **Adapted from Sifrim et al, Nature Genetics 2016 Sep [20].**

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CHAPTER 3.1

MEIS2 Involvement in Cardiac Development, Cleft Palate and Intellectual Disability

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ABSTRACT

MEIS2 has been associated with cleft palate and cardiac septal defects as well as varying degrees of intellectual disability. We present a female patient with a more severe phenotype compared to previous reported patients. She has multiple congenital malformations; cleft palate and congenital heart defect characterized by septal defects and aortic coarctation. She has severe feeding problems, facial dysmorphism, severely delayed gross motor and verbal development and autism spectrum disorder. Facial dysmorphism consisting of bitemporal narrowing, arched and laterally extended eyebrows, mild upslanting palpebral fissures, deep set eyes, a tented upper lip, thin upper vermilion, full lower vermilion, broad first ray of hands and feet, a gap between the first and second toes and syndactyly of toe II-III.

Exome sequencing revealed a non-frameshift deletion (c.998_1000del:p.Arg333del) of three base pairs in the *MEIS2* homeodomain. The more severe phenotype is most probably due to dominant-negative mechanisms. This is the first report showing a *de novo* small intragenic mutation in *MEIS2* and further confirms the important role of this gene in normal development.

KEYWORDS

MEIS2, cleft palate, cleft lip, cardiopathy, heart, intellectual disability, next generation sequencing (NGS)

INTRODUCTION

MEIS2 is a homeodomain-containing transcription factor of the TALE superfamily. Cytogenetic studies have identified *MEIS2* as a candidate gene for congenital malformations of the heart and palate. In 2007, a first patient with atrial septal defect type secundum (ASD II), cleft palate, moderate mental delay, severe speech delay and mild motor delay was described with a 5.3Mb deletion in 15q14 [Erdogan et al., 2007]. This was followed by two more patients with cleft palate and ventricle septal defect (VSD), respectively showing a deletion of 5.6Mb and 123kb in 15q14 [Chen et al., 2008; Crowley et al., 2010]. *MEIS2* was the only gene overlapping in all of these patients, making it the primary candidate gene for cleft palate, cardiac septal defects and varying degrees of developmental delay. More recently, nine patients, including a family with four patients, demonstrate that *MEIS2* disruption alone can cause cleft palate and cardiac defects (VSD) [Chen et al., 2008; Johansson et al., 2014; Crowley et al., 2010]. One of these also suffered from autism spectrum disorder, albeit with mild intellectual disability (ID) and without cleft palate or cardiopathy [Johansson et al., 2014]. Interestingly, most of these patients had delayed motor development, but varying degrees of mental disability, ranging from normal to moderate ID. It remains uncertain whether the ID observed in the other individuals is related to a deletion of nearby genes, or related to haploinsufficiency of the *MEIS2* gene. We here report the first patient with an intragenic *MEIS2* mutation detected by exome sequencing.

CLINICAL REPORT

A female patient is the second child of healthy, unrelated parents, and family history is negative regarding congenital malformations. She was born at term after an uneventful pregnancy, with weight 3650gram (25-50th centile), length 51cm (50th centile) and head circumference 36cm (50-75th centile). Multiple congenital malformations were present, a cleft of the soft and posterior part of the hard palate, a congenital heart defect (a large perimembranous, inlet-to-outlet VSD, an ASD II, a small left ventricular outflow tract without obstruction, and aortic coarctation). Surgical correction with coarctectomy and VSD closure was done. She also had congenital lobar emphysema of the left upper lobe for which a lobectomy was performed at the age of 1 month. There were severe feeding problems with gastro-esophageal reflux, oral aversion, aerophagia and achalasia necessitating gastrostomy and Botox infiltrations. She has arched and laterally extended

eyebrows, mild upslanting palpebral fissures, deep set eyes, a tented upper lip, thin upper vermilion, full lower vermilion. There was bitemporal narrowing. She had a broad first ray of hands and feet, a gap between the first and second toes and syndactyly of toe II-III (Figure 1). Biometry at the age of 5years 8months was height 108.5cm (10^{th} centile) and weight 15.8kg (3^{rd} centile).

Her gross motor and verbal development was severely delayed. She could sit at the age of 17 months and walk at the age of 30 months. At the age of 4 years she pronounces single words. Mentally she scored equivalent to age 25 months at the age of 50 months (4 years 2 months), with an IQ in the range of 35-49, placing her in the moderate group of intelligence disability. The diagnosis of autism spectrum disorder was made at the age of 3 years. Cerebral echography and MRI could not detect any abnormalities. Previously performed array-CGH (1Mb resolution) was normal.

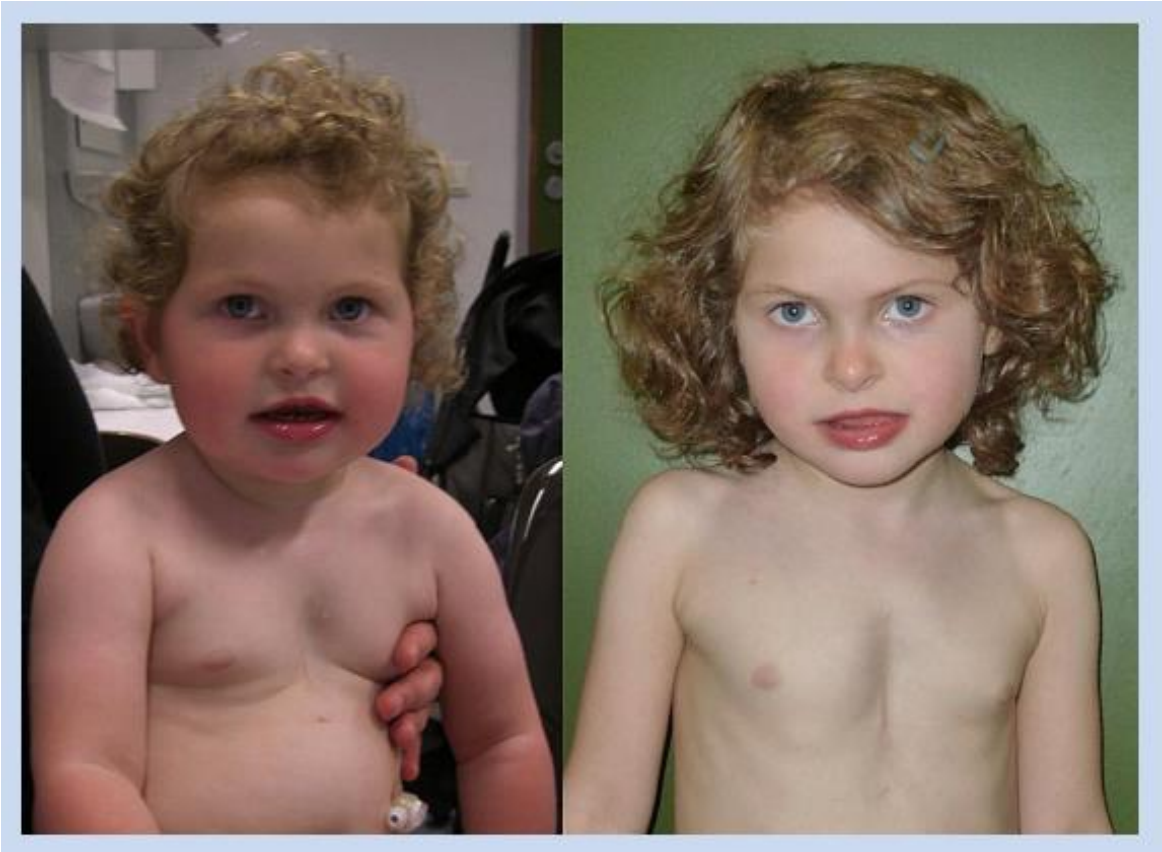

Figure 1

Clinical pictures at the age of 2 years and 5 years showing mild dysmorphic facial features: bitemporal narrowing, arched and laterally extended eyebrows, mild upslanting palpebral fissures, deep set eyes, a tented upper lip, thin upper vermilion and full lower vermilion. Pectus excavatum is most likely due to cardiac surgery performed at a young age.

MATERIALS AND METHODS

EXOME ANALYSIS

Informed consent for trio analysis by exome analysis was obtained from the parents. Library construction for all samples were prepared using *TruSeq DNA* Library Preparation Kit (Illumina, Inc., San Diego, CA, USA) in which platform-specific adaptors and unique DNA indexes were ligated. For each sample, 1 µg genomic DNA was sheared by sonication to approximately 300bp fragments, followed by end-repair, adenylation and adapter ligation steps. DNA sequencing libraries were subsequently enriched with the SeqCap EZ Human Exome Library v3.0 (Roche, NimbleGen), and 2 × 100-bp paired-end reads were generated on the Illumina HiSeq2000 platform with 3-4 exome-seq samples pooled per lane of a sequencing flow-cell. Sheared DNA, whole genome libraries and enriched exome-seq libraries were validated using DNA-1000 chips on the BioAnalyser (Agilent), and library concentrations were determined using the dsDNA Broad Range Assay using the Qubit (Invitrogen).

Data analysis was done using commercial and in-house developed software (Genomics Core/UZ Leuven). Exome sequences were obtained from both parents and the patient. As our hypothesis was a *de novo* mutation, the patient was filtered against all in-house (n=72) exomes, allowing the exclusion of local rare variants. All non-reference calls were excluded in the parents, reference calls were excluded in the patient. According to Ensembl (www.ensemble.org) only exonic, exonic/splicing and splicing variants were included. Synonymous variants were excluded. Variants occurring with a frequency of <1% in the 1000 genomes project or with an unknown frequency were included. Variants occurring in *HLA* and *MUC* genes were excluded. Splicing site changes occurring at less than 5 positions were considered as possible candidates.

RESULTS

EXOME SEQUENCING

After variant filtering as outlined in the methods section, we identified variants in 48 candidate genes (Table IS). This gene list was manually curated using functional data and genotype-phenotype correlations for the implicated genes. We thus identified a *de novo* non-frameshift deletion of three base pairs in the *MEIS2* gene NM_170674.2 c.998_1000del:p.Arg333del as the

most likely cause. This causes a deletion of three nucleotides GAA and thus deletion of the amino acid Arginine (Arg). Results were confirmed by Sanger sequencing (Figure 2).

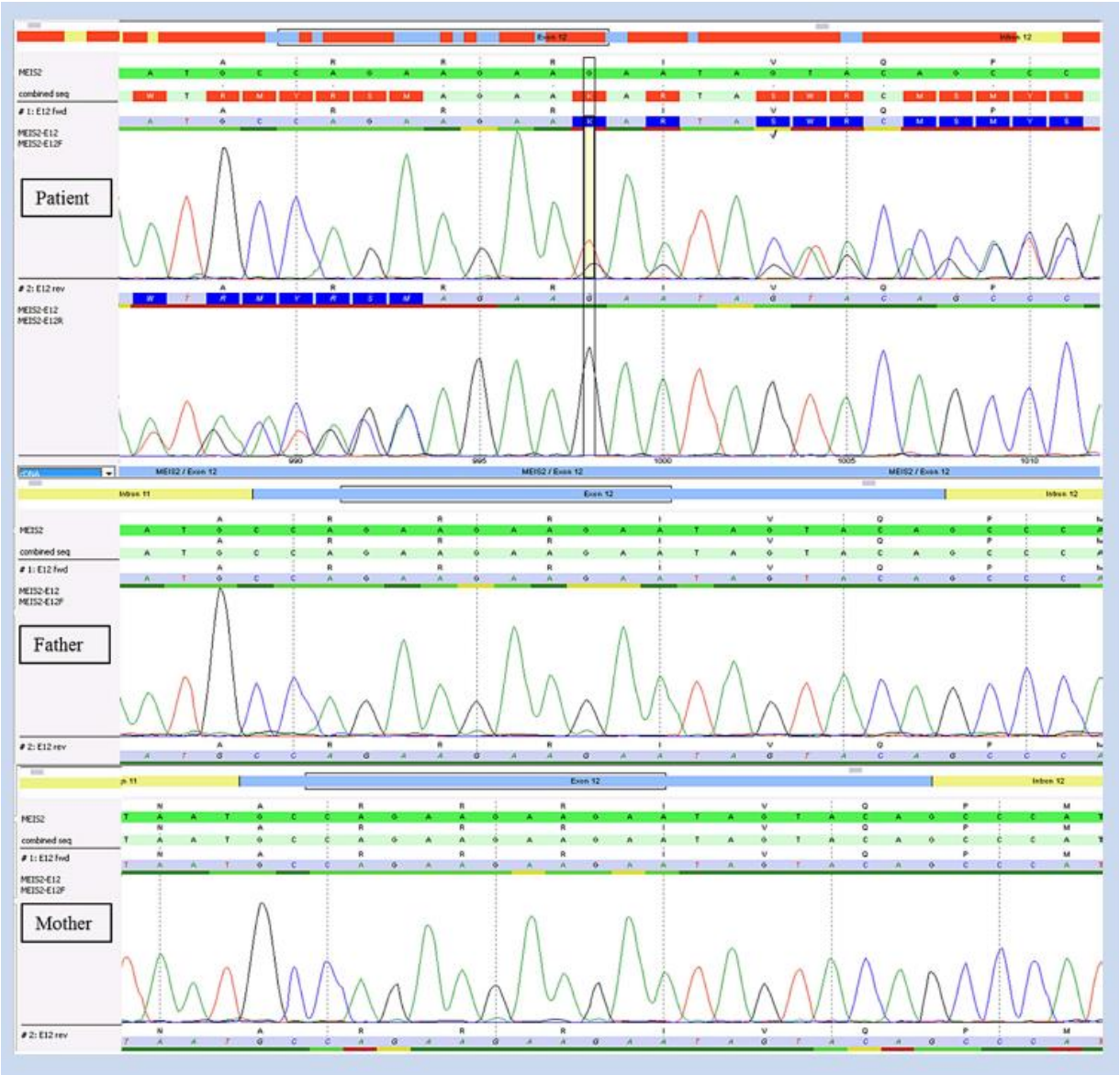

Figure 2

Sanger sequencing of the patient, father and mother showing a *de novo* non-frameshift deletion in the patient of three base pairs in the *MEIS2* gene.

DISCUSSION

We report the first patient who carries a small intragenic mutation in the *MEIS2* gene, confirming the previous association of *MEIS2* with cleft palate and cardiac septal defects. No other in-frame deletions or point mutations have been described until now. Of interest, the present patient had moderate ID, severe gross motor delay and autism spectrum disorder. Previously reported patients with an intragenic *MEIS2* deletion also had delayed motor development, but varying degrees of delay in mental development, ranging from normal to moderate. One of these patients also suffered from autism spectrum disorder as diagnosed in our patient, albeit with a mild ID and without cleft palate or cardiopathy [Johansson et al., 2014] Table 1.

MEIS2 is a transcription factor and most likely has a role in the stabilization of the Homeoprotein-DNA complex. It binds to HOX or PBX proteins to form dimers and multimers. The arginine residue deleted is located in the homeodomain. This single amino acid deletion could therefore interfere with DNA binding. Arg333 is highly conserved across all species and isoforms. In addition, according to the Protein Databank in Europe (PDBePISA) database, the Arg residue mutated in the present patient is involved in a multimer contact [Krissinel et al., 2007; (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html)]. Any hydrogen bonds that could be made by the wild type residue to other monomers would therefore be lost and affect the multimeric compound. Therefore, the more pronounced ID observed in the present patient may possibly be due to another mechanism than merely haploinsufficiency. Thus, the deletion of the Arg residue may have an additional dominant negative effect.

Case number	Sex	Chromosomal aberration	Genes involved	Cardiac malformation	Clefting	Mental development
1	F	5.3 Mb del	*ACTC, GREM1, CX36,* ***MEIS2****, ARHGAP11A, CHRNA7, CHRM5*	ASD II	palate	severe speech delay, mild motor delay
2	M	5.6 Mb del	***MEIS2***, many other genes	VSD	palate	delayed, epilepsy
3	M	123 kb del	***MEIS2***	VSD	cleft soft palate	bilateral moderate hearing loss
4	F	58 kb dup	***MEIS2***	none	submucous cleft palate	mild ID
5	F	58 kb dup	***MEIS2***	none	submucous cleft palate	delayed
6	M	58 kb dup	***MEIS2***	none	open cleft palate	delayed
7	F	58 kb dup	***MEIS2***	none	open cleft palate	mild ID
8	F	0.6 Mb del	*C15orf41, CSNK1A1P1,* ***MEIS2***	VSD	open cleft palate	normal
9	M	0.6 Mb del	*C15orf41, CSNK1A1P1, LOC145845,* ***MEIS2***	none	bilateral cleft lip and palate	delayed
10	M	1.0 Mb del	*C15orf41, CSNK1A1P1, LOC145845,* ***MEIS2***	none	none	mild ID, ASD
11	M	1.9 Mb del	*C15orf41, CSNK1A1P1, LOC145845,* ***MEIS2****, TMCO5A, SPRED1, FAM98B, RASGRP1, C15orf53*	VSD	none	delayed
12	F	4.8 Mb del	*C15orf41, CSNK1A1P1, LOC145845,* ***MEIS2****, TMCO5A, SPRED1, FAM98B, RASGRP1, C15orf53, C15orf54*	VSD	submucous cleft palate, bifid uvula	delayed
13	F	c.998_1000del: p.Arg333del	***MEIS2***	ASD II, VSD, LVOTO, CoA	soft and hard cleft palate	severe gross motor and verbal delay, ASD

Table 1
Main phenotypic features and involved genes of patients with *MEIS2* haploinsufficiency.

Functional studies showed that *MEIS2B,* one of two orthologs of *MEIS2* in zebrafish*,* is important in heart formation, regulation and function. Knockdown of *MEIS2B* in zebrafish embryos lead to defective cardiac morphogenesis with no midline formation of the linear heart tube, severe defects in heart looping, pericardial edema and a significantly reduced heart rate [Paige et al., 2012; Glickman et al., 2002]. Interestingly, expression of *MEIS2B* in the heart field of developing mutant zebrafish embryos closely resembles that of *GATA4*, a known cardiac transcription factor [Paige et al., 2012]. Mutations in *GATA4* are well-known in causing primarily cardiac septal defects, ranging from ASD and VSD to atrio-ventricular septal defects (AVSD) [Garg et al., 2003]. Regarding ID and developmental delay; *MEIS2B* expression is observed in the developing hindbrain of somites [Zerucha et al., 2001], and *MEIS2* is a principal key factor in patterning of the hindbrain [Waskiewicz et al., 2001] as well as normal mesencephalic development in mice and chick embryos [Shim et al., 2007; Agoston et al., 2009; Vennemann et al., 2008].

This case report builds on the previous publications that *MEIS2* should be considered in patients with cleft palate, septal cardiac defects and ID. Further functional studies regarding the role of *MEIS2* in neuronal pathways and mental development will be necessary to explore the exact mechanisms involved in this intricate mechanism.

CONFLICT OF INTEREST

The authors have no conflict of interest or disclosures to declare.

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SUPPLEMENTARY ONLINE MATERIAL

Table IS

Remaining variants after filtering for heterozygous calls in the index and reference calls in both parents.

Chr	Start	End	Reference	Observed	Gene	AA Change	Function	Exonic function
1	152276671	152276671	C	T	FLG	FLG:NM_002016:exon3:c.G10691A:p.R3564H	exonic	nonsynonymous SNV
1	111957553	111957553	G	T	OVGP1	OVGP1:NM_002557:exon11:c.C1570A:p.L524M	exonic	nonsynonymous SNV
1	111957558	111957558	T	G	OVGP1	OVGP1:NM_002557:exon11:c.A1565C:p.K522T	exonic	nonsynonymous SNV
1	111957561	111957561	T	C	OVGP1	OVGP1:NM_002557:exon11:c.A1562G:p.E521G	exonic	nonsynonymous SNV
1	11584023	11584023	G	T	PTCHD2	PTCHD2:NM_020780:exon11:c.G2387T:p.S796I	exonic	nonsynonymous SNV
2	233712209	233712209	TCAGCAGCAGCAGCTGCCACAG	T	GIGYF2	GIGYF2:NM_001103147:exon29:c.3676_3696del:p.1226_1232del	exonic	nonframeshift deletion
2	240982073	240982073	G	C	PRR21	PRR21:NM_001080835:exon1:c.C327G:p.C109W	exonic	nonsynonymous SNV
2	240982135	240982135	A	G	PRR21	PRR21:NM_001080835:exon1:c.T265C:p.S89P	exonic	nonsynonymous SNV
2	240982199	240982199	A	C	PRR21	PRR21:NM_001080835:exon1:c.T201G:p.C67W	exonic	nonsynonymous SNV
2	240982200	240982200	C	T	PRR21	PRR21:NM_001080835:exon1:c.G200A:p.C67Y	exonic	nonsynonymous SNV
3	171065010	171065010	G	GA	TNIK		splicing	
3	75832519	75832519	A	G	ZNF717		splicing	
3	75832522	75832522	G	T	ZNF717		splicing	
4	47033767	47033767	ATCTCGC	A	GABRB1		splicing	
4	144922436	144922436	T	G	GYPB	GYPB:NM_002100:exon2:c.A38C:p.E13A	exonic;splicing	nonsynonymous SNV
4	59469	59469	A	G	ZNF718		splicing	
7	142460428	142460428	CCAA	C	PRSS1		splicing	
8	144940706	144940706	C	T	EPPK1	EPPK1:NM_031308:exon1:c.G6716A:p.R2239H	exonic	nonsynonymous SNV
9	35809214	35809214	G	T	NPR2	NPR2:NM_003995:exon21:c.G3048T:p.Q1016H	exonic	nonsynonymous SNV
9	35683240	35683240	T	TG	TPM2		splicing	
10	135439049	135439049	G	C	FRG2B	FRG2B:NM_001080998:exon4:c.C391G:p.P131A	exonic	nonsynonymous SNV
10	50819952	50819952	G	T	SLC18A3	SLC18A3:NM_003055:exon1:c.G1166T:p.G389V	exonic	nonsynonymous SNV
11	48346541	48346541	A	T	OR4C3	OR4C3:NM_001004702:exon1:c.A49T:p.T17S	exonic	nonsynonymous SNV
11	48346547	48346547	C	T	OR4C3	OR4C3:NM_001004702:exon1:c.C55T:p.P19S	exonic	nonsynonymous SNV

Table 1S (continued)								
Chr	Start	End	Reference	Observed	Gene	AA Change	Function	Exonic function
11	56468440	56468440	G	T	OR9G9	OR9G9:NM_001013358:exon1:c.G577T:p.G193C	exonic	nonsynonymous SNV
12	49994257	49994257	T	G	FAM186B	FAM186B:NM_032130:exon4:c.A1166C:p.H389P	exonic	nonsynonymous SNV
12	53069222	53069222	CACCTCCGGAGCCGTAGCTGCT	C	KRT1	KRT1:NM_006121:exon9:c.1669_1689del:p.557_563del	exonic	nonframeshift deletion
12	11183541	11183541	T	C	TAS2R31	TAS2R31:NM_176885:exon1:c.A394G:p.M132V	exonic	nonsynonymous SNV
13	20279879	20279879	T	C	PSPC1	PSPC1:NM_001042414:exon9:c.A1309G:p.M437V	exonic	nonsynonymous SNV
14	19553586	19553586	T	C	POTEG	POTEG:NM_001005356:exon1:c.T170C:p.L57P	exonic	nonsynonymous SNV
15	79067065	79067065	G	A	ADAMTS7	ADAMTS7:NM_014272:exon12:c.C1777T:p.P593S	exonic	nonsynonymous SNV
15	72954577	72954577	C	G	GOLGA6B		splicing	
15	37188864	37188864	ATTC	A	MEIS2	MEIS2:NM_170674:exon10:c.998_1000del:p.333_334del	exonic	nonframeshift deletion
16	67424209	67424209	G	T	TPPP3	TPPP3:NM_016140:exon5:c.C399A:p.G133G	exonic	nonsynonymous SNV
17	38126911	38126911	AACACAC	A	GSDMA		splicing	
17	39296412	39296412	T	A	KRTAP4-6	KRTAP4-6:NM_030976:exon1:c.A328T:p.S110C	exonic	nonsynonymous SNV
17	39253949	39253949	T	A	KRTAP4-8	KRTAP4-8:NM_031960:exon1:c.A388T:p.S130C	exonic	nonsynonymous SNV
17	39253953	39253953	G	T	KRTAP4-8	KRTAP4-8:NM_031960:exon1:c.C384A:p.S128R	exonic	nonsynonymous SNV
17	39254126	39254126	C	T	KRTAP4-8	KRTAP4-8:NM_031960:exon1:c.G211A:p.V71M	exonic	nonsynonymous SNV
17	39254133	39254133	G	T	KRTAP4-8	KRTAP4-8:NM_031960:exon1:c.C204A:p.S68R	exonic	nonsynonymous SNV
17	78897395	78897395	C	A	RPTOR	RPTOR:NM_020761:exon23:c.C2730A:p.G910G	exonic	nonsynonymous SNV
19	42795827	42795827	G	C	CIC	CIC:NM_015125:exon11:c.G2816C:p.G939A	exonic	nonsynonymous SNV
19	7935863	7935863	G	T	FLJ22184	UNKNOWN	exonic	nonsynonymous SNV
19	50826910	50826910	G	T	KCNC3	KCNC3:NM_004977:exon2:c.C1300A:p.L434M	exonic	nonsynonymous SNV
19	13936534	13936534	G	T	ZSWIM4		splicing	
20	26061859	26061859	G	T	FAM182A		exonic	stopgain SNV
20	62655943	62655943	C	A	PRPF6	PRPF6:NM_012469:exon14:c.C1805A:p.A602D	exonic	nonsynonymous SNV
X	140993695	140993695	G	T	MAGEC1	MAGEC1:NM_005462:exon4:c.G505T:p.A169S	exonic	nonsynonymous SNV

CHAPTER 4

IDENTIFICATION OF THE GENETIC CAUSE OF FAMILIAL SYNDROMIC CARDIOPATHIES AND RARE TYPES OF CARDIOMYOPATHY

ABSTRACT

We studied five small families with two affected siblings but unaffected parents with a familial form of congenital syndromic cardiopathy or a rare cardiomyopathy.

Genetic analysis was done using linkage analysis in the parents and both the unaffected and affected siblings, followed by whole exome sequencing (WES). WES was done in the affected siblings only of Family 1 and 2, and in all family members of Families 3, 4 and 5. Data analysis was done using commercial and in-house developed software. Only variants in genes from the linkage regions were retained. Variant analysis was done according to the hypothesis of different possible inheritance patterns (autosomal recessive, dominant with parental mosaicism and X-linked).

In the two families with a rare type of cardiomyopathy, a genetic cause was identified. Homozygous mutation of *ALMS*1 explained the mitogenic cardiomyopathy phenotype, and was confirmed by another group studying unrelated families. We identified novel compound heterozygous mutations in *KIF20A* as the cause of a lethal form of restrictive cardiomyopathy of the right ventricle. Functional and zebrafish studies supported a role for this gene in the observed phenotype. In the three other families with a syndromic type of CHD, no genetic cause was identified, suggesting that current genetic analysis tools lack the sensitivity to identify all possible mutations.

INTRODUCTION

Familial CHD represents about 4% of all CHD's. The majority of these are non-syndromic [1-5], and genetic testing can often identify the causal genetic mutation, especially in large families [6]. Familial syndromic CHD is very rare and occurs in around 1% of all CHD. In some instances, the syndrome occurs in different generations: when it is mild and inherited as an autosomal dominant disorder (e.g. Holt-Oram syndrome or Noonan syndrome) or when transmitted through an unaffected female in X-linked recessive disorders (e.g. Barth syndrome, Duchenne or Becker muscular dystrophy). However, most syndromes are severe and have a reduced reproductive fitness, due to the severity of the disorder or the associated intellectual disability. This typically results in sporadic syndromic CHD (S-CHD) due to a *de novo* mutation, as discussed in chapter 3. In this chapter, we study five small families with two siblings affected with a severe, syndromic CHD or with a lethal, exceptional type of cardiomyopathy. These very rare and specific phenotypes are likely to have a monogenic cause. In these families, in contrast to large non-syndromic families, no clinical diagnosis could be reached which could orient our genetic analyses. This is because the combination of distinct anomalies observed in the siblings does not fit a known entity, and thus constitute a new unknown syndrome [7]. Alternatively, the cardiac phenotype is unique, e.g. an early onset/neonatal cardiomyopathy (CM) with unknown genetic cause. In these families, the inheritance pattern is most likely autosomal recessive (AR), especially when the parents are consanguineous and sometimes X-linked (when the affected siblings are males). Germline or low grade somatic mosaicism in one parent for an AD disorder should also be considered (Figure 1).

We evaluated to what extent exome sequencing in combination with linkage analysis allowed us to identify the causative genes in these small families.

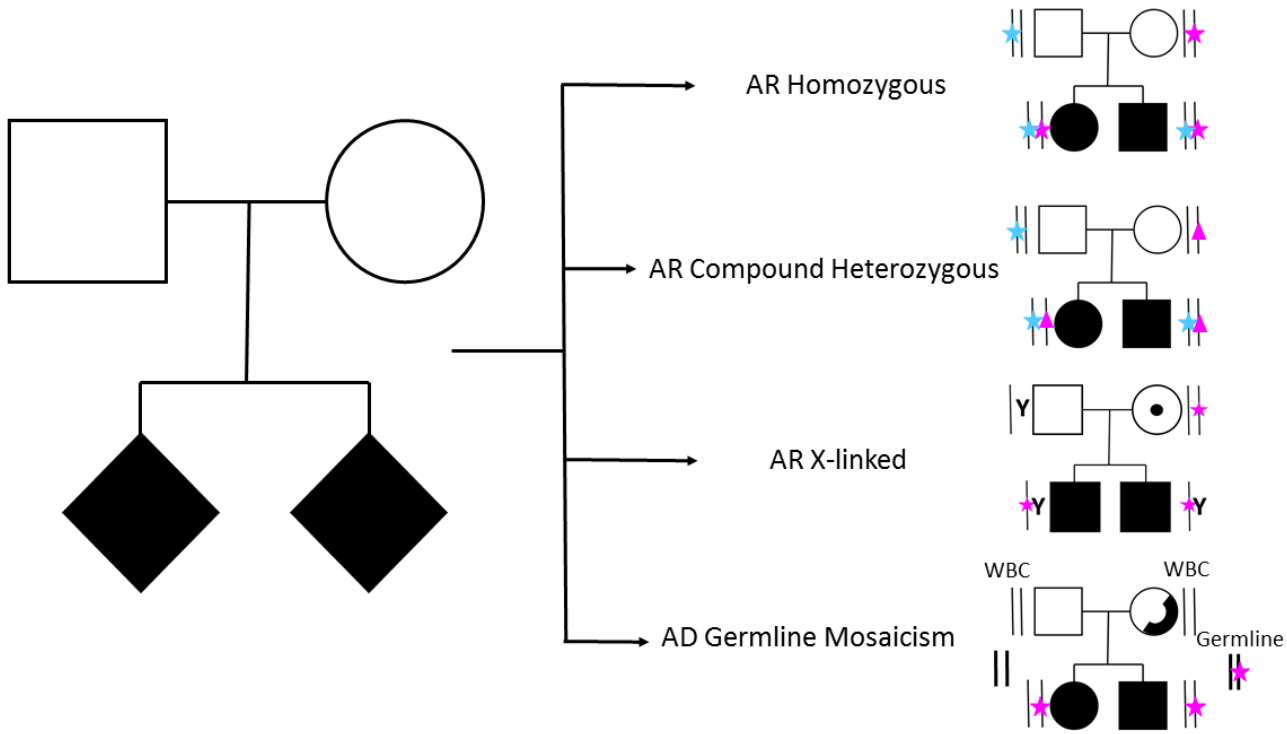

Figure 1. Possible modes of inheritance in families with unaffected parents and affected siblings. Mutations in genes are shown as paternal (blue) or maternal (pink). Mutations are detected in DNA of white blood cells (WBC) and inherited as autosomal recessive (AR) or X-linked recessive disorders, or can be present in DNA of germline cells and thus inherited as an autosomal dominant (AD) disorder.

MATERIALS AND METHODS

Patient selection and description

We analysed 5 families followed in the pediatric cardiology department and Center for Human Genetics in our tertiary hospital.

Inclusion criteria

1. **Syndromic CHD or a rare cardiomyopathy** with syndromic defined as the presence of 1 additional major abnormality apart from the CHD, or intellectual disability not otherwise explained and/or dysmorphism, defined as 3 or more minor anomalies.
2. **An unknown cause** after extensive evaluation:

* All patients were examined by an experienced clinical geneticist and clinical pictures were available for discussion with other clinical geneticists and for review of the phenotype in case a mutation was found. Clinical information on the parents and unaffected sibling were available.

* High resolution array-CGH was normal in the patients and parents and showed no unclassified variants.

3. **DNA was available** from the patients, both parents and the unaffected sibling.

5. **No external causes identified**: no teratogens during pregnancy, no other known cause for neurological damage.

The study was approved by the Ethical Committee University Hospital Leuven (S52853 – B322201010111). Informed consent was obtained from all parents.

Family	Sibling	Gender	Result	Age	CHD	ID	Other major malformations	Minor malformations	HC (SDS)	Weight (SDS)	Height (SDS)	Other
1	1	M	*ALMS1*	0	MCM	NA	none	none	2,5	-0,8	3,0	
1	2	F	*ALMS1*	0	MCM	NA	none	none	NA	1,6	-1,8	
2	1	M	*KIF20A*	0	RCM	NA	none	none	-0,5	-0,2	1,0	
2	2	F	*KIF20A*	0	RCM	NA	none	none	0,5	1,3	0,9	
3	1	M		4,9	MS,AS, LVOTO	Mild	unilateral renal agenesis, kyphosis	facial dysmorphism	0,9	-2,5	-3,4	strabismus
3	2	F		1,5	AS, LVOTO	Mild	Situs inversus totalis	facial dysmorphism	3,3	-0,2	-0,8	strabismus
4	1	F		5,2	VSD	S	growth retardation, bilateral postaxial polydactyly, atlanto-occipital fusion, coloboma, hypoplastic right kidney	facial dysmorphism	-2,5	-5	-3,5	frequent respiratory infections, nystagmus, ectopic neuropituitary gland
4	2	F		0,3	VSD, ASD II, PS	NA (deceased)	bilateral postaxial polydactyly, hypoplasia vermis	facial dysmorphism	NA	-1,4	NA	ectopic neuropituitary gland, enlarged cisterna magna
5	1	M		4,2	ASD II	Mild	agenesis ductus venosus	pectus carinatum, facial dysmorphism, bilateral palmar simian crease	-0,8	-3,7	-3,7	feeding problems, neutropenia during infancy, OSAS
5	2	M		0,2	ASDII	NL	none	facial dysmorphism, bilateral palmar simian crease	-2,3	-2,7	-3,6	

Table 1

Clinical data of the five families.

Columns show family number, sibling number, gender, genetic result, age at examination, type of CHD, level of ID, other major malformations, description of minor anomalies, biometry and other characteristics. Level of ID at age of examination was normal (NL), mild, moderate (M) and severe (S). CHD included mitogenic cardiomyopathy (MCM), restrictive cardiomyopathy (RCM), mitral stenosis (MS), aortic stenosis (AS), left ventricular outflow tract obstruction (LVOTO), atrial septal defect type secundum (ASD II), ventricle septal defect (VSD) and pulmonary stenosis (PS). Head Circumference (HC), weight and height is given as standard deviations. Obstructive sleep apnea syndrome (OSAS). Some data was not available (NA), mostly due to the young age at which evaluation of neurologic development was not possible.

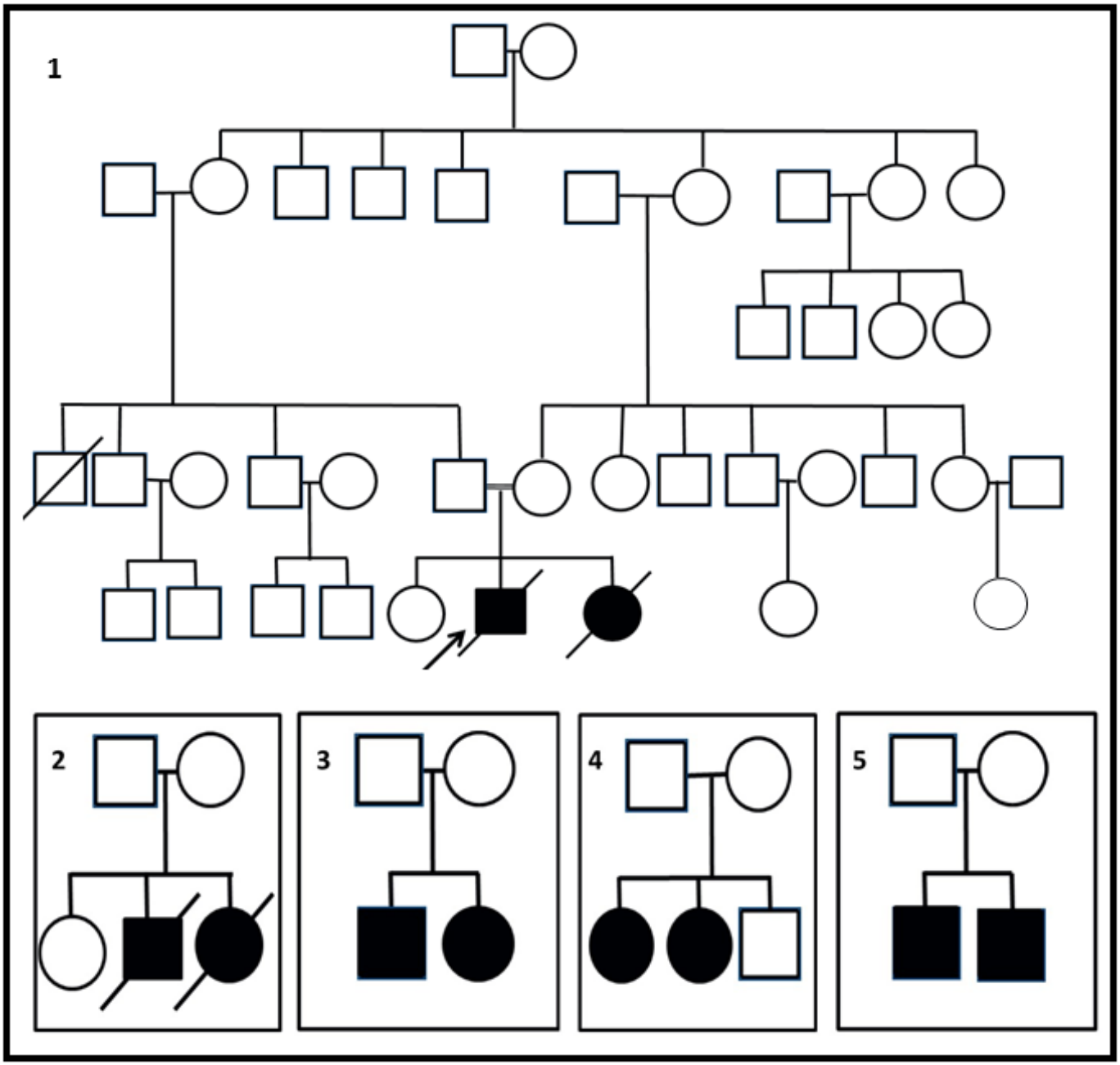

Figure 2. Pedigrees of the 5 families studied.

Family 1

Two siblings from consanguineous parents of Turkish descent (Figure 2, Family 1) presented with an isolated dilated cardiomyopathy leading to early death in infancy. The diagnosis of a rare cardiomyopathy i.e. mitogenic cardiomyopathy was made histologically. The clinical and genetic findings in this family are described in detail in annex Chapter 4.1.

Family 2

Two siblings, one male and one female (Figure 2, Family 2), were diagnosed in late fetal life with a CHD categorized as restrictive cardiomyopathy (RCM) of the right ventricle (RV). An older sibling and both non-consanguineous parents had a normal phenotype. Both affected children demised in infancy at the ages of respectively 3 and 2 months due to progressive and fatal cardiac failure. The clinical and genetic findings in this family are described in detail in annex Chapter 4.2.

Family 3

This was a non-consanguineous Caucasian family with two affected children (Figure 2, Family 3). The index patient had a complex CHD which required multiple cardiac interventions. The CHD consists of a Shone-complex characterized by an arcade mitral valve (MV) with dysplastic and thickened valve leaflets, a smaller annulus and short MV chordae. There was aortic stenosis due to a small bicuspid aortic valve, thickened leaflets as well as subvalvular aortic stenosis. He had mild ID and facial dysmorphism with anteversion of the nares, strabismus, bright blue irides, small ears, epicanthic folds, a tented upper lip and full cheeks. He had unilateral renal agenesis, lumbar kyphosis and a short neck. There was excessive keloid formation after cardiac surgery which required surgical resection.

His younger sister was born at term (PMA 39 weeks) with a weight of 3,56 kg (SD + 0,4) and a head circumference of 36 cm (SD – 0,5). Prenatally there was polyhydramnion; additionally the diagnosis of situs inversus totalis was made as well as a mild aortic stenosis. This progressed postnatally to a multilevel and complex CHD: a dysplastic mitral valve with thick, muscular chordae, a dysplastic tricuspid aortic valve, severe subvalvular aortic stenosis with a subvalvular ridge, and hypertrophic cardiomyopathy for which multiple and complex cardiac surgeries were necessary. She has mild ID and the same facial dysmorphic features and strabismus as her older brother as well as macrocrania with a head circumference of 51,5 cm (SD + 3.3) at the age of 1,5

years. In the two siblings, chromosomal analysis by means of array comparative genomic hybridization (CGH) was performed using the 180k CytoSure ISCA v2 microarray (Oxford Gene Technology, OGT, Oxford, UK). No pathogenic chromosomal variants or recurrent chromosomal variants of unknown significance were detected (Table 2, Family 3).

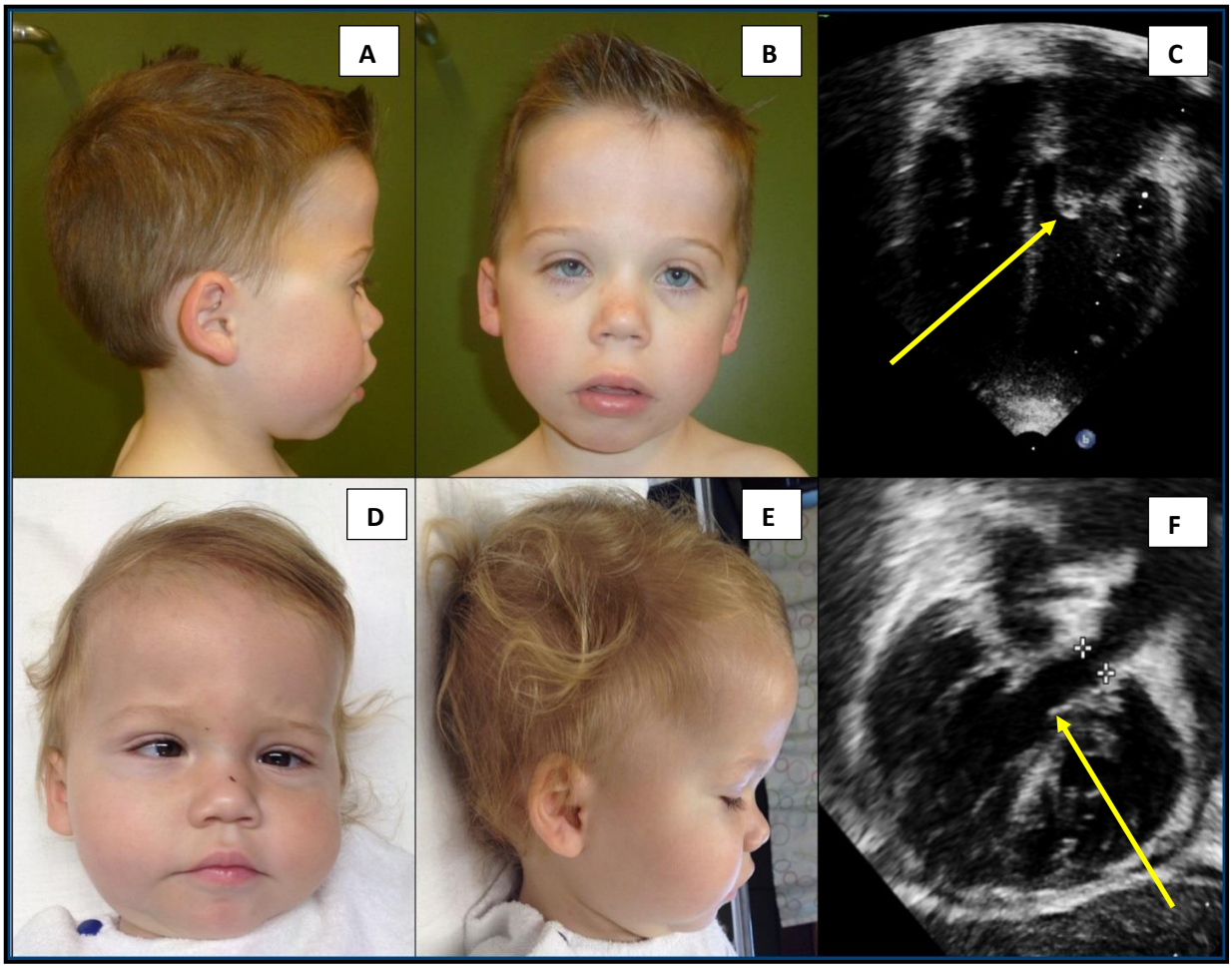

Figure 3. Clinical findings in two siblings of family 3.

Note the similar facial features in the index (A & B) and his younger sister (D & E). Cardiac ultrasound images show the mitral valve with dysplastic and thickened valve leaflets and chordae in the index (C) and multilevel left ventricular outflow tract obstruction with a small LVOT (crosses) and subvalvular ridge (arrows) as well as LV hypertrophy.

Family 4

The clinical findings in family 4 have been reported before [7]. In short, this was a non-consanguineous family (Figure 2, family 4) with three children. The index was born at term, with birth weight 2640 g (SD -2,1), length 44 cm (SD -3,5) and head circumference 31.5 cm (SD -3). She had bilateral postaxial polydactyly of the hands, a perimembranous VSD and a right renal hypoplasia. She had upslant of the eyes, hypoplasia of the nasal bridge, anteversion of the nostrils, a long and smooth philtrum, a narrow vermillion of the upper lip and a prominent lower lip vermillion with a swelling on the right side (Figure 3A). She had temporal balding with coarse and brittle hair as well as a hemangioma on the scalp. The nipples were wide set and the feet were oedematous. She had a severe growth delay: at age of 19 months, weight was 7.8 kg (SD -3,4), length 70.5 cm (SD -4,0) and head circumference 44.9 cm (SD -1,7). There was a small coloboma of the left eye. She had an ectopic neuropituitary gland but no endocrine disturbance was observed (Figure 4E). She had torticollis due to an atlanto-occipital fusion and partial fusion of C2-C3. She had a severe ID. She also had frequent respiratory infections. She had a viral myocarditis at the age of 5 years after surgical closure of the VSD with secondary dilated cardiomyopathy. This evolved to a chronic, but stable, mild cardiac dysfunction with a fractional shortening of 27% (normal >30%) under cardiac failure medication.

Her younger sister was born after an uneventful pregnancy at a gestational age of 32 weeks, with a birth weight of 1.2 kg (SD -1,4), length of 37.5 cm (SD -2,0) and head circumference of 26 cm (SD – 1,8). She had a VSD with pulmonary stenosis and an ASD II. Postaxial polydactyly of both hands was present. The facial features were almost identical to that of her sister (Figure 4B and 4D). Magnetic resonance imaging of the brain also revealed an ectopic neuropituitary gland, and a normal to slightly enlarged posterior fossa associated with an enlarged cisterna magna and mild hypoplasia of the cerebellar vermis. Endocrine studies during the first months of life revealed normal thyroid and adrenal function, and normal growth hormone levels. She died at the age of 4 months from postoperative complications after cardiac surgery.

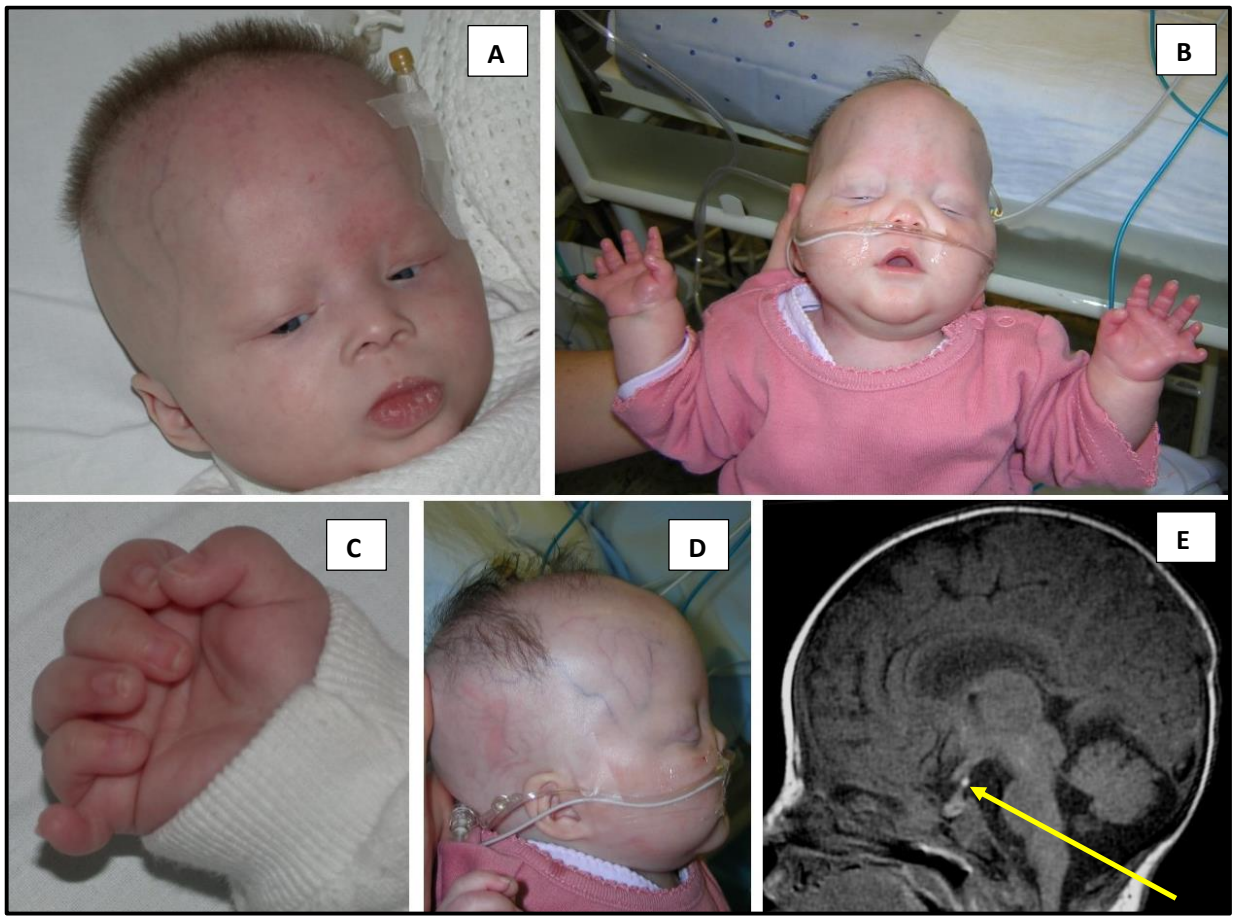

Figure 4. Clinical findings in the two affected siblings from family 4.

Note the similar features in the index (A&C) and her younger sister (B&D). Cerebral magnetic resonance imaging (E) of the index showing the ectopic neuropituitary gland as indicated by the arrow.

Family 5

The index patient was born at term as the first child of non-consanguineous Caucasian parents (Figure 2, family 5). At birth he had a weight of 2, 13kg (SD -3.3), length of 45 cm (SD -2,5) and head circumference of 32cm (SD -1,64). During infancy he had recurrent severe infections due to a transient neutropenia. There was a prenatal diagnosis of agenesis of the ductus venosus, drainage of the umbilical vein occurred through extrahepatic veins to the right atrium. The heart was displaced to the left, horizontal and posterior. The diagnosis of a fenestrated ASD II was made

which closed spontaneously. The umbilicus was implanted to the left and he had pectus carinatum. He had facial dysmorphic features with anteversion of the nostrils, retrognathia and short palpebral fissures. He had a bilateral palmar simian crease. He had mild intellectual disability, and more pronounced gross motor delay. He had obstructive sleep apnea syndrome (OSAS).

His younger brother had a milder phenotype. He had a small stature with weight 4,3 kg (SD -2,7), height 52cm (SD – 3,6) and head circumference of 38 cm (SD – 2,3) at the age of 2 months. He had the same facial dysmorphic features and a small ASD II which closed spontaneously in the first year of life. He had no neutropenia. At the age of 2 months, no signs of major developmental difficulties were observed.

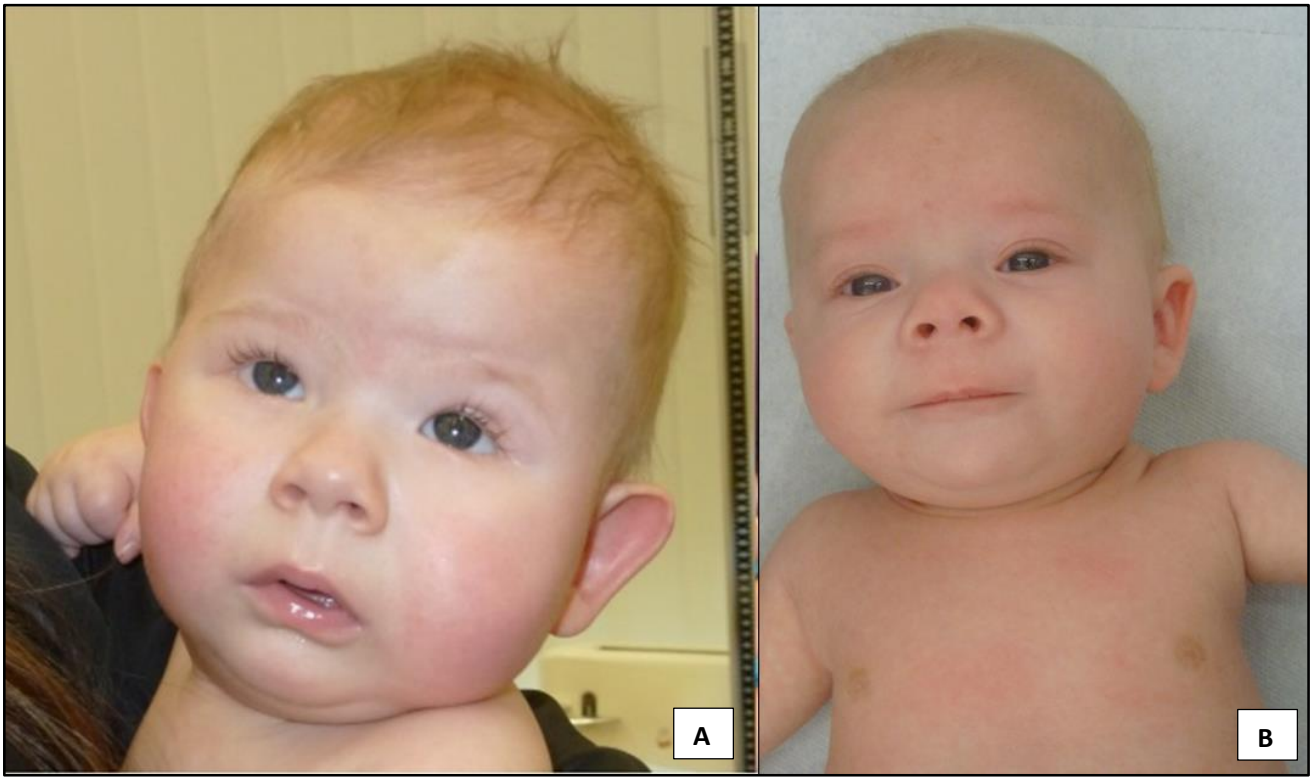

Figure 5. Clinical findings in the two brothers from Family 5.

Note the similar facial phenotype in the index (B) and his younger brother (A).

Exome sequencing

WES was done by the Genomics Core KU Leuven/UZ Leuven as described in Chapter 3.

In the affected siblings of family 4 and 5 the WES analysis was done in collaboration with the Human Genome Sequencing Center (HGSC) at Baylor College of Medicine through the Baylor-Hopkins Center for Mendelian Genomics initiative in an Index-only analysis. Whole exome sequencing was performed at the HGSC as described previously in chapter 3 [8-10].

Linkage analysis

Genotyping was done on DNA extracted from peripheral white blood cells, obtained from the parents and both the unaffected and affected siblings. A dense SNP marker set derived from the 250k Affymetrics SNP typing platform was used in a recessive model in all families and in the family with two affected males an X-linked model was also done. Genome wide parametric linkage analysis with Merlin software was performed (http://www.sph.umich.edu/csg/abecasis/Merlin/tour/parametric.html).

Variant filtering

Exome sequences were obtained from both parents and the patient. VCF files were converted into Excel files, all further filtering was done manually. We analyzed variants with quality class 1 and 2 as described in chapter 3. In the families with a cardiomyopathy, we first selected a panel of known CM genes from the exome to specifically exclude mutations in these genes. Likewise, in family 4 mutations in known ciliopathy genes were specifically evaluated [11].

We excluded all variants that were homozygous in one of the parents or in the unaffected sibling. Variants that were homozygous reference in the affected siblings were excluded. Only exonic and splicing variants were included, synonymous variants were excluded. Variants occurring with a frequency of <5% in the 1000 genomes project or with an unknown frequency were included.

1.1. Autosomal recessive inheritance

We only retained candidate genes located in the linkage regions for which the parents and the unaffected sibling were either heterozygous or reference.

1.1.1. Homozygous inheritance

The patients were filtered against 72 local exomes, i.e. retaining variants for which only the patients were homozygous. In-silico predictions and manual curation of the remaining variants were done as described previously.

1.1.2. Compound heterozygous inheritance

Firstly, the patients were filtered against 72 local exomes, i.e. excluding variants that were homozygous in these control exomes. All heterozygous calls were included in the parents, homozygous calls were excluded in the unaffected sibling. Remaining variants were manually filtered according to one paternal and one maternal inherited variant occurring in the same gene. In-silico predictions and manual curation of the remaining variants were done as described above.

1.2. X-linked hypothesis

In the case of two male affected patients (family 5) the patients were first filtered against all in-house (n=85) exomes, allowing the exclusion of local rare variants. All heterozygous variants occurring in other patients were thus excluded, only variants occurring in the X-chromosome were analyzed. Next, we filtered for variants that were wild-type (reference) in the father and heterozygous in the mother (obligatory heterozygous carrier). In a third step, we retained exonic nonsynonymous variants, exonic/splicing and intronic splicing variants occurring at less than 5 positions from the splicing site. Next, we only retained variants occurring with a frequency of <1%, or with an unknown frequency, in the 1000 Genomes Project. In-silico predictions by Polyphen, Mutation Taster and SIFT were used with discretion as an additional filter when necessary, only variants predicted as damaging or disease causing by 2 or more in-silico tools were further analysed. The remaining list was manually curated with literature using UCSC, OMIM, Pubmed and Genetools.

1.3. Autosomal dominant inheritance due to germline mosaicism

All variants were analysed and thus not only those in linkage regions, for the presence of a de novo variant shared by the two siblings. All heterozygous calls in the affected patients were included, only wild-type (reference) calls were included in the parents. We retained exonic nonsynonymous variants, exonic/splicing and intronic splicing variants occurring at less than 5 positions from the splicing site. Next, we only retained variants occurring with a frequency of <1%, or with an unknown frequency, in the 1000 Genomes Project. In-silico predictions by Polyphen, Mutation Taster and SIFT were used with discretion as an additional filter when necessary, only variants predicted as damaging or disease causing by 2 or more in-silico tools were further analysed. The remaining list was manually curated with literature using UCSC, OMIM, Pubmed and Genetools.

RESULTS

In the three families with syndromic CHD in two siblings, no causative mutation was identified. Following the hypothesis of autosomal recessive inheritance, we identified several candidate genes carrying two (mostly) missense variants inherited from both parents in all families, or one variant inherited from the mother in family 5 with two affected boys according to the X-linked autosomal recessive hypothesis (Table 2). For none of these genes, there was convincing evidence to pursue further functional testing. Under the hypothesis of *de novo* dominant inheritance with germline mosaicism in one of the parents, no good candidate genes were identified.

Table 2. Candidate genes and variants identified in three unsolved families.

Variants obtained in the possible inheritance mechanisms: autosomal recessive (AR), autosomal dominant (AD) and X-linked recessive (XL). Variants in the affected patients are either homozygous (Hom), heterozygous (Het) or hemizygous (Hemi). NS: nonsynonymous, FS: frameshift. In-silico scores are given as a score out of 3 as predicted by AV-SIFT, Polyphen and Mutation Taster. OMIM disease indicates whether mutations in this gene have been implicated in a recognized OMIM disorder.

Family	Mode of inheritance	Gene	Mutation	Mutation	Variant Function	In-silico (N)	OMIM disease
3	AR	*CIDEC*	NM_001199551:exon7:c.C630T:p.H210H	Hom	NS- SNV	1	Lipodystrophy, partial familial type 5 (AR)
		ENGASE	NM_001042573:exon7:c.G962A:p.R321H	Hom	NS- SNV	3	
		EXPH5	EXPH5:NM_015065:exon6:c.T3931C:p.C1311R	Hom	NS- SNV	1	Epidermolysis bullosa (AR)
		PKD1	NM_001009944:exon45:c.C12176T:p.A4059V	Het	NS- SNV	1	Adult polycystic kidney disease 1 (AD)
		PKD1	NM_001009944:exon10:c.C2081T:p.P694L	Het	NS- SNV	3	
		GSTM5	NM_000851:exon4:c.G199A:p.A67T	Het	NS- SNV	0	
		GSTM5	GSTM5:NM_000851:exon8:c.A649G:p.S217G	Het	NS- SNV	0	
	AD	*BCR*	NM_004327:exon19:c.3274_3275insCCGG:p.S1092fs	Het	FS- insertion	0	
		OR2T2	NM_001004136:exon1:c.C310T:p.L104F	Het	NS- SNV	0	
		TPTE2	NM_001141968:exon5:c.T98G:p.L33R	Het	NS- SNV	1	
		VCX3A	NM_016379:exon3:c.475_504del:p.159_168del	Het	nonFS- deletion	0	
4	AR	*CCDC138*	NM_144978:exon4:c.G295T:p.D99Y	Hom	NS- SNV	1	
		COL15A1	NM_001855:exon11:c.C1592G:p.P531R	Hom	NS- SNV	2	
		CRIPAK	NM_175918:exon1:c.295_323del:p.99_108del	Hom	FS- deletion	0	
		DNAJC13	NM_015268:exon43:c.A5018G:p.Y1673C	Hom	NS- SNV	0	
		HLA-DRB5	NM_002125:exon5:c.C780A:p.H260Q	Hom	NS- SNV	0	
		KRTAP5-10	NM_001012710:exon1:c.276_277insGGCTGTGGCTCCTG TGGGGGCTCCAAGGGA:p.G92delinsGGCGSCGGSKG	Hom	nonFS- insertion	0	
		MAPK6	NM_002748:exon5:c.C868G:p.L290V	Hom	NS- SNV	2	
		PRAMEF13	PRAMEF13:NM_001024661:exon4:c.G1276A:p.D426N, PRAMEF14:NM_001099854:exon4:c.G1132A:p.D378N	Hom	NS- SNV	0	
		PRDM9	PRDM9:NM_020227:exon11:c.C2042G:p.T681S	Hom	NS- SNV	0	
		TNXB	TNXB:NM_019105:exon43:c.G12541A:p.A4181T	Het	NS- SNV	2	Ehlers-Danlos S. due to Tenascin-X deficiency (AR; AD)
		TNXB	TNXB:NM_019105:exon43:c.G12514A:p.D4172N	Het	NS- SNV	2	
		KMT2C	MLL3:NM_170606:exon18:c.G2963T:p.C988F	Het	NS- SNV	3	
		KMT2C	MLL3:NM_170606:exon18:c.T2959C:p.Y987H	Het	NS- SNV	3	
		KMT2C	MLL3:NM_170606:exon14:c.G2512A:p.G838S	Het	NS- SNV	3	
		KMT2C	MLL3:NM_170606:exon7:c.C871T:p.L291F	Het	NS- SNV	2	
	AD	*OR4A16*	NM_001005274:exon1:c.C958A:p.P320T	Het	NS- SNV	0	
5	AR	*BEND2*	NM_153346:exon5:c.G586A:p.E196K	Hom	NS- SNV	3	
		VSIG4	NM_007268:exon2:c.G274T:p.V92F	Hom	NS- SNV	2	
		SHROOM3	NM_020859:exon5:c.G3160T:p.V1054L	Hom	NS- SNV	2	
		COL4A6	NM_033641:exon42:c.C4232G:p.P1411R	Hom	NS- SNV	2	
		ARSD	NM_001669:exon6:c.G992A:p.W331X	Het	stopgain SNV	2	
		ARSD	NM_001669:exon6:c.G959A:p.G320D	Het	NS- SNV	2	
		PABPC3	NM_030979:exon1:c.G532A:p.E178K	Het	NS- SNV	2	
		PABPC3	NM_030979:exon1:c.G541A:p.A181T	Het	NS- SNV	2	
		PABPC3	NM_030979:exon1:c.G617A:p.R206H	Het	NS- SNV	2	
		PABPC3	NM_030979:exon1:c.C619T:p.L207F	Het	NS- SNV	2	
		PABPC3	NM_030979:exon1:c.A691G:p.K231E	Het	NS- SNV	2	
		PABPC3	NM_030979:exon1:c.C832T:p.R278C	Het	NS- SNV	3	
		PABPC3	NM_030979:exon1:c.C874T:p.Q292X	Het	stopgain SNV	2	
		PABPC3	NM_030979:exon1:c.C956T:p.T319I	Het	NS- SNV	2	
		PABPC3	NM_030979:exon1:c.G1033T:p.E345X	Het	stopgain SNV	2	
		PRSS3	NM_001197097:exon4:c.A367C:p.M123L	Het	NS- SNV	2	
		PRSS3	NM_001197097:exon4:c.G458C:p.C153S	Het	NS- SNV	3	
		GXYLT1	NM_173601:exon3:c.T444G:p.H148Q	Het	NS- SNV	3	
		GXYLT1	NM_173601:exon3:c.A378T:p.R126S	Het	NS- SNV	3	
		SLC25A5	NM_001152:exon2:c.A230C:p.N77T	Het	NS- SNV	2	
		SLC25A5	NM_001152:exon2:c.A235T:p.I79F	Het	NS- SNV	2	
		SLC25A5	NM_001152:exon3:c.G707C:p.R236P	Het	NS- SNV	3	
		NOTCH2	NM_024408:exon2:c.G112A:p.E38K	Het	NS- SNV	2	Alagille S (AD); Hadju-Cheney (AD)
		PPIAL4G	NM_001123068:exon1:c.C302T:p.A101V	Het	NS- SNV	2	
		NOTCH2NL	NM_203458:exon4:c.C338T:p.P113L	Het	NS- SNV	2	
		NOTCH2NL	NM_203458:exon4:c.C473T:p.T158I	Het	NS- SNV	3	
		NOTCH2NL	NM_203458:exon4:c.C563A:p.P188H	Het	NS- SNV	2	
		NOTCH2NL	NM_203458:exon4:c.A586T:p.T196S	Het	NS- SNV	2	
	AD	*HLA-B*	NM_005514:exon4:c.G703A:p.A235T	Het	NS- SNV	1	
		VCX3B	NM_001001888:exon2:c.C7T:p.P3S	Het	NS- SNV	0	
	XL	*BEND2*	NM_153346:exon5:c.G586A:p.E196K	Hemi	NS- SNV	3	
		COL4A6	NM_033641:exon42:c.C4232G:p.P1411R	Hemi	NS- SNV	2	
		RP2	NM_006915:exon3:c.C844T:p.R282W	Hemi	NS- SNV	0	
		TFDP3	NM_016521:exon1:c.T101C:p.V34A	Hemi	NS- SNV	0	
		UTP14A	NM_006649:exon9:c.G766A:p.V256M	Hemi	NS- SNV	0	
		VCX	NM_013452:exon2:c.C7T:p.P3S	Hemi	NS- SNV	1	
		VSIG4	NM_007268:exon8:c.C1148T:p.T383I	Hemi	NS- SNV	0	
		VSIG4	NM_007268:exon2:c.G274T:p.V92F	Hemi	NS- SNV	2	
		ZNF81	NM_007137:exon5:c.C554T:p.S185L	Hemi	NS- SNV	1	

A detailed description of the results in Family 1 and 2 as well as the additional functional and animal model studies are found in the annex to this chapter, 4.1 and 4.2 respectively.

In Family 1, we identified 6 candidate genes in the linkage regions with homozygous mutations in the patient, inherited from both parents, and for which the unaffected sibling was heterozygous or reference. This gene list was manually curated using functional data and genotype-phenotype correlations for the implicated genes. We thus identified a deleterious mutation in the *ALMS1* gene as the most likely cause. No additional mutations were found in known hypertrophic or dilated cardiomyopathy genes. The affected siblings were homozygous for a frameshift deletion of one basepair in the *ALMS1* gene NM_015120.4:c.7760delG, p.Cys2587Phefs*5 (NC_000002.11:g.73716849delG or NG_011690.1:g.108964del). This is predicted to cause a premature stop at position 5 downstream. The unaffected sister and parents are heterozygotes. Results were confirmed by Sanger sequencing.

In Family 2 WES was performed on both affected siblings and the unaffected sibling. After filtering the variants in the 1273 genes in the linkage regions, under a hypothesis of autosomal recessive inheritance, we identified in *KIF20A* a maternal missense variant (c.544C>T: p.R182W), and a paternal frameshift mutation, creating a premature stop codon (c.1905delT: p.S635Tfs*15). The missense variant c.544C>T: p.R182W was predicted to be damaging by in silico tools SIFT, Polyphen and MutationTaster. These observations suggest that both variants are likely to affect protein function. Further functional testing was thus initiated to confirm pathogenicity, as described in Chapter 4.2.

DISCUSSION

In this project, we were able to identify the genetic cause in 2 out of 5 small, likely autosomal or X-linked recessive families with an almost certain monogenetic cause of a syndromic CHD or distinct, lethal type of severe cardiomyopathy. We thus identified two novel genetic causes of cardiomyopathy. First, mitogenic cardiomyopathy is the severe end of the spectrum of a well-known disorder, Alström syndrome. This is a very rare disorder (estimated incidence <1/1 000 000) [12], and cardiomyopathy is a known feature. In many academic hospitals targeted WES panels exist to screen for most known cardiomyopathies. These gene panels include known genes in hypertrophic-, dilated-, restrictive- and Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC). However, the *ALMS1* gene usually is not in this panel, and therefore, this diagnosis would be missed.
Next, we identified a novel type of restrictive cardiomyopathy of the right ventricle, and we provide extensive genetic and functional evidence that near complete loss-of-function mutations in the *KIF20A* gene cause this phenotype.

In three families with a distinct phenotype, we did not identify a causal mutation. Several candidates were identified, but for none of them, functional knowledge on the gene indicated a causal mutation fitting the phenotype. One of the limitations is that these are unique families. The replication of a mutation in the same gene in unrelated individuals with the same phenotype is the cornerstone of gene identification. When no other families are known with the same disorder, replication is not possible, and we rely on functional tests. However, given the often large number of remaining candidate genes, this was not obvious.

The question then remains how the genetic cause in these families will be resolved in the future? First, it is possible that, as for the mitogenic cardiomyopathy, other families will emerge with mutations in one of the implicated genes. Large collaborative studies generate a vast amount of genetic data, which can be interrogated through tools such as Gene Matcher [13, 14]. Also, the Deciphering Developmental Disorders consortium provides, in a Decipher Research track, all mutations found in genes for which currently there is not yet sufficient evidence that they are indeed developmental disorder genes. As an example, in the Decipher research track, two splice mutations are present in the *MEIS2* gene, in patients with ID and cleft palate. Contacting the contributing clinicians can thus advance knowledge in this field. No mutations in *KIF20A* are listed.

An alternative explanation is that the true genetic cause has been missed by the used technology. This is discussed in more detail in the final discussion of this thesis. WES is far from perfect to identify

all exonic mutations. In these three families, the average coverage of 20X was obtained in 82% of the target region (range 40-94%) meaning that around 18% of exons are not sufficiently covered. In addition, indels are often not called appropriately. Moreover, CNV's too small to be detected by array-CGH but too large to be detected in WES will also be missed. We anticipate that future re-assessment of these families using WGS has a good chance of identifying the causative mutation.

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CHAPTER 4.1

Homozygous loss-of-function mutation in *ALMS1* causes the lethal disorder mitogenic cardiomyopathy in two siblings

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ABSTRACT

Background: Two siblings from consanguineous parents of Turkish descent presented with isolated dilated cardiomyopathy, leading to early death in infancy. The diagnosis of mitogenic cardiomyopathy was made histologically.

Methods and results: Linkage analysis combined with exome sequencing identified a homozygous deleterious mutation in the *ALMS1* gene as the cause of this phenotype.

Conclusions: Alström syndrome is characterized by a typically transient dilating cardiomyopathy in infancy, suggesting that mitogenic cardiomyopathy represents the extreme phenotype, resulting in demise before the other clinical symptoms become evident. This observation further illustrates the role of *ALMS1* and cell cycle regulation.

KEYWORDS

Cardiomyopathy, Alström syndrome, *ALMS1*, exome sequencing, ciliopathy

INTRODUCTION

Cardiomyopathies are a heterogeneous group of primary myocardial disorders in which the heart muscle is structurally and functionally abnormal, in the absence of other causes including coronary artery disease, hypertension, valvular or congenital heart disease [1].The annual incidence of pediatric cardiomyopathy is low, 1/100 000 children, with the highest incidence in the first year of life [2, 3]. Four major types are distinguished, i.e., dilated, hypertrophic, restrictive, and arrhythmogenic right ventricular cardiomyopathy [4]. Other unclassified types, which do not meet the criteria of one of the above, include endocardial fibroelastosis and ventricular non-compaction.

Mitogenic cardiomyopathy is an extremely rare type of dilated cardiomyopathy leading to death in early infancy. To date, only 6 cases have been reported in 4 families [5, 6]. *Zerbini et al.* described this condition in an 8-day-old infant, who died suddenly. Pathological examination revealed normal cardiac anatomy. The right ventricle was slightly dilated and endocardial fibroelastosis was present. Histology of the myocardium showed numerous mitoses and frequently enlarged myocardial nuclei with condensed chromatin forming a serrated thread running in the long axis, termed caterpillar nuclei. They observed an increased DNA ploidy of myocardial cells. The 1-month-old sibling of this patient also presented with heart failure and severe, dilated cardiomyopathy. An endomyocardial biopsy revealed endocardial fibroelastosis, but no increased mitoses. DNA ploidy analysis, on the other hand, showed an increased ploidy of the myocardial cells. This patient responded positively to intensive treatment; however, no long term follow-up data are available. In 2010 *Chang et al.* described 5 cases with an identical disorder, including 2 pairs of siblings. They all presented during early infancy with symptoms of cardiac failure and died soon thereafter. There were no associated extracardiac anomalies. Autopsy showed an enlarged, dilated heart, mostly ventricular, with endocardial fibroelastosis in all cases. Distinct findings were nuclear hypertrophy of the cardiomyocytes and a markedly increased mitotic activity with a proliferative index of 10 to 20% (normal < 1%), as well as caterpillar nuclei. In 1 of the 2 pairs of siblings there was parental consanguinity. This, and the observation of affected males and females strongly suggested autosomal recessive inheritance. We here report a novel family with an identical disorder. By a combination of linkage analysis and exome sequencing, we identify mutations in the *ALMS1* gene as the cause of this distinct type of cardiomyopathy.

PATIENT DATA

Patient 1

The index is the second child of healthy, consanguineous parents of Turkish descent. He was born at 41 weeks of gestation after an uneventful pregnancy. Weight was 3200g (3^{rd}-10^{th} centile). He presented at age 20 days with excessive crying and feeding difficulties. On clinical examination, an inguinal hernia was noted. On reducing the hernia, cardio-circulatory arrest occurred. He was resuscitated and transferred to the university hospital. Despite continuous and prolonged resuscitation, the infant demised. Postmortem echocardiography revealed a structurally normal heart.

The child was not dysmorphic. Weight was 4180 g (50^{th} centile), length 56,5 cm (75^{th}-90^{th} centile) and head circumference 37,5 cm (50^{th} centile). Pathological examination revealed signs of congestive cardiac failure (Figure 1). The weight of the heart was 31,1g (75^{th}-95^{th} centile) which is within normal range for age[7]. There was cardiomegaly, caused by globular dilatation of the left ventricle [8]. The endocardium was pale and thickened, indicative of endocardial fibroelastosis, which was confirmed histologically. Apart from this “dilated cardiomyopathy”, the heart was structurally normal, including normal origin of the coronary arteries and normal aortic and mitral valves. There were no signs of non-compaction cardiomyopathy. Histology showed no signs of myocarditis, nor was there any evidence for a metabolic disorder. Myofibrillar disarray was absent. The most striking phenomenon was a marked mitotic activity in the cardiac myocytes (Figure 2). The myocardium also showed myocyte nuclear hypertrophy with the frequent occurrence of binuclear and even trinuclear myocytes. Some myocytes contained caterpillar nuclei (Figure 3), thus named due to condensed chromatin forming a serrated thread in the long axis. Immunohistochemical staining for Ki-67 (Mib1) showed a markedly increased proliferative activity of the myocardium. The proliferation index was 20% (normal value <1%). These findings lead to the diagnosis of mitogenic cardiomyopathy.

Left ventricular dilatation

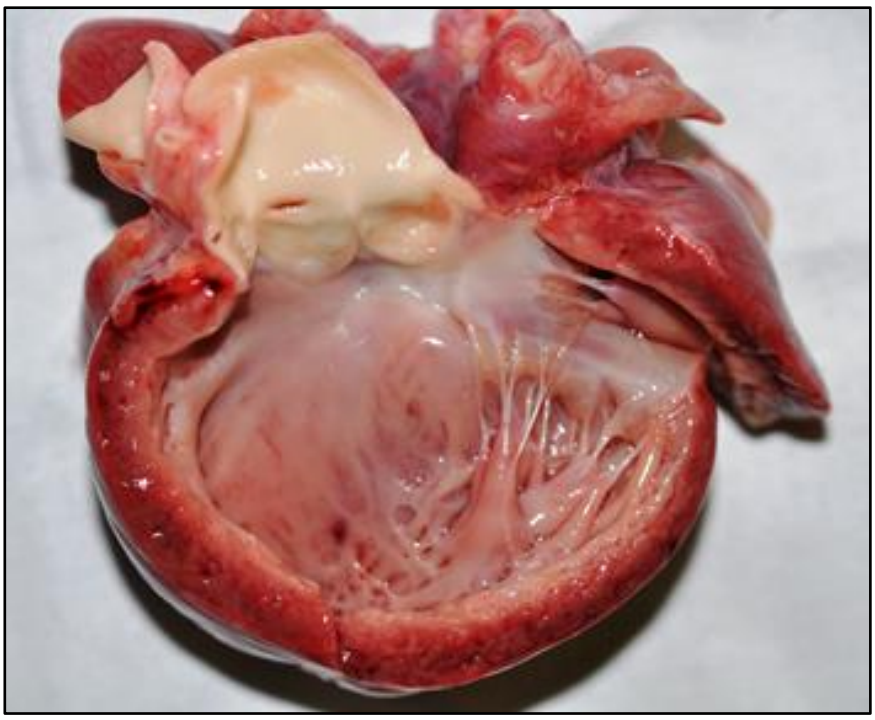

Figure 1. This specimen (patient 1) of the left ventricle clearly shows left ventricular dilatation and endocardial fibroelastosis. The heart was structurally normal.

Increased myocardial proliferation

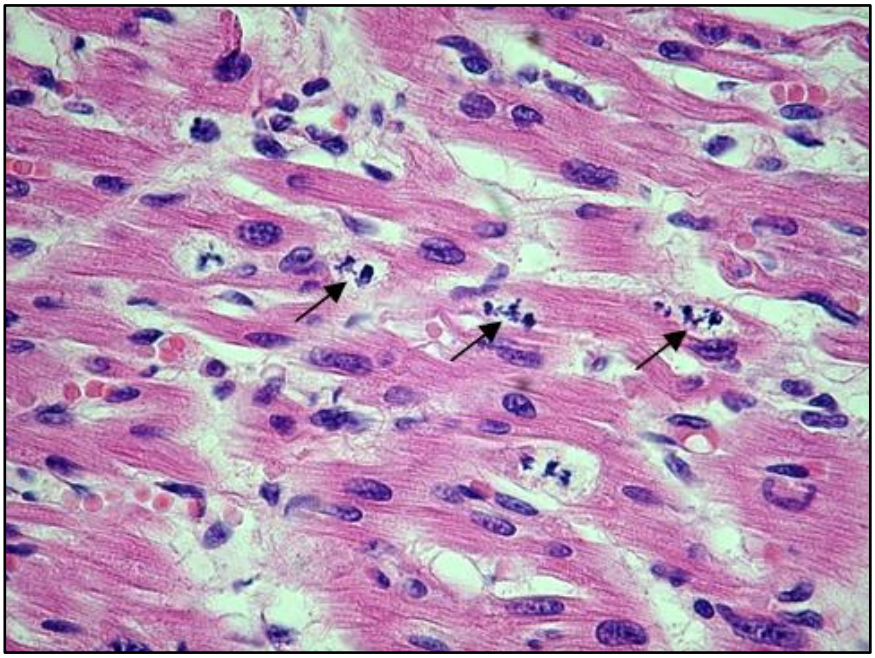

Figure 2. This image shows markedly increased proliferative activity of the myocardium.

Caterpillar nuclei

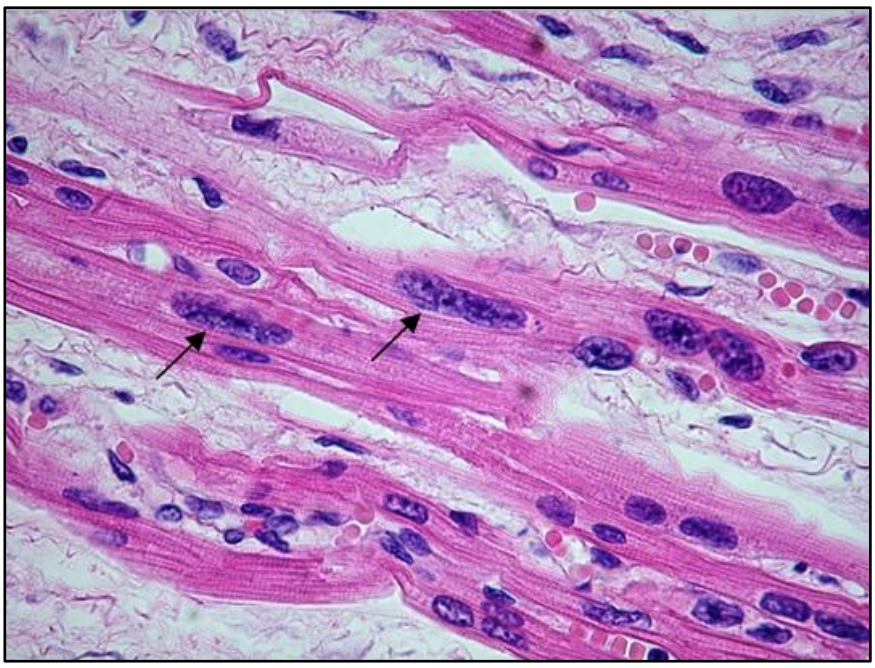

Figure 3. Caterpillar nuclei with condensed chromatin

Patient 2

Because of the high recurrence risk, the following pregnancy was closely followed. Repeated prenatal cardiac ultrasound investigations remained normal. A female infant was born at 41 weeks gestation, weighing 2840 g (3^{rd}-10^{th} centile). Early neonatal echocardiography was normal. However, at day 19, she was admitted with overt heart failure. Echocardiography (figure 4) revealed a dilated cardiomyopathy with left ventricular inner dimension at end-diastolic (LVIDd) of 25mm (normal 12.9-19.1mm) [8]. There was endocardial fibroelastosis and severe mitral and tricuspid regurgitation. Cardiac function was poor, with a fractional shortening (FS) of 12% (normal ≥30%) and retrograde pulmonary hypertension. Despite respiratory and circulatory support, she progressively deteriorated and demised at the age of 22 days. No autopsy was performed. Family history was otherwise negative; cardiac investigations of both parents and the 5 year old sibling were normal (figure 4). Informed consent was given by the family for further genetic studies.

Echocardiography

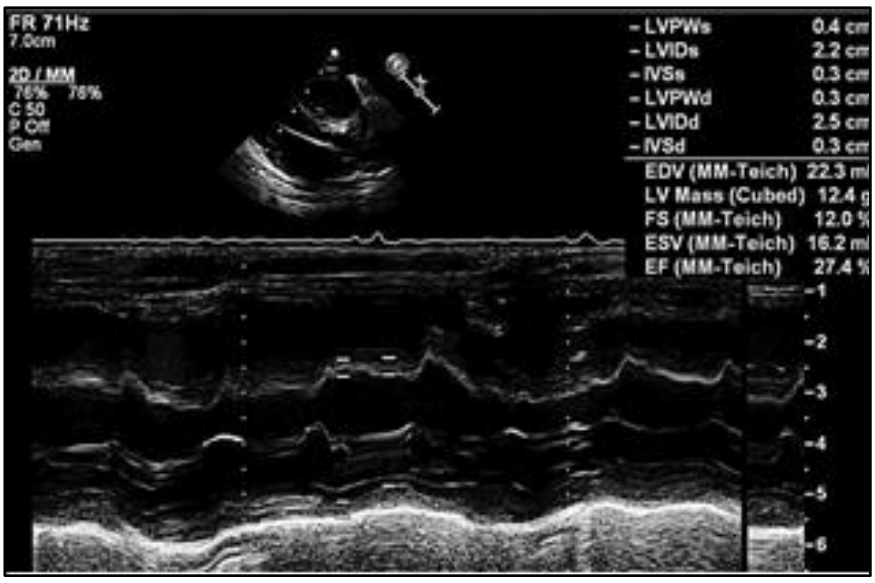

Figure 4. Echocardiography using M-mode showing poor cardiac function (FS 12%, normal >30%) and dilated left ventricle. Left ventricle posterior wall at end- diastole (LVPWd), left ventricle inner dimension at end-diastole (LVIDd), Interventricular septum at end-diastole (IVSd), Left ventricular fractional shortening (FS).

METHODS

Linkage analysis

Genomewide parametric linkage analysis with Merlin software was performed [9]. A dense SNP marker set derived from the 250k Affymetrics SNP typing platform was used in a recessive model. Genotyping was done on DNA extracted from peripheral white blood cells, obtained from the parents and both the unaffected and affected siblings.

Exome analysis

Library construction for all samples were prepared using *TruSeq DNA* Library Preparation Kit (Illumina, Inc., San Diego, CA, USA) in which platform-specific adaptors and unique DNA indexes *were ligated*. For each sample, 1 µg genomic DNA was sheared by sonication to approximately 300bp fragments, followed by end-repair, adenylation and adapter ligation steps. *DNA* sequencing libraries were subsequently enriched with the SeqCap EZ Human Exome Library v3.0 (Roche, NimbleGen), and 2 × 100-bp paired-end reads were generated on the Illumina HiSeq2000 platform

with 3-4 exome-seq samples pooled per lane of a sequencing flow-cell. Sheared DNA, whole genome libraries and enriched exome-seq libraries were validated using DNA-1000 chips on the BioAnalyser (Agilent), and library concentrations were determined using the dsDNA Broad Range Assay using the Qubit (Invitrogen).

Data analysis was done using commercial and in-house developed software (Genomics Core/UZ Leuven). Exome sequences were obtained from both parents, patient 1 and the unaffected sister. Filtering was done with a High-quality depth of 5. From the variant files, we only retained variants in genes from the linkage regions. Additional mutations in all known hypertrophic and dilated cardiomyopathy genes were excluded. All homozygous calls were excluded in the parents and the unaffected sibling, reference calls were excluded in the affected sibling. According to Ensembl (www.ensembl.org) only exonic and splicing variants were included. Synonymous variants were excluded. Variants occurring with a frequency of <1% in the 1000 genomes project or with an unknown frequency were included. All remaining calls were checked for correct calling using Integrative Genomics Viewer (IGV, Broad Institute, Cambridge, MA, USA).

RESULTS

Linkage analysis

In 8 regions the maximum LOD score of 1.9 was reached. All together these regions were spanning 76, 769 163 Mb and contained 487 Human Genome Organization (HUGO) genes.

Exome sequencing

After variant filtering as outlined in the methods section, we identified 6 candidate genes in the linkage region with homozygous mutations in the patient, inherited from both parents, and for which the unaffected sibling is heterozygous or reference (supplementary table 1S). This gene list was manually curated using functional data and genotype-phenotype correlations for the implicated genes. We thus identified a deleterious mutation in the *ALMS1* gene as the most likely cause. No additional mutations were found in known hypertrophic or dilated cardiomyopathy genes.

Results were confirmed by Sanger sequencing (Figure 2S). The two affected siblings are homozygous for a frameshift deletion of one basepair in the *ALMS1* gene NM_015120.4:c.7760delG, p.Cys2587Phefs*5 (NC_000002.11:g.73716849delG or NG_011690.1:g.108964del). This is predicted to cause a premature stop at position 5 downstream. The unaffected sister and parents are heterozygotes.

DISCUSSION

We report a novel family with an extremely rare and lethal disorder: mitogenic cardiomyopathy. It is characterized by infantile-onset dilated cardiomyopathy resulting in irreversible heart failure and death. Histologically, there is a dramatically increased mitotic activity in the cardiomyocytes. Given the likely autosomal recessive inheritance and parental consanguinity in this family, the combination of linkage analysis and exome sequencing allowed us to identify mutations in the *ALMS1* gene as the most likely cause of the condition in this family. The homozygous mutation in *ALMS1* was located in a 3, 435 478 MB region on chromosome 2 (Supplemental figure 1S).
The cardiac phenotype of dilated cardiomyopathy, although more severe, fits with Alström syndrome. In Alström syndrome, more than 60% of individuals develop congestive heart failure, most often dilated cardiomyopathy. An episode of heart failure due to dilated cardiomyopathy occurs in about 40% of cases during early infancy, between 2 and 16 weeks of age [10]. In most cases the initial poor cardiac function improves and patients remain stable for many years. In about 15% a recurrence of restrictive heart failure occurs during adolescence or adulthood. In addition, 20% of patients present in adolescence or adulthood with progressive restrictive cardiomyopathy. Besides this interfamilial variability, pronounced intra-familial variability has been observed regarding the occurrence and severity of cardiomyopathy [11]. Thus, the severe dilated cardiomyopathy observed in the present family can be regarded as an extreme presentation of Alström syndrome. The clinical presentation in these infants is dominated by heart failure, at an age when the additional manifestations such as nystagmus were not yet evident. For this reason, the diagnosis of Alström was not suspected. It is currently not known whether mitogenic cardiomyopathy is a feature of Alström syndrome related to dilated cardiomyopathy during infancy. We were unable to find reports or pathological data in infants with proven Alström syndrome. Also, we currently do not know whether the other cases with mitogenic cardiomyopathy are also caused by mutations in *ALMS1*. No biological material could be obtained from the previously reported cases.

There is no immediate explanation for the more severe phenotype observed in this family. On the one hand, this may be due to ascertainment bias. On the other hand, the marked intra-familial variability, and interfamilial variability observed in individuals sharing the same *ALMS1* mutations suggests the presence of genetic and/or environmental modifiers [12]. The mutation occurs in a region where many other mutations have been reported before, and, similarly to most of them

result in a (predicted) truncated protein [13]. Additional mutations in known hypertrophic or dilated cardiomyopathy genes were excluded.

The present observation indicates a link between *ALMS1* and the mechanisms underlying the neonatal structural and functional changes in the heart. In the different developmental stages from fetus to adult, the ventricular myocardium differs morphologically, quantitatively and qualitatively in function and structure. In the adult myocardium the myocytes are organized in parallel and there is little interstitial tissue between myocytes. Polyploidy is frequently noted and the nucleus is relatively small. In the fetal heart, myocytes are less well organized and the intercellular space is greater. Nuclei are large and polyploidy is unusual. In the immediate weeks following birth, a rapid decrease in myocyte mitosis is noted; essentially all growth beyond the early neonatal period is due to hypertrophy. The stimulus is the normal developmental increase in mural stress and work. The workload of the left ventricle increases postnatally due to increased left ventricular output. This causes a rapid increase in thickness and weight of the left ventricle due to an increase in myocytes [14, 15].

The increased mitotic activity observed in cardiomyocytes in the present condition suggests a defect in cell cycle regulation. *ALMS1* localizes to centrosome and to the ciliary basal bodies. There is a well-established role of the primary cilium in regulating cell cycle [16]. Of interest, in a mouse model for Alström syndrome, carrying a homozygous truncating mutation in exon 10 (*alms1* $^{L2131X/L2131X}$), [17] loss of cilia in the kidney was observed in older mice, associated with increased proliferation and cyst formation as well as apoptosis. In these mice, no cardiac phenotype was described. Also in the heart, cilia may play a role in cardiomyocyte development and proliferation. Abolishing the function of the primary cilium in the pluripotent mouse stem cells P19.CL6 prevents further differentiation of these cells into beating cardiomyocytes [18].

Reaching a genetic diagnosis in rare disorders remains a challenge. We illustrate that even in a single family with only two affected individuals, the identification of the underlying defect is feasible, using a combination of the sophisticated genetic tools. As in this family, we anticipate that the unbiased whole exome screens for mutation is likely to reveal further phenotypic heterogeneity in previously well delineated monogenic conditions.

DISCLOSURES

None.

ACKNOWLEDGEMENTS AND FUNDING SOURCES

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SUPPLEMENTAL MATERIAL

Linkage region

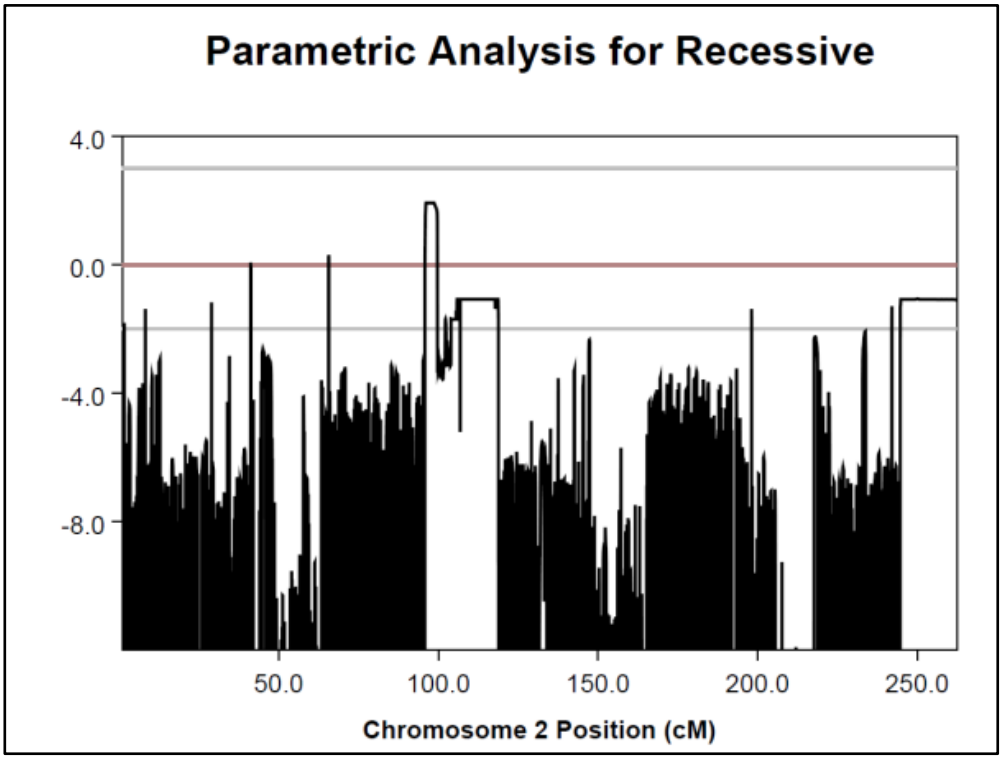

Figure 1S. Linkage region in chromosome 2.

Sanger sequencing

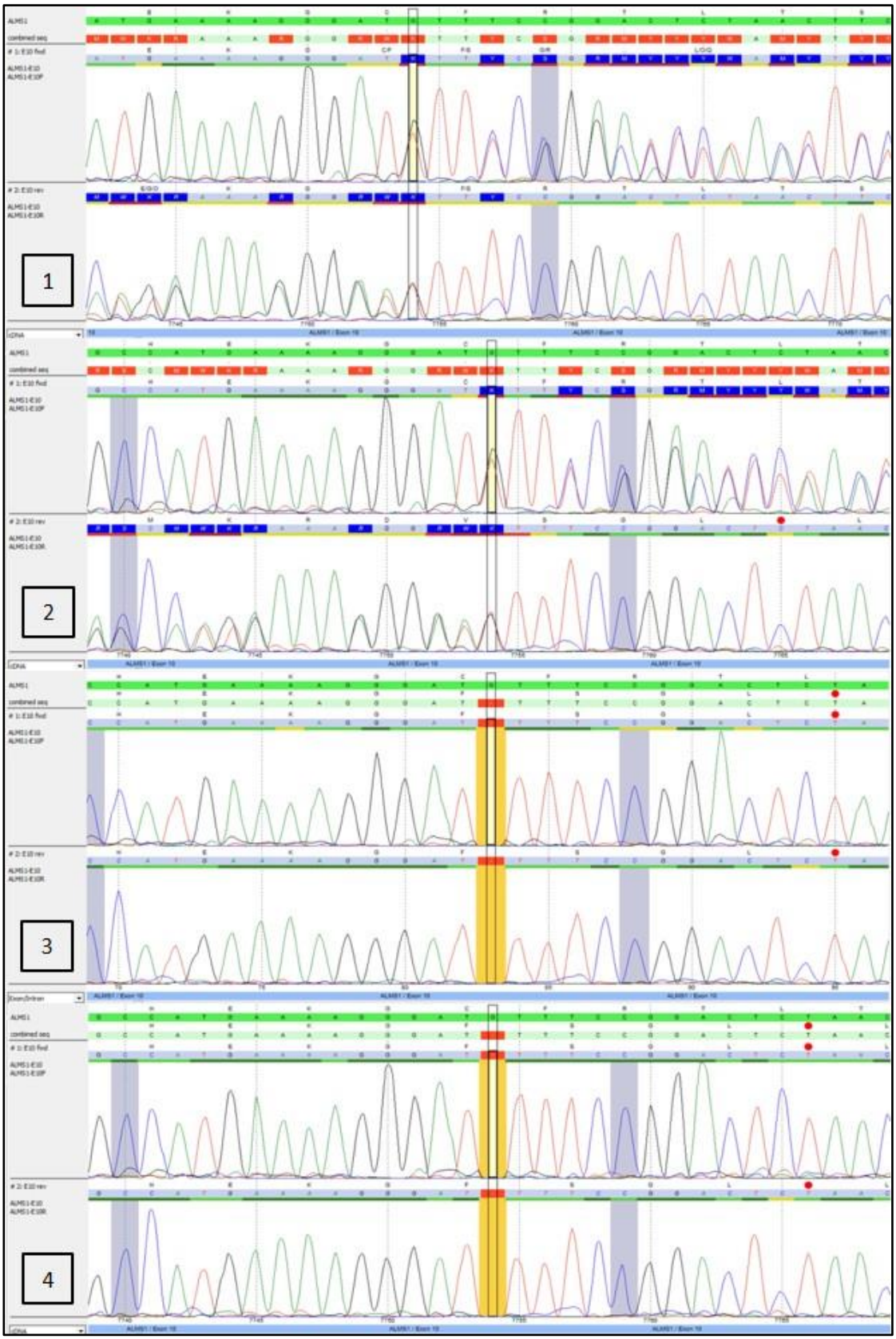

Figure 2S. Sanger sequencing results showing a homozygous deletion of one basepair in the *ALMS1* gene in the two affected siblings (3, 4). The parents are heterozygous (1, 2).

Variants after filtering

Chr	Start	End	Ref	Obs	Index	Sister	Mother	Father	Gene	Omim nr.	Phenotype	AA Change	Function	Exonic function
2	73716849	73716849	G	-	hom	het	het	het	*ALMS1*	606844	Alström syndrome	ALMS1:NM_015120:exon10:c.7760delG:p.Cys2587Phefs*5	exonic	frameshift deletion
3	40085754	40085754	G	A	hom	het	het	het	*MYRIP*	611790	NA	MYRIP:NM_015460:exon3:c.G324A:p.Q108Q	exonic	nonsynonymous SNV
8	110393640	110393640	G	T	hom	het	het	het	*PKHD1L1*	607843	NA	PKHD1L1:NM_177531:exon3:c.G205T:p.A69S	exonic	nonsynonymous SNV
8	110460416	110460416	T	C	hom	het	het	het	*PKHD1L1*	607843	NA	PKHD1L1:NM_177531:exon39:c.T5821C:p.F1941L	exonic	nonsynonymous SNV
8	105010471	105010471	G	A	hom	het	het	het	*RIMS2*	606630	NA	RIMS2:NM_014677:exon15:c.G2479A:p.D827N	exonic	nonsynonymous SNV
8	116632203	116632203	C	G	het	ref	ref	ref	*TRPS1*	604386	TRPS type I and III	TRPS1:NM_014112:exon3:c.G122C:p.S41T	exonic	nonsynonymous SNV

Table 1S. Remaining variants after filtering. Variants are classified as homozygous (hom), heterozygous (het) and reference (ref). Phenotype is classified as not applicable (NA) if no specific known phenotype is associated with the gene.

CHAPTER 4.2

Compound heterozygous loss-of-function mutations in *KIF20A* are associated with a novel lethal congenital cardiomyopathy in two siblings.

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(Manuscript in review)

ABSTRACT

Aims

Congenital or neonatal cardiomyopathies are commonly associated with a poor prognosis and have multiple etiologies. In two siblings, a male and female, we identified an undescribed type of lethal congenital restrictive cardiomyopathy affecting the right ventricle. We hypothesized a novel autosomal recessive condition. To identify the cause, we performed genetic, in vitro and in vivo studies.

Methods and Results

Genome-wide SNP typing and parametric linkage analysis was done in a recessive model to identify candidate regions. Exome sequencing analysis was done in unaffected and affected siblings. In the linkage regions, we selected candidate genes that harbor two rare variants with predicted functional effects in the patients and for which the unaffected sibling is either heterozygous or homozygous reference. We identified two compound heterozygous variants in *KIF20A*; a maternal missense variant (c.544C>T: p.R182W) and a paternal frameshift mutation (c.1905delT: p.S635Tfs*15). Functional studies confirmed that the R182W mutation creates an ATPase defective form of KIF20A which is not able to support efficient transport of Aurora B as part of the chromosomal passenger complex. Due to this Aurora B remains trapped on chromatin in dividing cells and fails to translocate to the spindle midzone during cytokinesis. Translational blocking of KIF20A in a zebrafish model resulted in a cardiomyopathy phenotype.

Conclusion

We identified a novel autosomal recessive congenital restrictive cardiomyopathy, caused by a near complete loss-of-function of KIF20A. This finding further illustrates the relationship of cytokinesis and congenital cardiomyopathy.

Keywords

Congenital cardiomyopathy, *KIF20A*, cytokinesis, exome sequencing, linkage analysis

INTRODUCTION

Cardiomyopathies are a heterogeneous group of primary myocardial disorders in which the heart muscle is structurally and functionally abnormal, in the absence of other causes such as coronary artery disease, hypertension, valvular or congenital heart disease [1]. The annual incidence of paediatric cardiomyopathy is low, 1/100 000 children, with the highest incidence in the first year of life [2, 3].

Congenital or neonatal cardiomyopathies are commonly associated with a poor prognosis and have multiple etiologies. These etiologies differ considerably from cardiomyopathies in older children and adults[4]. Congenital cardiomyopathies can be divided into different groups according to the clinical presentation and echocardiographic criteria: hypertrophic (HCM), dilated (DCM), restrictive (RCM), or unclassified including ventricular non-compaction cardiomyopathy and endocardial fibroelastosis. Genetically, most cardiomyopathies are caused by pathogenic mutations in genes coding for sarcomeric proteins [5, 6].

The etiological landscape in congenital hypertrophic cardiomyopathy is heterogeneous; including various cellular mechanisms such as storage of metabolites as in Pompe disease, disturbed energy metabolism (e.g. fatty acid oxidation defects and mitochondrial diseases), altered signal transduction pathways (e.g. rasopathies due to mutations in genes altering the Ras subfamily and mitogen-activated protein kinases as in Noonan syndrome) or altered cell proliferation (e.g. as in Beckwith-Wiedemann syndrome). Congenital dilated cardiomyopathy is most frequently caused by myocarditis, but mitochondrial diseases can also present as DCM.

Restrictive cardiomyopathy (RCM) is very rare and mostly affects older people. It accounts for 2.5-5% of all diagnosed cardiomyopathies in children and occurs in less than 1 per million children. It is characterized by the replacement of normal heart muscle by abnormal tissue, such as scar tissue which makes the ventricles become stiff and rigid [7, 8]. The systolic function is usually normal, but the diastolic function and relaxation of the ventricles are abnormal. The stiff ventricles do not allow the atria to empty normally, resulting in dilated atria and signs of heart failure. Often, there can be a lack of symptoms which makes the diagnosis difficult. Therapeutic options are limited resulting in a high morbidity and mortality. Several systemic and myocardial diseases, e.g. amyloidosis, metabolic diseases, sarcoidosis

and scleroderma, are associated with RCM; but idiopathic RCM remains the most common [9].

We report a small family with an undescribed type of congenital cardiomyopathy resulting in a lethal restrictive cardiomyopathy. Clinical, genetic and functional studies were performed which led to the identification of a near complete loss-of-function of KIF20A as the most likely cause of this disorder.

CLINICAL DESCRIPTION

We present a small Caucasian family with three children (Figure S1). The parents are not consanguineous. Two of the children, one male (II-2) and one female (II-3), were diagnosed in late fetal life with a congenital heart defect categorized as restrictive cardiomyopathy of the right ventricle (RV). In the male index patient, the diagnosis of a small RV with severe pulmonary stenosis was made at the postmenstrual age (PMA) of 35 weeks. Due to secondary hydrops foetalis, with chylothorax and ascites, labour was induced at 35 weeks and 2 days. At birth, weight was 2400g (25^{th}-50^{th} centile), length 48cm (75^{th} centile) and head circumference 31,8cm (25^{th}-50^{th} centile). Postnatal echocardiography (Figure 1) confirmed the diagnosis of a bipartite RV with agenesis of the apex, a functional pulmonary stenosis, moderate pulmonary insufficiency (grade 2/4) and severe tricuspid insufficiency (grade 3/4). Due to a pulmonary circulation dependent on a patent ductus arteriosus, IV prostaglandin was started. On day 1 percutaneous dilatation of the pulmonary valve was performed and the ductus arteriosus was stented. On day 5 a Rashkind balloon septostomy of the intra-atrial septum was performed. Due to persistent ascites, pronounced hepatomegaly and increased transaminases, an MRI of the liver and liver biopsy was performed at 40 days postnatal age. This showed billirubinostasis, hypoplasia of the portal veins and associated hyperplasia of the portal arteries. A preliminary diagnosis of a ductal plate abnormality was made. During the subsequent weeks, the heart function of both ventricles progressively decreased. At the age of 3 months the decision was made to start palliative care and the patient demised at the age of 93 days. Autopsy confirmed the cardiac diagnosis. There was pronounced sub-endocardial to transmural ischemic fibrosis of the myocardium. The myocardial tissue was hypertrophic with hydropic swelling and myocytolysis. The endocardium showed fibrous thickening. The

myocardium of the left ventricle (LV) was grossly normal, except for endocardial fibrosis which was clearly less pronounced compared to the RV. Pronounced chronic venous congestion of the liver was noted with cardiac fibrosis. The venous centrolobular walls were severely thickened with formation of centro-central fibrous septae. Ductal proliferation was present, but ductal plate malformation could not be confirmed given the normal central bilious ducts in the larger portal fields.

In a following pregnancy, at the PMA of 32 weeks, the diagnosis of restrictive right ventricular cardiomyopathy with RV dysfunction was made in the female fetus. She was born at the PMA of 37 weeks. Her weight, length and head circumference at birth were within normal range; 3,450 kg (25^{th}-50^{th} centile), 49,5cm (10^{th}-25^{th} centile) and 33,8 cm (3^{rd}-10^{th} centile) respectively. She was admitted in NICU due to cyanosis and cardiac decompensation with pronounced ascites. Postnatal echocardiography confirmed the diagnosis of a restrictive cardiomyopathy. The pulmonary valve was morphologically normal, but decreased anterograde flow as well as moderate tricuspid insufficiency was present (grade 2/4) secondary to RV dysfunction. No hepatic abnormalities were present. In the following weeks the heart function progressively decreased, at the age of 71 days (2 months) the patient demised. An autopsy was not performed.

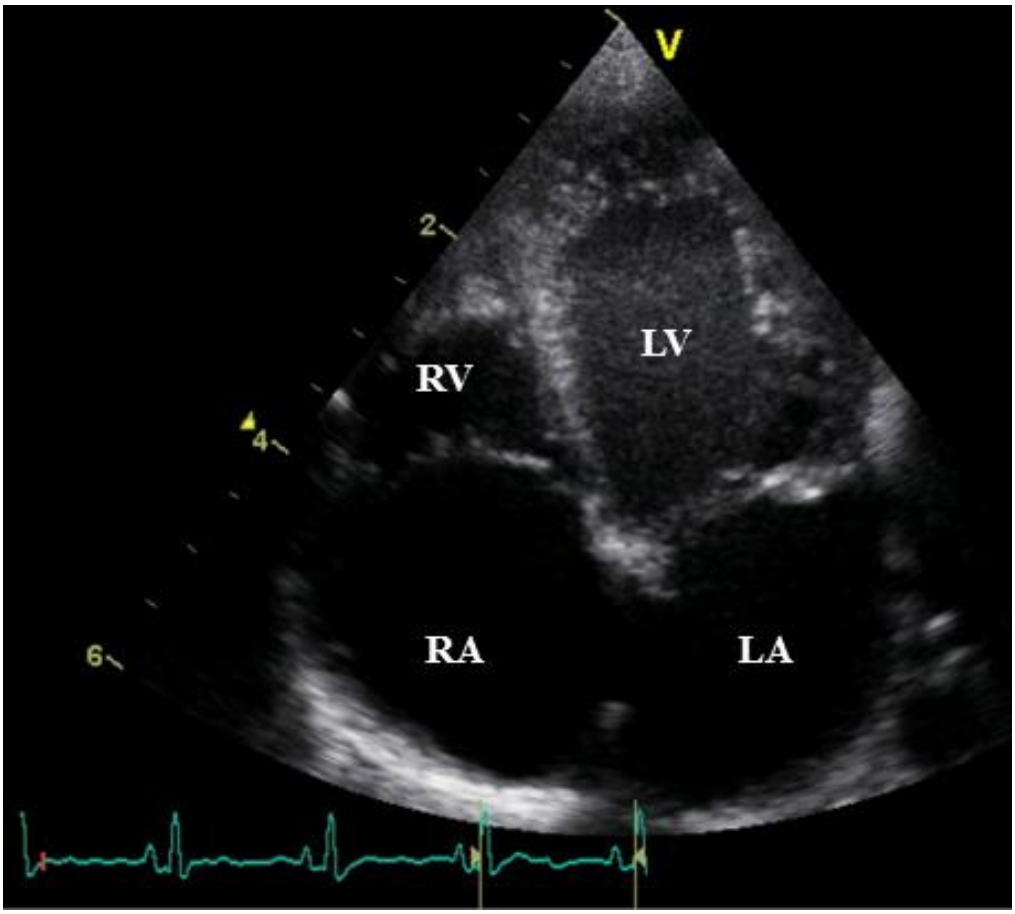


Figure 1. Echocardiography of index patient

Postnatal echocardiography of patient II-2 showing a bipartite RV with agenesis of the apex. Marked dilatation of the RA due to severe tricuspid insufficiency (grade 3/4). RA, right atrium; RV, right ventricle; LV, left ventricle; LA, left atrium.

METHODS

Linkage Analysis

Genotyping was done on DNA extracted from peripheral white blood cells, obtained from the parents and both the unaffected and affected siblings. A dense SNP marker set derived from the 250k Affymetrics SNP typing platform was used in a recessive model. Genome wide parametric linkage analysis with Merlin software was performed (http://www.sph.umich.edu/csg/abecasis/Merlin/tour/parametric.html).

Whole Exome Sequencing

Whole exome sequencing was done on both patients and the unaffected sibling. Genomic DNA was sheared by sonication, platform-specific adaptors were ligated, and the resulting fragments were size selected. The library was captured using the SeqCap EZ Human Exome Library v2.0 (Roche NimbleGen®), and 2 x 76 bp paired-end reads were generated on a HiSeq2000 (Illumina®). Reads that did not pass Illumina's standard filters were removed prior to alignment. Remaining reads were aligned to the reference human genome (hg19), using the Genome Analysis ToolKit (GATK) pipeline. After duplicate removal, local realignment and base quality score recalibration, the data were used for variant calling with GATK Unified Genotyper (2.4-9). Annovar was used for functional annotation of detected variants. Quality filtering was applied by excluding variants found in less than 5 reads and variants detected in less than 15% variant reads.

From the variant files, we only retained variants in genes from the linkage regions. Exonic variants and only intronic variants located less than 6 bp from the intron-exon boundary were included. Synonymous variants were excluded. Variants occurring with a frequency of <1% in the 1000 genomes project or with an unknown frequency were included. Variant filtering was done under the hypothesis of autosomal recessive inheritance, thus retaining only homozygous or compound heterozygous variants in both affected siblings, but not in the unaffected sibling. All remaining calls were checked for correct calling using Integrative Genomics Viewer (IGV, Broad Institute, Cambridge, MA, USA).

Real-Time Quantitative PCR

Primary fibroblasts from patients and unrelated controls were grown from skin biopsy and cultured in Dulbecco's modified Eagle medium DMEM/F12 (Life Technologies®) supplemented with 10% fetal bovine serum (Clone III, HyClones), 1% streptomycine and 0,02% Fungizone at 37°C under 5% CO2.

The PCR was performed for *KIF20A* (GenBank NM_005733) and the house-keeping gene *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase, GenBank NM_002046), which was used as an endogenous control for normalization. qPCR primers were designed using Genscript

software (www.genscript.com/ssl-bin/app/primer). All primers were synthetized by Integrated DNA Technologies.

Reagents and antibodies

General laboratory chemicals and reagents were obtained from Sigma-Aldrich and Thermo Fisher Scientific. Sheep antibodies were raised to the *KIF20A* motor domain (N) or neck plus C-terminus (C) domains. Rabbit antibodies to *KIF4A*, *KIF23*, *PRC1* and *KIF23* pS911 peptide were described previously [10-13]. Specific antibodies were purified using the antigens conjugated to Affi-Gel 15, eluted with 0.2 M glycine-HCl, pH 2.8, and then dialyzed against PBS before storage at −80°C. Commercially available antibodies were used to *AIM1* (mouse 611083; BD). Affinity-purified primary and secondary antibodies were used at a final concentration of 1 µg/ml. Secondary antibodies conjugated to Horseradish Peroxidase (HRP) were obtained from Jackson ImmunoResearch Laboratories, Inc. Secondary antibodies for microscopy conjugated to Alexa Fluor 488, 555, and 647 were obtained from Invitrogen. DNA was stained with DAPI (Sigma-Aldrich).

Molecular biology

Human *KIF20A* was amplified directly from human testis cDNA. The *KIF20A* R182W mutant was created using QuikChange mutagenesis according to the instructions from Agilent Technologies. Mammalian expression constructs for N-terminally GFP-tagged *KIF20A* was made using pcDNA5/FRT/TO vector (Invitrogen). Hexahistidine-tagged bacterial expression constructs for the motor domain (1-507) of wild type *KIF20A* or R187W *KIF20A* were made in pQE32 (QIAGEN).

Cell culture and microscopy

HeLa cells were cultured in DMEM containing 10% (vol/vol) bovine calf serum (Invitrogen) at 37°C and 5% CO2. For synchronization, cells were treated for 18 hours with 2mM thymidine, washed three times in PBS, and twice with growth medium. For plasmid transfection and siRNA transfection Mirus LT1 (Mirus Bio LLC) and Oligofectamine (Invitrogen) were respectively used. The siRNA duplexes used targeted the following sequences: control 5'-CGTACGCGGAATACTTCGA-3', *KIF20A* 3'-UTR 5'-CCACCTATGTAATCTCATG-3'. Microscopy was performed as described previously[13].

Protein expression and purification

The motor domains of *KIF20A* wild type and R187W were expressed in *Escherichia coli* strain JM109 and purified after induction for 3 hours with 0.5mM IPTG. Cell pellets were washed once in ice-cold PBS, and then lysed in 20 ml of IMAC20 (20mM Tris-HCl, pH 8.0, 300mM NaCl, 20mM imidazole) and protease inhibitor cocktail (Sigma-Aldrich) for 20 minutes on ice. Cell lysis was performed using an Emulsifex C5 cell breaker system (Avestin Europe GmbH). Cell lysate was clarified by centrifugation and loaded onto a 1-ml HisTrap FF column (GE Healthcare) at 0.5 ml/min. The column was then washed with 30 ml of IMAC20, and eluted with a 20-ml linear gradient from 20 to 200mM imidazole in IMAC20 collecting 1 ml fractions. Peak fractions were buffer exchanged using 5 ml Zeba Desalt Spin columns (Perbio) into TND (20mM Tris-HCl, pH 8, 300mM NaCl, and 1mM DTT). Protein samples were snap-frozen in 15-µl aliquots and stored at −80°C for further use.

Kinesin motor ATPase assays

A commercial enzyme–linked inorganic phosphate assay was used to measure kinesin ATPase activity (Cytoskeleton, Inc.) as described previously[13]. In brief, a microtubule premix was created at room temperature by mixing 1ml of reaction buffer (15mM PIPES-KOH pH 7 and 5mM $MgCl_2$), 10µl of 2mM paclitaxel, 80µl of preassembled microtubules (1mg/ml tubulin, 15mM PIPES-KOH pH 7, 5mM $MgCl_2$, 1mM GTP, and 20µM paclitaxel), 240µl of 1mM 2-amino-6-mercapto-7-methylpurine riboside, and 12µl of 0.1U/µl purine nucleoside phosphorylase. Reactions were set up in 96-well plates by mixing the protein of interest in a total volume of 7.5µl TND with 147.5µl of the microtubule premix at room temperature. To start the assay,

10µl of 10mM ATP was added to each well. Final assay volume was 165µl of 12mM PIPES-KOH pH 7, 4mM $MgCl_2$, 0.61mM ATP, and 14.5mM NaCl. This was then rapidly transferred to a 37°C plate reader (Tristar LB 941; Berthold Technologies) set to read absorbance at 360nm. Readings were acquired every 30 seconds during 1 hour. An inorganic phosphate standard curve was created in the same assay buffer and used to convert absorbance to nmol hydrolysed ATP.

Zebrafish model

A. Zebrafish maintenance and transgenic lines.

Wild-type, Tg*(kdrl:EGFP)*^{s843} and double transgenic Tg(*gata1:DsRed2;kdrl:EGFP*) zebrafish lines were maintained as previously described[14]. Embryos were collected by natural matings of zebrafish adults and incubated in egg water at 28.5°C according to The Zebrafish Book. Embryos at different developmental stages were presented as hours post fertilization (hpf) or days post fertilization (dpf)[15].

B. Antisense morpholino oligonucleotides and zebrafish embryo microinjection.

The translational blocking morpholino oligomer (MO) for zebrafish *kif20a* was designed and ordered from Gene-Tools, LLC (OR, USA). Its sequence is 5'-GCATGGAGACGCCAGAGCCATTATA-3'. Lyophilized MOs were diluted in water according to the protocol of Gene Tools. Stocks were further diluted to different working concentrations with phenol red added as indicator. 15 ng *kif20a*-MO was injected into 1-2 cell stage embryos. Co-injection of p53 MO was performed to rule out a possible toxicity of MO off-target effect. The dose dependent effect of *kif20a* morpholino was performed by injection of different concentrations of MO into 1-2 cell stage embryos. The efficiency of MO on inhibiting *kif20a* expression was checked by Western blot assay. The antibody for human KIF20A protein was home-made by immunizing sheep and purified. The proteins were extracted from 48 hpf embryos in RIPA buffer (Thermo Scientific, USA) with protease inhibitor (Roche, Germany). The same amount of protein samples were loaded in the Bis-Tris 4–12% SDS-PAGE denaturing gel (The sequence of antigen will be provided upon request.).

For the rescue experiment embryos were injected with *kif20a*-MO together with 10 pg pcDNA3.1 plasmids carrying either a human KIF20A WT cDNA or KIF20A R182W cDNA fragment. Cardiac phenotypes were analyzed at 3dpf.

C. Imaging and data analysis.

Live embryos were photographed with a Nikon SMZ18 microscope. Confocal images of live embryos embedded in 0.9% low melting agarose were made with a Nikon Spinning-Disk confocal microscope, images were generated by ImageJ software.

D. Zebrafish embryonic cardiac function evaluation.

Live confocal imaging was done to quantify cardiac function using a Nikon Spinning-Disk confocal microscope. Five to nine embryos of WT and *kif20a*-atgMO respectively were imaged at 72 hpf (day 3) and 96 hpf (day 4). This was repeated totaling 54 zebrafish larvae, respectively 28 WT and 26 *kif20a*-atgMO. The atrium and ventricle was imaged separately, images were processed by ImageJ software. Heart rate was calculated by counting the number of beats in 15 seconds and multiplying by 4 to obtain beats per minute. Fractional shortening (%) was calculated using the formula (100)(width at diastole–width at systole)/(width at diastole) for the atrium and ventricle as described before by Hoage et al[16]. Statistical analysis was done using SPSS software. P-values were calculated using Mann-Whitney U test, the significance threshold was set at .001.

E. Histology

Embryos at 4dpf were stored in 4% PFA in PBS and transferred to warm DEPC treated saline on a heating plate. Sequential transfer of embryos from the saline through the increasing agarose concentrations (0.25% - 1.5%) was done every 10 seconds on a heating plate of 60°C. The final 1.5% agarose is cooled to room temperature after positioning and orientation of the embryos. Agarose is dehydrated in 70% EtOH/saline while kept on ice. Samples are imbedded in paraffin after graded dehydration in methanol. Transverse sections of 4µM were made using wet mounting with a RN2255 microtome (Leica Technology). Staining was done with Harris hematoxylin and special eosin II (BBC Biochemical, Mount Vernon, WA, USA), the stained sections were imaged with a Motic AE31 TrinocularAE30 Inverted Microscopeswith Leica MC170 HD camera.

RESULTS

Linkage analysis

Linkage analysis was performed on the entire family, and maximal LOD-score (MLS) of 0,727 was obtained in 27 regions (Figure S2). These regions contained a total of 1273 genes, obtained from Ensemble (www.ensembl.org).

Whole exome sequencing and gene identification

Whole exome sequencing was performed on both affected siblings and the unaffected sibling. After filtering the variants in the genes in the linkage regions, under a hypothesis of autosomal recessive inheritance, we identified 1 gene with a homozygous variant (*PHCDA9*) and 2 genes (*ZNF587* and *KIF20A*) with compound heterozygous variants (Table 1S). The *PCHDA9* gene contained a nonsynonymous variant (c.1006C>G: p.L336V) which was absent in the 1000 genomes, but with an allele frequency of 51% in local exomes and common in the ExAC database. In *ZNF587* two missense variants were detected, c.956C>G (p.T319S) and c.1676G>A (p.R559Q) with an allele frequency of respectively 1% and 6% in local exomes.

In *KIF20A* we identified a missense variant (c.544C>T: p.R182W), changing an arginine to a tryptophan, and a frameshift mutation, creating a premature stop codon (c.1905delT: p.S635Tfs*15). The c.544C>T substitution in exon 6 results in a single amino acid substitution (p.R182W) within the motor domain of the protein. Arginine and tryptophan are members of different chemical amino acid groups, and the R182 amino acid is highly conserved across mammalian species. The variant c.544C>T: p.R182W was predicted to be damaging by in silico tools SIFT, Polyphen and MutationTaster. The c.1905delT in exon 15 results in a frameshift that introduces a premature stop codon 15 amino acids downstream. These observations suggest that both variants are likely to affect protein function.

These variants were absent in the population control exomes. In the ExAC Browser database, containing genetic data of 60 706 humans of various ethnicities, the missense variant was found in 2 individuals, respectively of South Asian and European origin. The frameshift variant was present in 32 individuals of African descent. All of the *KIF20A* variants in this database are heterozygous [17]. Sanger sequencing validated the presence of both variants in the affected

siblings and confirmed a heterozygous carrier status in both parents (maternal c.544C>T and paternal c.1905delT). Both variants were absent in the unaffected sibling. Mutations in other known cardiomyopathy genes were absent in the two affected siblings.

Quantitative real-time PCR (qPCR) was used to investigate the effects of the *KIF20A* variants on its expression by comparing *KIF20A* cDNA-levels amplified from mRNA isolated from patient and control fibroblasts. Both patients had a significantly reduced expression level to 40-60 % of control levels (Figure 2A). To investigate the effect of the variants on KIF20A protein levels, immunoblotting was performed using unrelated controls and patient fibroblasts. Both affected individuals had a reduced amount of endogenous KIF20A protein compared to controls (Figure 2B). Antibodies for the N-terminal and C-terminal part of the protein gave identical results, indicating that the frameshift mutation leads to elimination of the transcript by nonsense-mediated mRNA decay.

The localization of the remaining KIF20A in dividing patient fibroblasts (c.544C>T: p.R182W) was then examined. These cells have approximately half the levels of KIF20A when compared to control fibroblasts, but retain normal levels of other cell division proteins (Figure 2C). In control cells, KIF20A localizes to the spindle midzone in anaphase and telophase of dividing cells where it promotes recruitment of the Aurora B kinase (Figure 3A). In both patients KIF20A was aberrantly targeted to chromatin and failed to support translocation of Aurora B to the spindle midzone (Figure 3A).

As a consequence of the inability to relocate Aurora B to the spindle midzone, Aurora B phosphorylation of a key anaphase central spindle protein KIF23 was reduced (Figure 3B, arrows).

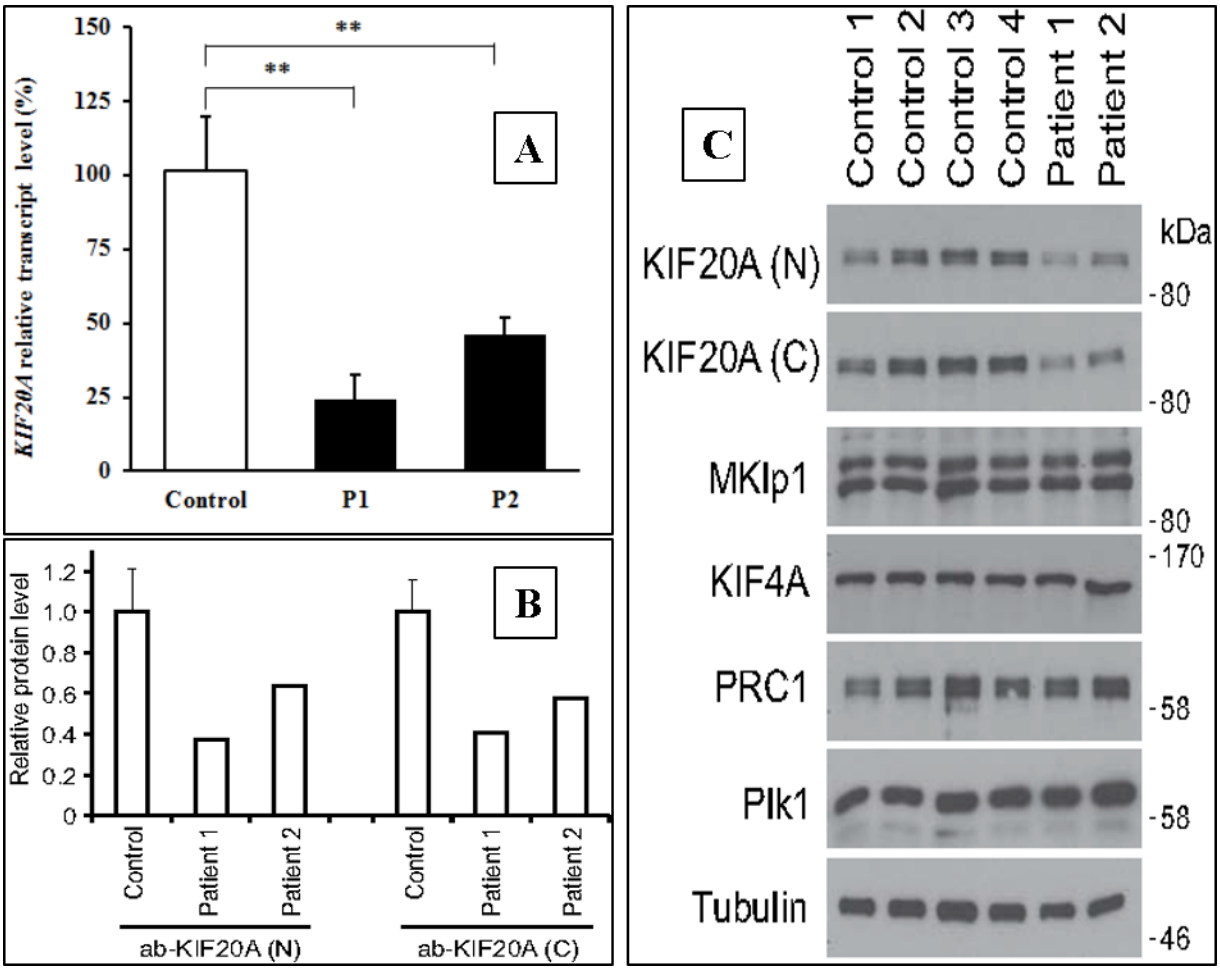


Figure 2. **KIF20A mutations affect Aurora B transport during cell division in patient fibroblasts.**

Figure 2A shows quantification of *KIF20A* transcript level in unrelated controls and patients by qPCR. *KIF20A* expression was normalized to the expression of the house-keeping gene GAPDH. ** indicates $p < 0{,}01$. Figure 2B shows KIF20A levels in unrelated control and patient fibroblasts undergoing cell division. Figure 2C shows western blot analysis of KIF20A and other anaphase spindle protein levels in unrelated control and patient fibroblasts.

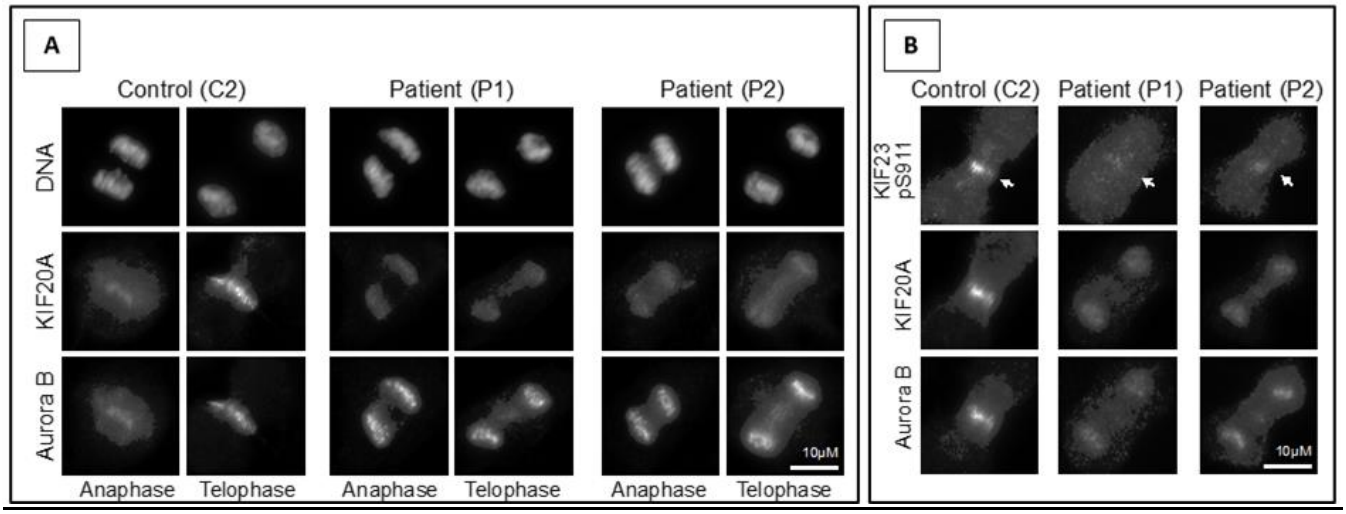


Figure 3. KIF20A mutations affect Aurora B transport during cell division in patient fibroblasts.

Localization of KIF20A in control (C2) and two patient (P1 and P2) fibroblasts undergoing cell division. Cells were stained with antibodies for KIF20A, Aurora B and the Aurora B pS911 phosphorylation site on KIF23 (marked with arrows).

This failure to move from chromatin to the anaphase spindle microtubules suggested that the missense mutation (c.544C>T: p.R182W) perturbed the kinesin motor activity. This possibility was therefore tested using microtubule-stimulated ATPase assays. Purified wild type or R182W mutant KIF20A proteins were tested over a range of concentrations in microtubule-stimulated ATPase assays. Plots of the initial rate of ATPase hydrolysis as a function of the concentration of motor domain show that the KIF20A R182W missense mutation has greatly reduced microtubule activated motor activity (Figure 4A). This reduction in ATPase activity is typical of kinesin “rigor” mutants, which can bind to microtubules but cannot dissociate or move along them. To further pursue this idea, a wild type KIF20A E245A “rigor” mutant and the missense mutation present in the patient cells R182W were transfected into HeLa cells where the endogenous copy of KIF20A was removed by siRNA. In the absence of any KIF20A, Aurora B is trapped on chromatin and is not present on the central spindle Figure 4B. Expression of wild type KIF20A rescues the transport of Aurora B to the central spindle. However, neither the patient R182W mutation nor the rigor E245A supported efficient Aurora B transport and this remains trapped on chromatin in dividing cells. Together these results

indicate that the missense variant (c.544C>T: p.R182W) is a near-complete loss-of-function mutation creating an ATPase defective form of KIF20A.

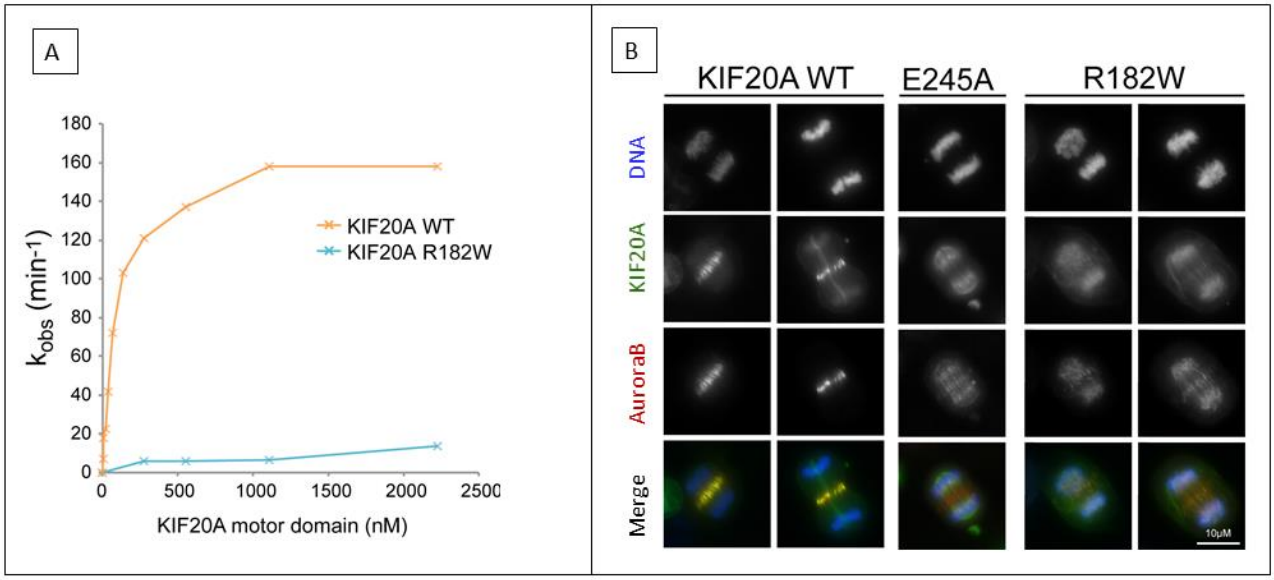


Figure 4. Functional studies of the KIF20A R182W mutant.

(A) Microtubule stimulated ATPase assays for control wild type (WT) and patient (R182W) KIF20A proteins revealed a near complete loss-of-function. (B) Localization of wild type KIF20A (WT) and an engineered "rigor" mutant (E245A) and the patient-associated R182W mutant in HeLa cells revealed that Aurora B remains trapped on chromatin and is not present on the central spindle.

Zebrafish model

Knockdown analysis

The R182 amino acid was conserved in zebrafish and human. Amino acid sequence alignment of human KIF20A and the zebrafish *kif20a* protein showed a 46% identity and 64% similarity (Figure S3), suggesting that zebrafish *kif20a* may have similar function as the human ortholog. The gene was expressed in all early zebrafish stages from 1-2 cell to 6 dpf (Figure 5A). Using a *kif20a*-atgMO, a 74% reduction in protein production was obtained at 48hpf (Figure 5B). Zebrafish hearts started beating at the expected 24 hpf. At 48hpf, cerebral oedema was observed, as well as a smaller trunk shorter total body length. From 2dpf onward a progressive cardiac phenotype was seen in the morphants with pooling of red blood cells proximal to the

atrium, relative tachycardia and cardiac oedema (Figure 5C). At 144 hpf (day 6) the abnormal phenotype was present in 90% of the 80 embryos in 4 independent experiments (Figure 6). Co-injection of P53 MO to rule a possible toxicity effect did not influence the phenotype. A dose dependent effect of *kif20a*-MO was evident (Figure 5D). Partial rescue was obtained by cDNA bearing human KIF20A WT, confirming kif20a was the gene responsible for the cardiac phenotype (Figure 5E). No rescue was seen with the R182W mutant. Together these results indicate that *kif20a* was essential for zebrafish heart development, an evolutionally conserved function.

Histology of the heart and cardiac function

The whole heart of *kif20a* morphants was smaller and significant pericardial edema was evident in all transverse sections of the morphants when compared with controls (Figure 6A). The atrioventricular (AV) valve in morphants appeared morphologically normal, the bulbus arteriosus (BA) was smaller and the atrial and ventricular walls were thicker compared to controls. A clear looping defect was present in the *kif20a* morphants (Figure 6A), where the atrium was located laterally at the same lever of the ventricle as opposed to dorsally in controls. Pooling of blood was present anterior to the atrium of the morphants, suggesting a decreased function.

To better characterize the heart phenotype in *kif20a* morphants, we evaluated several cardiac parameters including heart rate and fractional shortening (FS). At 3dpf the heart rates were similar in the controls and *kif20a* morphants. At 4dpf the morphants showed a significant increase in heart rate (p = 0.009, Figure 6B), most likely as a response to progressive heart failure and decreased stroke volume, since cardiac output equals heart rate x stroke volume. Although an increased ventricular fractional shortening was seen in the *kif20a* morphants compared to the controls (40.6 % ± 10.38 vs. 30.49% ± 4.91, p = 1,62774E-05); more outliers were observed in the *kif20a* morphants which suggested systolic failure following diastolic failure (Table 1 and Figure 6B). The end-systolic diameter (ESD) of the atrium in *kif20a* morphants was significantly smaller (p = 0.001); this is most likely due to increased force necessary to empty the atrium into a more rigid ventricle. These findings suggest that *kif20a* is required for normal heart function in zebrafish embryos.

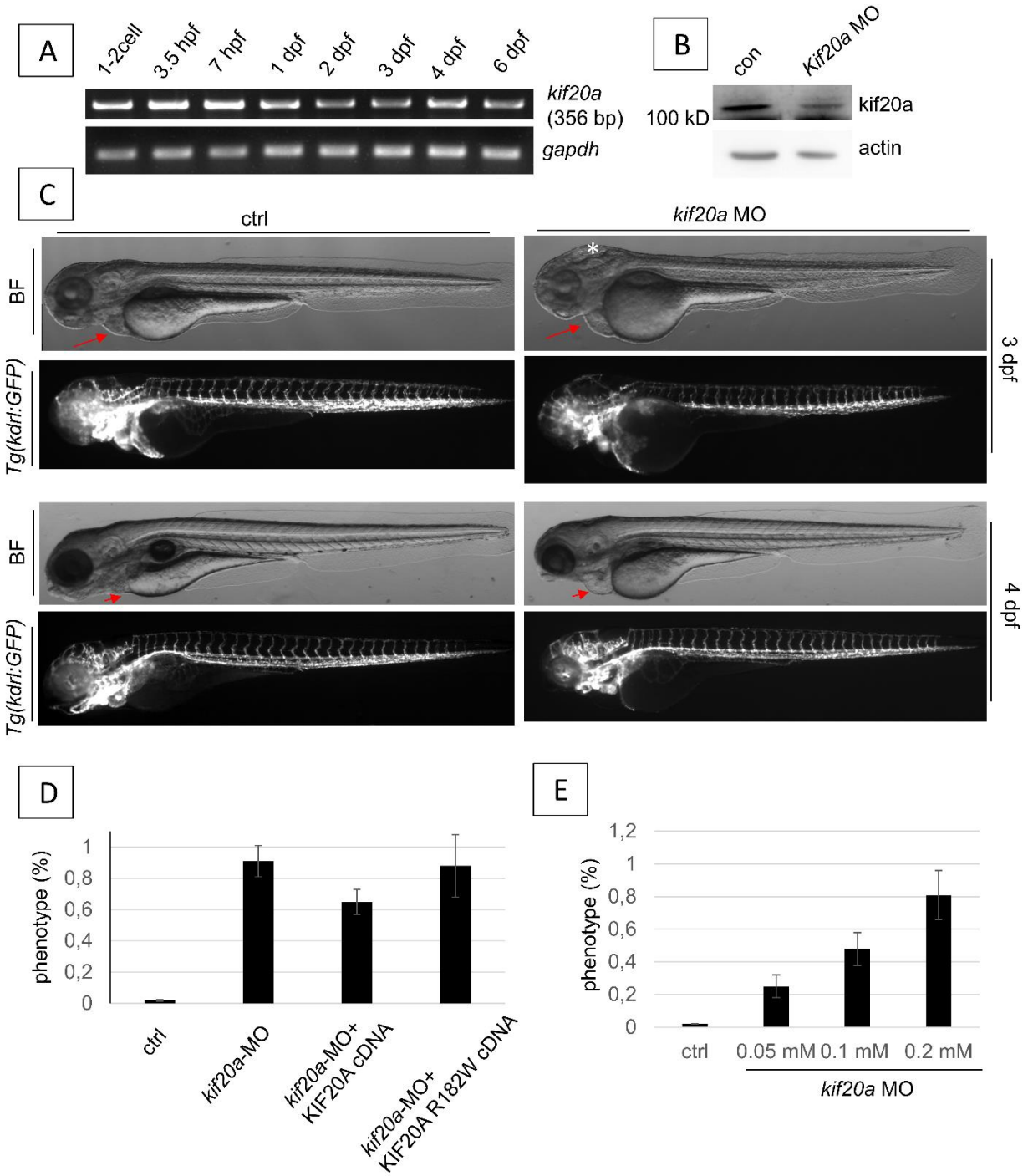

A
1-2cell
3.5 hpf
7 hpf
1 dpf
2 dpf
3 dpf
4 dpf
6 dpf
kif20a
(356 bp)
gapdh
B
con
Kif20a MO
100 kD
kif20a
actin
C
ctrl
kif20a MO
BF
Tg(kdrl:GFP)
3 dpf
4 dpf
D
phenotype (%)
1
0,8
0,6
0,4
0,2
0
ctrl
kif20a-MO
kif20a-MO+ KIF20A cDNA
kif20a-MO+ KIF20A R182W cDNA
E
phenotype (%)
1,2
1
0,8
0,6
0,4
0,2
0
ctrl
0.05 mM
0.1 mM
0.2 mM
kif20a MO

Figure 5. Zebrafish *kif20a* knockdown studies.

(A) RT-PCR analysis of zebrafish *kif20a* gene expression during early stages. *Gapdh* was used as a housekeeping gene.

(B) Western blot analysis of whole lysates from control and *kif20a* morphants showing a 74 % protein reduction. Actin was used as a loading control.

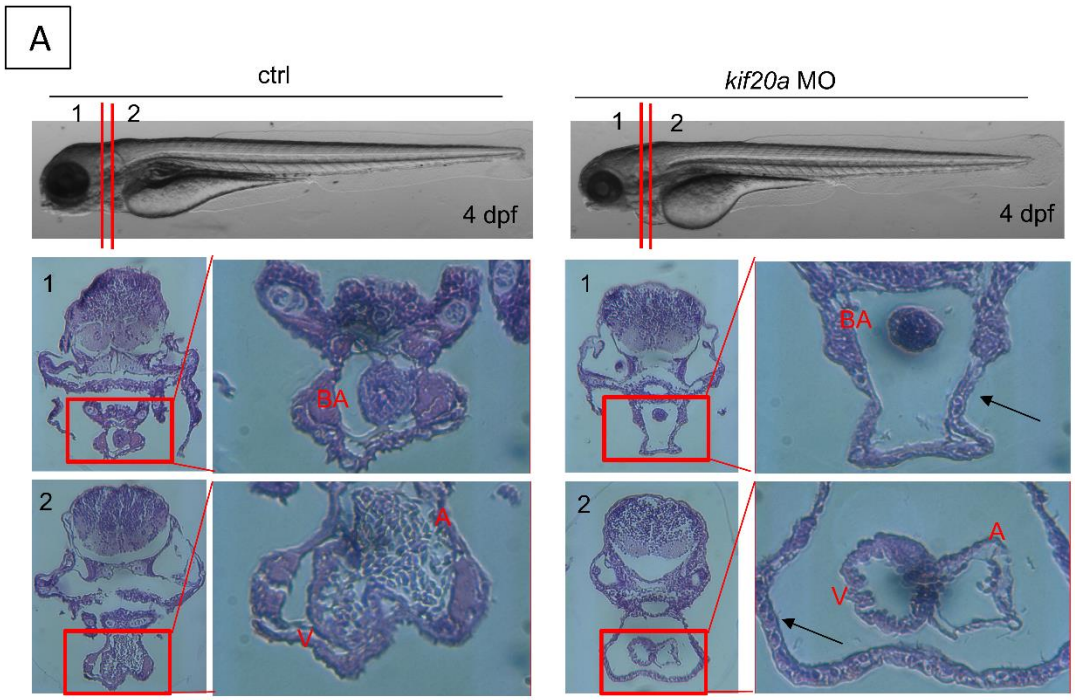
(C) Morphological analysis of zebrafish control and kif20a morphants at 3-4 dpf.

Upper panel: Bright-field and fluorescence images of zebrafish control and *kif20a* morphants at 3 dpf. The white star indicates cerebral oedema.

Lower panel: Bright-field and fluorescence images of zebrafish control and *kif20a* morphants at 4 dpf. The red arrows indicate cardiac oedema.

(D) Rescue experiments where embryos were injected with *kif20a*-MO only, together with human KIF20A WT cDNA or KIF20A R182W cDNA. The percentage of cardiac phenotype in each groups at 3 dpf is presented.

(E) Dose dependent effect of *kif20a*-MO with varying concentrations of *kif20a*-MO (range 0-2 mM), injection dose was 4,6 nl. The percentage of cardiac phenotype at 3 dpf is shown.


A
ctrl
kif20a MO
1
2
4 dpf
BA
A
V

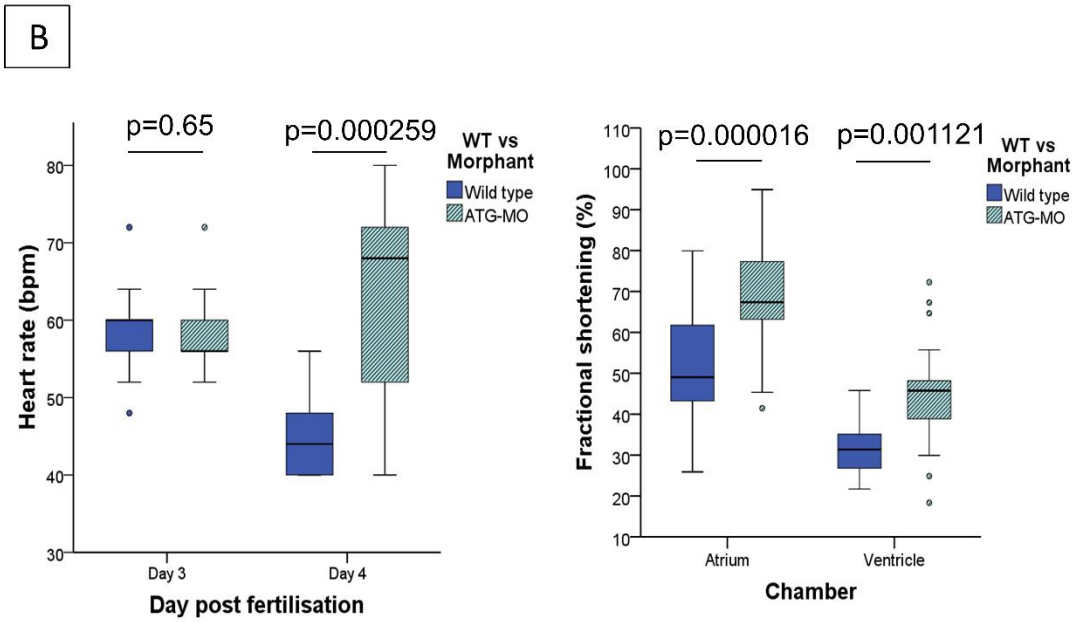

B
p=0.65
p=0.000259
WT vs Morphant
Wild type
ATG-MO
Heart rate (bpm)
Day 3
Day 4
Day post fertilisation
p=0.000016
p=0.001121
Fractional shortening (%)
Atrium
Ventricle
Chamber

Figure 6. Histology and cardiac function evaluation of zebrafish *kif20a* morphants.

(A) H&E staining of zebrafish embryos injected with control MO and *kif20a* MO at 4 dpf, magnification of 10x and 40x. Arrows indicate cardiac oedema. Upper panel: Bright-field images of control and *kif20a* morphants at 4 dpf showing the section locations (1 and 2).
Lower panel: H&E staining images of control and *kif20a* morphants at position 1 and 2.

(B) Cardiac function analysis.

Left panel: Heart rate in beats per minute is compared in control and kif20a morphants at 3 dpf and 4 dpf. A significant increase in heart rate is observed in the morphants at 4 dpf, most likely due to progressive cardiac failure.

Right panel: Fractional shortening is compared in control and *kif20a* morphants respectively in the atrium and ventricle. Although a significant increased fractional shortening is present in the morphants, more outliers are seen, suggesting systolic failure.

DISCUSSION

We report a family with two siblings presenting with a novel lethal congenital heart disease. It was characterized by fetal-onset restrictive cardiomyopathy predominantly affecting the right ventricle and leading to irreversible heart failure and early death. Given the occurrence of the same distinct phenotype in siblings of both sexes with unaffected parents, autosomal recessive inheritance was likely. This phenotype is unique and to our knowledge has not been reported in literature previously. After exclusion of mutations in known CM genes, linkage analysis and exome sequencing was performed to identify the genetic basis. We were able to identify functional variants in the *KIF20A* gene as the most likely cause. Two compound heterozygous variants were found; one variant was a missense mutation (c.544C>T: p.R182W), the other a frameshift mutation, creating a premature stop codon (c.1905delT: p.S635Tfs*15). There is no known phenotype of constitutional *KIF20A* mutations in humans. In mice, homozygosity is lethal in all pups at an early age of 3-4 weeks, but no phenotypic details have been reported [18]. In a zebrafish model we showed that translational blocking of the zebrafish *kif20a* gene resulted in a cardiomyopathy phenotype and that kif20a is required for proper heart patterning and function, suggesting KIF20A has an evolutionary

conserved function in heart development. Future studies using more specific genetic knockout models could provide additional valuable information about its function.

Kinesin family member 20A (*KIF20A),* a.k.a. Mitotic kinesin-like protein 2 (*MKLP2*) or Rab6-interacting protein (*RAB6KIFL*) is one of the kinesin-like proteins. These proteins are microtubule-associated motors that play important roles in intracellular transport and cell division [19]. It is required for chromosomal passenger complex-mediated cytokinesis and translocation of the chromosomal passenger complex (CPC) from the chromatin to the central spindle in metaphase, anaphase and telophase[20]. Functional studies in patient fibroblasts revealed reduced protein levels associated with deficient transport of the Aurora B and the CPC which remains trapped on chromatin in dividing cells. This was due to the missense variant causing a near complete loss-of-function of the ATPase function of KIF20A. It is not excluded that a complete loss-of-function is embryonically lethal, and that the minimal residual function of one allele in this family allowed survival beyond fetal life.

KIF20A is highly expressed in cardiac myocytes, fetal liver and thymus; and to a lesser extent in fetal heart, kidney, spleen and lung. The crucial role of PLK1 in cardiomyocyte proliferation has been shown in zebrafish. This cardiomyopathy phenotype predominantly affected the right ventricle. Currently, it is not known why this occurs. It might be related to a different origin of the right ventricle which is formed by the second heart field, compared to the left ventricle which originates from the primary heart field. However, this might also be secondary to distinct differences in function of the fetal left and right heart. Unlike the adult circulation, in the fetus, the stroke volume of the fetal LV is not equal to the stroke volume of the RV as a result of intracardiac and extracardiac shunting. The RV receives around 65% of the venous return and the LV about 35% [21]. A cardiomyopathy affecting predominantly the RV could thus lead to significant morbidity and possible mortality during the fetal or early neonatal period. This could lead to early unexplained mortality and underreporting of this specific phenotype. Additional phenotypic features becoming apparent at a later age would also be difficult to detect.

The specific link with the cell cycle and this cardiopathy in humans is still unclear. Previously we and others reported mutations in the *ALMS1* gene as a cause of mitogenic cardiomyopathy. This links cardiomyopathy to ciliopathy and the cell cycle [22, 23]. These reports open a new mechanism for future research in cardiomyopathies and cytokinesis.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

None declared.

SUPPLEMENTAL MATERIAL

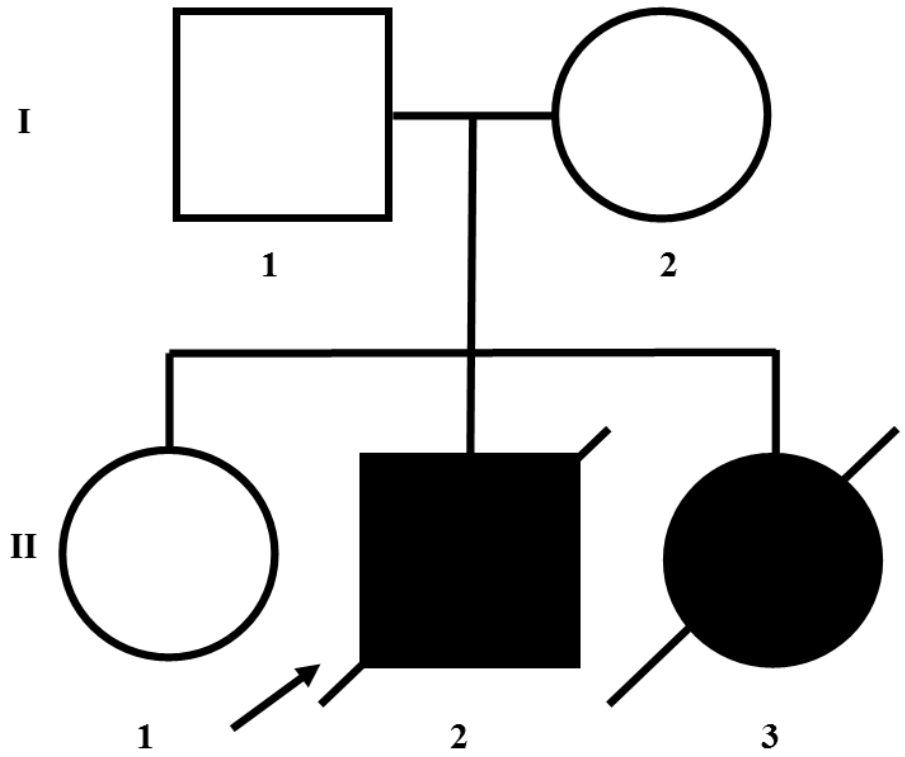
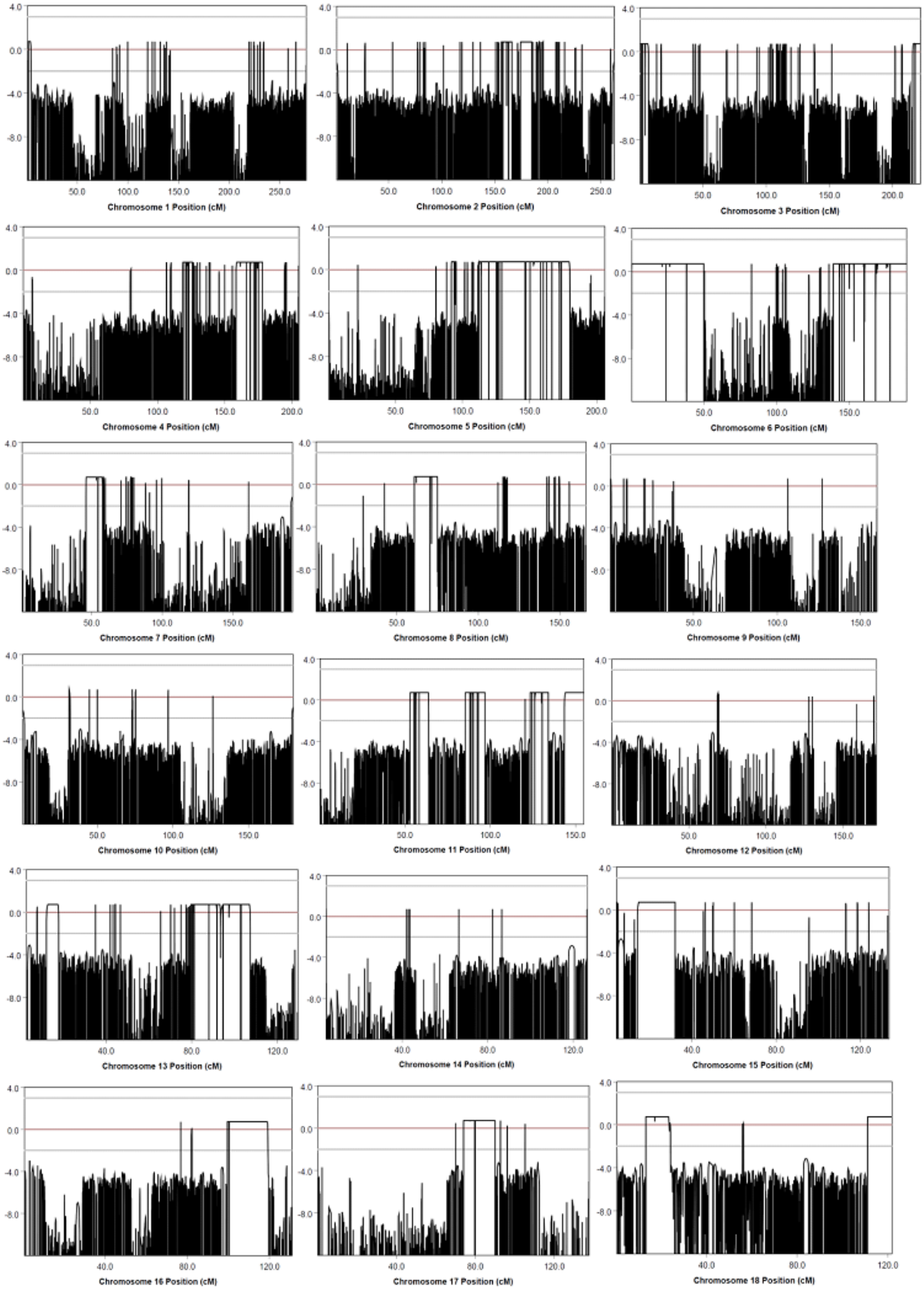


Figure S1. Pedigree of the family.

II-2 and II-3 were diagnosed with restrictive cardiomyopathy, patients demised at the age of 6 and 3 months respectively. The parents and older sibling have a normal phenotype.


Chromosome 1 Position (cM)
Chromosome 2 Position (cM)
Chromosome 3 Position (cM)
Chromosome 4 Position (cM)
Chromosome 5 Position (cM)
Chromosome 6 Position (cM)
Chromosome 7 Position (cM)
Chromosome 8 Position (cM)
Chromosome 9 Position (cM)
Chromosome 10 Position (cM)
Chromosome 11 Position (cM)
Chromosome 12 Position (cM)
Chromosome 13 Position (cM)
Chromosome 14 Position (cM)
Chromosome 15 Position (cM)
Chromosome 16 Position (cM)
Chromosome 17 Position (cM)
Chromosome 18 Position (cM)

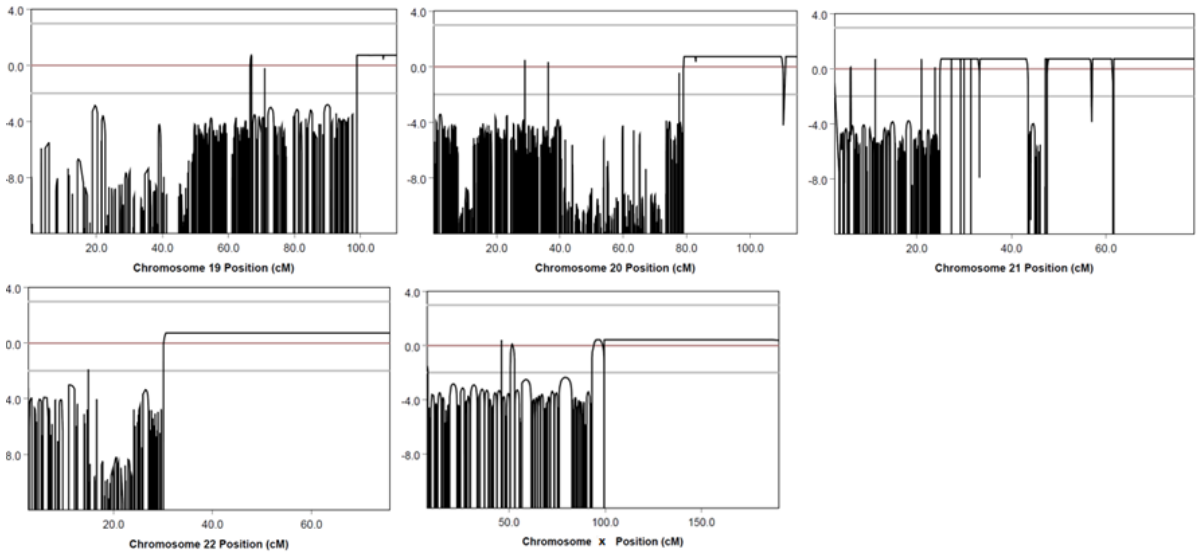


Figure S2. Represents the LOD score in function of the chromosomal position in centimorgan (cM).

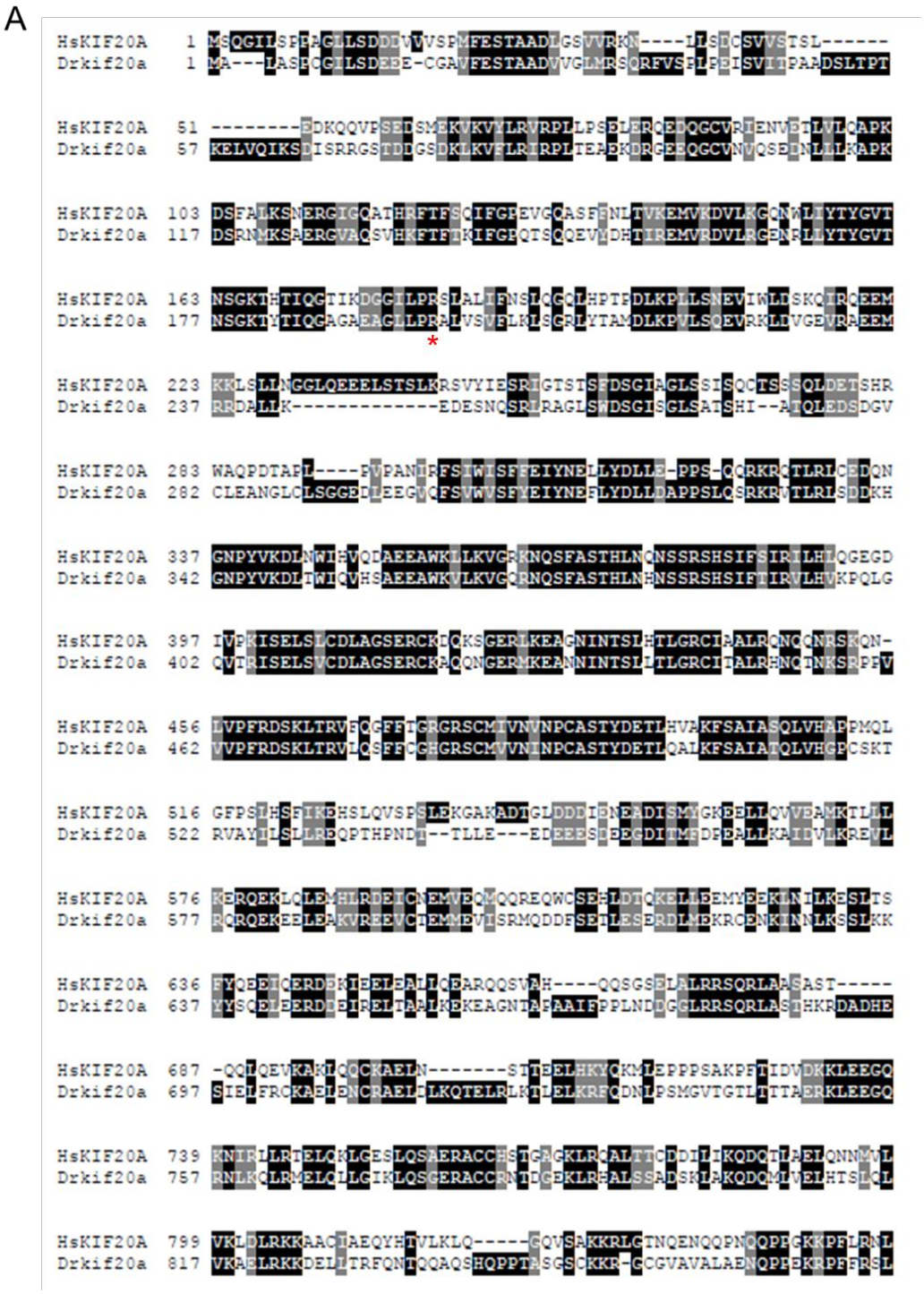


Figure S3. Amino acid sequence alignment of human and zebrafish KIF20A genes.

HsKIF20A, human KIF20A gene and Drkif20a, zebrafish kif20a gene are shown in alignment. Black highlighting shows identical residues with the red star indicating the conserved R182 residue in human and zebrafish.

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CHAPTER 5

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

1. GENE IDENTIFICATION IN CONGENITAL HEART DISEASE

The general objective of this study was to contribute to understanding the genetics of congenital heart defects (CHD) and congenital cardiomyopathies. One way to make progress is to identify novel genes and mechanisms involved in congenital heart disease, through the study of well-selected patients and families. We applied state-of-the art genetic technologies to screen the genome for intragenic mutations in sporadic and familial forms of unique types of syndromic CHD or cardiomyopathies.

In two families with suspected autosomal recessive inheritance of a highly distinct type of lethal cardiomyopathy, we identified the underlying genetic cause. In one family, with mitogenic cardiomyopathy, a homozygous mutation in the *ALMS1* gene was identified [1], a gene previously associated to Alström disease, a multisystem disorder also featuring cardiomyopathy. Shortly after submitting our observation, another group reported the same finding in other, unrelated families with mitogenic cardiomyopathy [2]. In a second family, with a thus far undescribed phenotype of restrictive cardiomyopathy of the right ventricle, compound heterozygous mutations in the *KIF20A* gene were identified. Extensive functional data provide strong support to a causative role of these mutations in the phenotype. However, definitive proof would require confirmation in a second family with the same phenotype. No such family has been identified so far, despite many calls for patients at different cardiology meetings and searches in the literature and genetic databases.

The finding of a novel genetic explanation for specific phenotypes has an important impact on the families involved. In the first family with *ALMS1* mutations, we were confident enough of a causal relationship between the mutations and the phenotype to offer prenatal diagnosis in a subsequent pregnancy. However, opinions differed amongst geneticists whether in the second family with the *KIF20A* mutations, the data are sufficiently certain to allow the use in prenatal diagnosis, if requested.

However, these results are also interesting from a more fundamental point of view. These two genes have a role in cell cycle regulation, and for the first time link mutations involved in this process to congenital cardiomyopathies. This is not unexpected. Fetal cardiomyocytes are mitotically active, but soon after birth, cardiomyocytes rapidly lose their capacity to divide and they undergo terminal differentiation. The ALMS1 protein plays a central role in this process

of mitotic arrest. In humans, lack of functional ALMS1 results in a dilated cardiomyopathy characterized by increased mitotic activity. Increased cardiomyocyte proliferation is also observed in cultured mouse cardiomyocytes where the *Alms1* mRNA has been knocked-down. Likewise, postnatally, increased cardiomyocyte proliferation is seen in homozygous *Alms1* knockout mice [2]. How this leads to dilated cardiomyopathy is currently not known.

ALMS1 is spatially associated with key components of the mitotic machinery: during mitosis, it localizes at the centrosomal spindle poles and during late mitosis also at the cleavage furrow and the contractile ring [3]. KIF20A, as one of the kinesin-like proteins also has a key role in regulating mitosis [4], through intracellular transport and cell division [5]. It is required for the translocation of a key player, the chromosomal passenger complex, from the chromatin to the central spindle in metaphase, anaphase and telophase [6].

These insights will contribute to a better understanding of the cellular processes mediating the fetal to postnatal transition of the cardiomyocyte.

Besides these cardiomyopathies, we also studied syndromic forms of structural heart defects, CHD. In the cardiology clinic, many patients with syndromic CHD remain without diagnosis. Over the last years the introduction of NGS has resulted in a spectacular increase in the number of new genes causing a wide variety of developmental disorders [7-10]. Recent studies indicate that persons with a syndromic type of CHD carry an excess of *de novo* mutations in other genes besides those already associated to CHD or ID [11]. This indicates that many genes still remain to be discovered in this field. In our (small) cohort of patients, we identified the *MEIS2* gene as a novel gene associated with a syndromic form of CHD, associated with cleft palate and ID. Since then, a number of other reports have confirmed this finding [12-14]. Likewise, we observed for the first time that CHD may belong to the phenotypic spectrum of *DYRK1A* mutations. Since then, this has also been confirmed by other research groups [11].

2. NGS: POSSIBILITIES AND LIMITATIONS

A second aim of this study was to provide updated guidance for genetic testing in the congenital cardiology clinic, in view of the recent introduction of diagnostic NGS.

Exome sequencing in families with a clear monogenic disorder often fails to identify the causal mutation, as we experienced in 5 of the sporadic patients investigated in a trio approach, but also in three of our five familial cases. This indicates that WES has its limitations, which can be both technical and biological. It is therefore crucial that clinicians are aware of the limitations of NGS as a diagnostic tool, including missed mutations, difficulties in variant classification and the importance of good clinical phenotyping.

1. TECHNICAL ASPECTS

1.1. Failure to detect intragenic mutations

A first step in exome analysis is capture of the exons of all coding genes [15, 16].

The efficiency at which exons are captured and sequenced is not equally distributed, resulting in regions that are poorly covered, meaning that the number of reads at this locus is too low to allow a reliable calling of variants sequences. For instance, the first exon of many genes is GC-rich, and is often poorly covered. A good quality experiment is 80% of the target region being covered at 20X or higher [17], meaning that on average, 20% of all exons in the target region are not high-quality calls analysed.

Different bioinformatics tools exist that identify variants in the sequences. However, the concordance between these different tools is low [17]. This implies that with any variant calling tool used, true variants will be missed, even though a variant is present in a sufficient number of reads. In particular, calling of indels (small insertions or deletions, with a size below 1kb) is challenging.

WGS is expected to give a more even coverage, since there is no capture nor any PCR amplification step which may introduce a bias [18]. Thus, it has been proposed that WGS yields 5% extra exonic mutations compared to exome [19].

In a trio approach (depending on the stringency of the filter) unequal coverage may result in some calls and thus candidate genes not being taken into consideration, e.g. when a maternally inherited variant is not sufficiently covered in the mother.

1.2. Exome sequencing will miss certain classes of mutations

1.2.1. Structural variations including deletion or duplication CNV's (size >1 kb) cannot be detected reliably by exome sequencing [15]. However, bioinformatics tools are being developed and it is expected that WGS will overcome this limitation. Balanced structural anomalies such as inversions or translocations cannot be detected by WES, but again, WGS offers the opportunity to detect these [20].

1.2.2. Epigenetic mutations

Currently, this category of mutations fall within the group of imprinting disorders. For the vast majority of autosomal genes, expression occurs simultaneously from both alleles. In mammals, however, a small proportion (<1%) of genes are imprinted, meaning that gene expression occurs from only one allele, either maternal or paternal. This is mediated by epigenetic alterations of these genes. Amongst other mechanisms (deletion, point mutations), epigenetic changes, associated with altered methylation profiles, can result in a developmental disorders such as Beckwith-Wiedemann syndrome, Prader-Willi syndrome, or Angelman syndrome. The diagnosis of this class of disorders requires dedicated technologies targeted at analysing the methylation profile of the sequences involved.

1.2.3. Uniparental disomy

Uniparental disomy occurs when both homologous chromosomes are inherited from a single parent, and the other parent's chromosome for that pair is missing. For most chromosomes, this is without consequence, but for a few chromosomes, it can result in a disorder when the chromosome harbours an imprinted gene. Also, when the region harbours a mutation in a gene for an autosomal recessive disorder, uniparental isodisomy (meaning that both chromosomes are identical) will result in a recessive disorder. In a trio analysis, UPD will readily be detected since in the region absence of alleles from one parent will be detected.

1.2.4. Mosaicism

Mosaicism results when a mutation occurs post-zygotically, and is present only in a proportion of cells. The first challenge is to detect a low grade somatic mosaicism, meaning that the variant is only present in a small proportion of cells and thus of the obtained sequences. This requires a much deeper sequencing, many more reads need to be obtained (with increasing cost) [21]. Second, the mutation may only occur in certain tissues, and thus escape detection in white blood cells. This has been observed for certain cortical dysplasias [22] and for Proteus syndrome [23].

1.2.5. Non-coding regions

In some cases with a well-defined genetic condition no mutations can be detected in the coding parts of the gene causing this disorder. It is assumed that mutations in non-coding regulatory sequences can result in the disorder as well. To date, only very few examples exist, probably because it is still challenging to identify such mutations. Examples of mutations in non-coding sequencing resulting in a distinct phenotype include the *SOX9* gene and Robin sequence [24], Cooks syndrome [25], and possibly also CHD [26], the *FOXL2* gene and BPES [27], the *SHH* gene in polydactyly [28], and the IRF6 gene and Van der Woude syndrome [29].

The identification of such variants will require WGS analysis, but functional validation of found variants will be very challenging, and require extensive validation studies.

1.2.6. Tandem repeats

Expansion of tandem repeats are a cause of some monogenic conditions, such as the Fragile-X syndrome, myotonic dystrophy and Huntington's disease. Most of the current sequencing technologies are not well suited to detect long repeats [30].

2. PITFALLS IN INTERPRETATION AND VARIANT FILTERING

Variant classification is one of the main challenges in contemporary genetics, as discussed already in chapter 3. Given the vast amount of very rare genetic variants, it remains often impossible to unambiguously interpret genetic variants. Classification according to certainty has been proposed, ranging from class 1 (certainly not functional) to class 5 (certainly pathogenic) [31]. However, this classification is still associated with much subjectivity. Moreover, it creates novel questions. For instance, which categories should be communicated to the patients, and under which circumstances? Should they be followed clinically or excluded from follow-up? Do we need to report unclassified variants (class 3), for which there is not sufficient evidence to classify them as either likely pathogenic or non-functional?

Also, the knowledge in this field is growing rapidly, with novel genes being identified continuously for different phenotypes. This may leave the clinician with the feeling of always running behind, in the sense that the genetic test offered may not have incorporated the latest findings. Can we expect the genetic laboratory to regularly re-analyse the data in view of novel insights? And if we do, is this practically feasible and economically valid?

Careful correlation of the genetic findings with the patient's phenotype is crucial in correct interpretation. First, patients may carry more than one pathogenic mutation, which may explain why the phenotype wasn't recognized clinically. In different series, patients with two separate *de novo* mutations were detected in 17/317 patients (5.3%) [8], 8/226 (3.5%) [32] and 101/2076 (4.9%) [33]. In one of the patients we studied, a *SALL1* mutation was detected. However, it remains uncertain whether this is the sole explanation of her condition, as her phenotype is atypical and the ID is too severe compared to what is observed in other patients with Townes-Brocks syndrome.

Also, under the hypothesis of a dominant cause of the disorder, variants found in a (supposedly) healthy parent will be discarded in a trio approach. For most severe syndromes, this is acceptable, since it is expected that these conditions occur *de novo*. However, many dominant syndromes exist with variable expressivity or even reduced penetrance. In these circumstances, a parent may be carrier of a pathogenic mutation with only mild or no manifestations. Examples include Noonan syndrome and Holt-Oram syndrome. It is therefore crucial that parents are also assessed clinically and counselled before genetic testing is initiated.

Another possible pitfall when filtering against inherited mutations are inherited mutations in imprinted genes, where the expression depends on the parental origin on which the mutation is located [34].

3. GUIDELINES FOR GENETIC TESTING IN PATIENTS WITH CHD

From a clinical view, the indication for genetic testing can best be evaluated by classification of the patient according to a sporadic or familial occurrence and a syndromic or non-syndromic appearance.

3.1. Syndromic and sporadic

This is the group where genetic testing is most frequently requested.

It is now generally accepted that chromosomal microarray analysis is a first tier test given a high diagnostic yield of 15-20% in clinically unclassified syndromic patients [35-39]. Parental analysis can aid in the interpretation of a variant of unknown significance, but equally important is the message that parental karyotype analysis with FISH is required when a *de novo* duplication or deletion is found. This serves to exclude the small chance (2%) that one of them is carrier of a balanced insertional translocation of the fragment involved [40]. This situation is associated with a 50% recurrence risk, compared to a very low risk when this can be excluded (1% or less, due to possible germline mosaicism).

WES is a second tier test, ideally in a trio design. The diagnostic yield of WES in cases with unknown syndromic CHD in the study by Homsy et al. [41] was 20%. This compares fittingly to other large studies in individuals with a developmental disorder, where the diagnostic yield varied from 16-50% [7, 42-44].

It is likely that in the future, WGS will replace both tests, since it will allow a comprehensive mutation scanning for both CNV's and SNV's.

3.2. Syndromic and familial

In this exceptional situation, microarray followed by WES is indicated as for sporadic syndromic cases. The optimal approach for WES depends on the suspected inheritance

pattern, which may be autosomal recessive or X-linked. However, in exceptional cases, the underlying cause will in fact be autosomal dominant, with gonadal mosaicism in one of the parents. It has been common practice in clinical genetics to counsel parents of a child with a *de novo* dominant disorder for a low (1%) recurrence risk. This has recently been substantiated in genetic family studies, where in a quad approach (two parents and two children) 1% of all *de novo* mutations were shared by the two siblings [45]

3.3. Familial non-syndromic

Four studies have performed screening of a panel of genes in a cohort of familial CHD [46-49] (Table 1).

Study	N° genes	N° families	Selection	N° solved	Solved (%)
Blue 2014	57	16	2 or more	5	31%
Jia 2015	57	13	3 or more	6	46%
El Malti 2016	4	154	2 or more	16	10%
LaHaye 2016	WES	9	2 or more	3	33%

Table 1. Comparison of 4 different studies in familial CHD with columns showing number of genes analysed, number of families included, selection of family based on number of individuals per family affected, number of families solved in total study and percentage of families solved in total study.

Taken together, these results show that the chance of identifying the causal gene increases in large families. In most instances, the candidate gene corresponded to known genotype-phenotype associations e.g. *NOTCH1* in left outflow tract obstruction, *TBX5* in atrial septal defects type II, *NKX2.5* in septal defects, *MYH6* in ASD II, and *ELN* in supravalvular stenosis of

peripheral pulmonary stenosis. These results indicate that testing a panel of known CHD genes is feasible in large CHD families with 3 or more affected individuals. However, as for many autosomal dominant disorders, genetic counselling is complicated by variable expressivity and reduced penetrance.

3.4. Sporadic non-syndromic

The majority of CHD patients occur sporadically and are non-syndromic. In this group, the contribution of genetic testing is limited.

Several studies have shown an increased burden of rare CNV's in cohorts of NS-CHD patients compared to the control population, on average about two fold increase [50]. Other studies reported an increased *de novo* incidence of CNV in 3.9%-15.4% of cases [51]. It is difficult to translate these findings to the clinic for a number of reasons. First, many studies did not include appropriate controls, but on average, a *de novo* CNV is found in 2% of the population. Second, different studies are difficult to compare, because of the inclusion of different types of CHD or different cut-off values for the size of a CNV (ranging from >100 kb to > 500 kb). Third, one of the complicating factors of such large cohort studies is that, in retrospect, many of the cases with a CNV were found to be in fact syndromic. Finally, given the occurrence or rare or *de novo* CNV's in the normal population, the finding of a novel CNV in an individual with a NS-CHD does not implicate causality. With the exception of known pathogenic CNV's, for most novel CNV's it will not be possible to unambiguously assign causality [52].

Before the advent of NGS, several studies have evaluated the incidence of mutations in single candidate genes in large cohorts of sporadic patients. Examples include *NKX2-5, GATA4,* and *TBX20*. The overall conclusion is in line with the results from more recent trio exome studies in NS patients that *de novo* mutations are in fact very rare in sporadic NS-CHD. Homsy et al [41] reported a low incidence of excess *de novo* PTV in 2% in NS-CHD cases (compared to 20% of cases in S-CHD). Likewise, Sifrim et al. [11] also found a small excess of *de novo* protein truncating in CHD genes and of missense mutations in CHD genes and non-DD genes.
As for CNV's, also an excess of inherited rare SNV's (minor allele frequency <1%) was observed in known CHD genes in NS-CHD, but not in S-CHD. This indicates that a small proportion of sporadic NS-CHD has an oligogenic cause, due to variants with a moderate effect and thus non-penetrance in one of the parents. In NS-CHD, because of reduced penetrance and variable

expressivity, patients can inherit mutations from an unaffected or mildly affected parent. Of interest, again an excess of inherited rare variants was observed in the genes not associated with CHD or DD, indicating that additional CHD genes remain to be identified. However, despite the statistically significant findings, the questions remains whether this is of clinical diagnostic interest as it only concerns a small proportion of cases (2% in the study of Homsy et al.) [41]. This proportion further decreases when considering that in the study of Sifrim et al. one case had an inherited mutation in a gene associated with a recognisable cardiac phenotype (e.g. the *ELN* gene) and more than half had a mutation in a gene that typically causes a syndromic CHD, be it with variable expressivity: *SOS1* and Noonan syndrome, *FBN2* and contractural arachnodactyly, *SALL4* and Townes-Brocks syndrome, as well as *COL1A1* and osteogenesis imperfecta. Therefore, from a diagnostic point of view, trio exome analysis in sporadic and apparently non-syndromic cases is unlikely to change the currently used empiric recurrence risks in the majority of cases, with the exception of a few very distinct types of CHD (*ELN* and SVAS/PPS, *NKX2.5* and ASD2 with conduction disturbances).

Thus, genetic testing in the majority of sporadic cases does not reveal a mutation, which is compatible with the generally accepted idea that sporadic NS-CHD has a multifactorial etiology.

CLASSIFICATION OF CHD

The distinction between these different categories is not always strict. First, certain CNV's can present as either of these four categories. There are CNV's that are associated with an increased risk for various medical, developmental and psychiatric manifestations, with variable expressivity and reduced penetrance. These CNV's are initially detected in an affected child, but can be inherited from a clinically unaffected parent. The classical example in cardiology is the duplication in chromosome 1q21.1. This CNV is associated with Tetralogy of Fallot, and found in almost 1% of all ToF cases [51, 53]. Moreover, there is a risk for borderline intelligence, gross and fine motor disturbance, Autism Spectrum Disorder and increased prevalence of macrocephaly. Thus, the presentation can be either isolated or syndromic [54]. In addition, more than 80% of 1q21.1 duplications are inherited, from a parent with variable manifestations [55]. It can thus occur sporadically or familial. Other CNV's that are associated to CHD include the 22q11.2 duplication syndrome [56, 57] and possibly the del15q11.2.

Counselling for such CNV's is especially challenging with regard to recurrence risk of manifestations in a sibling or when detected antenatally. These are variants with a moderate effect, and are situated in a spectrum with monogenic and multifactorial at the ends. It is likely that in the future, WES and WGS will identify other genes that are susceptibility loci for CHD, either isolated or syndromic. These will present the same challenges in interpretation and counselling.

Second, mutations in certain genes can be associated to either syndromic or non-syndromic CHD. Obviously, this classification depends on a very careful and expert clinical evaluation. For instance, in a large series of so-called non-syndromic CHD, mutations are found in several syndromic genes [11, 58]. However, currently it is generally accepted that mutations in *TBX5* and *JAG1* can be associated with either an isolated CHD as well as a syndromic [48, 59].

4. ECONOMIC ASPECTS OF NEXT GENERATION SEQUENCING

Finally, translation of this novel NGS technology into the clinic still comes at a high financial cost. Developmental disorders caused by *de novo* mutations have an estimated prevalence of 1/213 to 1/448 live births. Globally this would equate to almost 400,000 children being born per year with developmental disorders. For Belgium, with an annual birth rate of approximately 120.000, this means 270-560 children per year [60].

Previous studies have indicated that first tier testing using microarray analysis is cost effective for individuals with unexplained ID [61]. For NGS, several studies suggest cost-effectiveness of WES performed at an early stage of the diagnostic odyssey in developmental disorders [62-66], but other studies state that it is too early to say whether NGS technology offers value for money [67].

Diagnostic testing including genetic testing are indicated when clinical utility is obvious: the results needs to be actionable, e.g. with regard to reproductive counselling, improved treatment options or prediction of outcome). However, clinical utility is difficult to define. In the field of clinical genetics, there is a strong tradition of believe in personal utility, i.e. the value of information about the cause of the disorder for the patient and, his family [68, 69].

NGS is a great leap in accelerating our knowledge on genetics of CHD. This is already applied in research and diagnostic settings to the benefit of patients and their families. However, many limitations still exist. The next challenge is resolving the remaining gaps in our knowledge, and WGS will be the first next step in current and future studies.

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LIST OF ABBREVIATIONS

AD	Autosomal Dominant
AR	Autosomal Recessive
Array-CGH	Array Comparative Genomic Hybridization
CH	Compound Heterozygous
CHD	Congenital Heart Defects
CM	Cardiomyopathy
CNV	Copy Number Variation
DNM	De Novo Mutation
FISH	Fluorescence In Situ Hybridization
FS	Fractional Shortening
ID	Intellectual Disability
IUGR	Intra Uterine Growth Retardation
LV	Left Ventricle
LINE	Long Interspersed Nuclear Elements
MLPA	Multiplex Ligation-dependent Probe Amplification
MO	Morpholino Oligonucleotides
NGS	Next-Generation Sequencing
RCM	Restrictive Cardiomyopathy
RV	Right Ventricle
SINE	Short Interspersed Nuclear Elements
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing

CURRICULUM VITAE

Full names	Jacoba Johanna Louw
Date of Birth	9 September 1979
Nationality	South African, Belgian
Civil status	Married, spouse Jo Goethals
Children	Isabelle Goethals, Arne Goethals
Language	Afrikaans - Native Speaker English - Native Speaker Flemish - Fluent French - Advanced

QUALIFICATIONS AND TERTIARY EDUCATION

MBChB, MD	27 November 2003, University of Pretoria
Pediatrician	7 June 2010, Katholieke Universiteit Leuven (KUL), UZ Leuven
Pediatric Cardiologist	31 July 2014, UZ Leuven

EDUCATION

Primary School	Laerskool Pietersburg Oos
Grade 1-Grade 7	One of "Top Ten" pupils based on Academic Achievements
	Numerous subject-prizes
	Prestige colours in Academics, Choir, Culture and Athletics
	Prefect in final year (Grade 7)
High School	Hoërskool Pietersburg (PHS)
	Grade 12: 6 subjects, 6 distinctions cum laude
	Grade 8 – Grade 12: One of "Top Ten" pupils based on Academic Achievements.
	Best Academic Achievement in the following subjects: German, English, and Accountancy
	Prestige colours in Academics, Choir, Culture and Drama
	Excelled in Hockey, Cross-country running and Athletics

LIST OF PUBLICATIONS

First author

SERUM PROCALCITONIN IS NOT AN EARLY MARKER OF PULMONARY EXACERBATION IN CHILDREN WITH CYSTIC FIBROSIS
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Submitted for publication, 2017

Co-author

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Bjorn Cools, Stephen C. Brown, Jacoba Louw, Ruth Heying, Bart Meyns, Marc Gewillig
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Oral Presentations

March 2011: 39ste Jaarlijks Congres van de Belgische Vereniging voor Kindergeneeskunde
ACUTE RENAL FAILURE EARLY AFTER CARDIAC SURGERY WITH EXTRACORPOREAL CIRCULATION: LONG TERM OUTCOME?
Louw J.J., Mekahli D., Lombaerts R., Levtchenko E., Eyskens B., Heying R., Cools B., Gewillig M.

May 2011: 45th Annual Meeting of the Association of European Paediatric Cardiology
ACUTE NEONATAL HYPERTENSIVE CRISIS WITH CIRCULATORY FAILURE:
BE AWARE OF THE WOLF IN DISGUISE
Louw J. J., Thewissen L., Smits A., Eyskens B., Heying R., Cools B. , Levtchenko E., Allegaert K., Gewillig M.

December 2011: Fetal and Pediatric Cardiology Seminar: 9-10 December 2011, Paris, France
PREMATURE CLOSURE AND CONSTRICTION OF THE ARTERIAL DUCT
Louw, J J, Gewillig M.

September 2014: 25th European Dysmorphology Meeting, 10-12 September 2014, Strasbourg, France
SOLVING UNCOMMON LETHAL CARDIOMYOPATHIES IN SMALL FAMILIES
Louw, J J, Devriendt K.

6 March 2015: Belgian Society of Human Genetics, Gosselies, Belgium
EXOME SEQUENCING AND LINKAGE ANALYSIS AS TOOLS IN SOLVING AN UNCOMMON CARDIOMYOPATHY IN A SMALL FAMILY.
Jacoba Louw, Celine Verdood, Anniek Corveleyn, Yaojuan Jia, Marc Gewillig, Hilde Peeters, Koen Devriendt

12 March 2015: Human and Developmental Genetics Meeting, Leuven, Belgium
SOLVING UNCOMMON LETHAL CARDIOMYOPATHIES IN SMALL FAMILIES
Jacoba Louw, Celine Verdood, Anniek Corveleyn, Yaojuan Jia, Derize Boshoff, Marc Gewillig, Hilde Peeters, Koen Devriendt

9 March 2016: Human and Developmental Genetics Meeting, Leuven, Belgium
ANALYSIS OF WES DATA IN FAMILIAL SYNDROMIC CARDIOMYOPATHIES
Jacoba Louw

22 June 2016: Research PhD seminar, Leuven, Belgium
GENETICS OF CONGENITAL HEART DEFECTS: IDENTIFICATION THROUGH NGS
Jacoba Louw

March 2017: 51st Annual Meeting of the Association of European Paediatric Cardiology, Lyon, France
A NOVEL COMPOUND HETEROZYGOUS LOSS-OF-FUNCTION MUTATION IN KIF20A IS ASSOCIATED WITH A RARE, LETHAL CARDIOMYOPATHY IN TWO SIBLINGS
Louw J.J., Verdood C., Nunes Bastos R., Chen X., Corveleyn A., Jia Y., Breckpot J., Gewillig M., Peeters H., Santoro M., Barr F., Devriendt K.

USING A ZEBRAFISH MODEL TO QUANTIFY A CARDIOMYOPATHY PHENOTYPE IN A CAUSAL NOVEL GENE
Louw J.J., Verdood C., Nunes Bastos R., Chen X., Corveleyn A., Jia Y., Breckpot J., Gewillig M., Peeters H., Santoro M., Barr F., Devriendt K.

Co-author
May 2012: 46th Annual Meeting AEPC, Istanbul 2012.
SELF-EXPANDING STENT SINUS SUPERFLEX VISUAL TO CREATE AN UNRESTRICTIVE ATRIAL COMMUNICATION IN INFANTS.
Cools B., Louw J., Heying R., Boshoff D., Eyskens B., Gewillig M.

May 2012: 46th Annual Meeting AEPC, Istanbul 2012.
OFF-LABEL USE OF PERCUTANEOUS PULMONARY VALVED STENTS IN THE RVOT.
Boshoff D, Cools B., Heying R., Troost E., Budts W., Louw J., Eyskens B., Gewillig M.

March 2014: 48th Annual Meeting AEPC, Helsinki 2014.
THE MELODY VALVED STENT IS MORE VULNERABLE FOR ENDOCARDITIS THAN HOMOGRAFTS OR CONTEGRA CONDUITS IN RVOT.
Van Dijck I., Budts W., Eyskens B., Cools B., Heying R., Louw J., Boshoff D., Frerich S., Vanagt W., Troost E., Meyns B., Rega F., Gewillig M.

Posters First author
October 2009: NACFC (North American Cystic Fibrosis Congress) in Minneapolis
SERUM PROCALCITONIN IN YOUNG CF SUBJECTS: VALUES AT BASELINE AND AT ONSET OF RESPIRATORY EXACERBATION
Louw, J. J. Toelen J., de Boeck K., Proesmans M., Vermeulen F., Billen J.

May 2011: 45th Annual Meeting of the Association of European Paediatric Cardiology in Granada, Spain
ACUTE RENAL FAILURE EARLY AFTER CARDIAC SURGERY WITH EXTRACORPOREAL CIRCULATION: LONG TERM OUTCOME?
Louw J.J., Mekahli D., Lombaerts R., Levtchenko E., Eyskens B., Heying R., Cools B., Gewillig M.

September 2011: 44th Annual Scientific Meeting of the Association of European Paediatric Nephrology in Dubrovnik, Croatia
ACUTE RENAL FAILURE EARLY AFTER CARDIAC SURGERY WITH EXTRACORPOREAL CIRCULATION: LONG TERM OUTCOME?
Louw J.J., Mekahli D., Lombaerts R., Levtchenko E., Eyskens B., Heying R., Cools B., Gewillig M.

May 2012: 46th Annual Meeting AEPC, Istanbul 2012
EXOME SEQUENCING IN SYNDROMIC PATIENTS WITH CONGENITAL HEART DISEASE PERFORMING TRIO ANALYSIS.
Louw J, Jia Y., Corveleyn A, Gewillig M, Devriendt K

March 2014: 48th Annual Meeting AEPC, Helsinki 2014
HOMOZYGOUS LOSS-OF-FUNCTION MUTATION CAUSES THE LETHAL DISORDER MITOGENIC CARDIOMYOPATHY IN TWO SIBLINGS
Louw J.J., Corveleyn A ., Jia Y., Iqbal S., Boshoff D., Gewillig M., Peeters H., Moerman P., Devriendt K.

March 2014: 48th Annual Meeting AEPC, Helsinki 2014
TRIO ANALYSIS USING NEXT GENERATION SEQUENCING TECHNOLOGY TO IDENTIFY DE NOVO MUTATIONS IN INDIVIDUALS WITH SYNDROMIC CARDIOPATHY
Louw, J.J., Corveleyn A., Jia Y., Gewillig M., Devriendt K.

February 2016: Human and Developmental Genetics Meeting, Leuven, Belgium
EXOME SEQUENCING AND LINKAGE ANALYSIS AS TOOLS IN SYNDROMIC CARDIOPATHIES IN SMALL FAMILIES
J. J. Louw, A. Corveleyn, J. Breckpot, N. Cosemans, H. Masset, M. Gewillig, J. Vermeesch, H. Peeters & K. Devriendt

June 2016: 50th Annual Meeting of AEPC, Rome, Italy
EXOME SEQUENCING AND LINKAGE ANALYSIS AS TOOLS IN SYNDROMIC CARDIOPATHIES IN SMALL FAMILIES
J. J. Louw, A. Corveleyn, J. Breckpot, N. Cosemans, H. Masset, M. Gewillig, J. Vermeesch, H. Peeters & K. Devriendt

March 2017: 51st Annual Meeting of the Association of European Paediatric Cardiology, Lyon, France
SOLVING SYNDROMIC CONGENITAL HEART DEFECTS USING WES IN A TRIO OR INDEX-ONLY APPROACH
Louw J.J., Corveleyn A., Jia Y., Breckpot J., Gewillig M., Devriendt K.

July 2017: 7th World Congress of Pediatric Cardiology and Cardiac Surgery (WCPCCS), Barcelona, Spain
COMPOUND HETEROZYGOUS LOSS-OF-FUNCTION MUTATIONS IN KIF20A ARE ASSOCIATED WITH A NOVEL LETHAL CONGENITAL CARDIOMYOPATHY IN TWO SIBLINGS
Louw J.J., Verdood C., Nunes Bastos R., Chen X., Corveleyn A., Jia Y., Breckpot J., Gewillig M., Peeters H., Santoro M., Barr F., Devriendt K.

Posters Co-author

March 2011: 39ste Jaarlijks Congres van de Belgische Vereniging voor Kindergeneeskunde
Sutureless anastomosis with covered stents during hybrid surgery for pulmonary arteries
B. Cools, J. Louw, W. Vanagt, B. Eyskens, R. Heying, D. Boshoff, M. Gewillig

Double wire/balloon, single stent delivery technique broadens interventional possibilities.
R. Heying, D. E. Boshoff, B. Cools, J.J. Louw, W. Vanagt, B. Eyskens, W. Budts, M. Gewillig

Unusual Late presentation of Giant Coronary Aneurysm and Arthritis in Kawasaki disease
L. Wouters, C. Wouters, B. Cools, J. Louw, M. Gewillig

Dilatable Banding of the PA as An «open end » Palliation for Right Systemic Ventricle
B. Cools, B. Eyskens, R. Heying, J. Louw, W. Vanagt, F. Rega, B. Meyns, M. Gewillig

Can a volume challenge pinpoint the limiting factor in a Fontan circulation?
W. De Mey, B. Cools, R. Heying, W. Budts, J. Louw, D.E. Boshoff, M. Gewillig

Transient Hyperaldosteronism in Severe Neonatal Arterial Hypertension.
Smits, L. Thewissen, J. Louw, M. Gewillig, D. Mekahli, K. Allegaert, E. Levtchenko

May 2012: 46th Annual Meeting AEPC, Istanbul 2012.
Fetal Dysfunction of the Arterial Duct: Clinical Spectrum and Outcome.
Eyskens B., De Catte L., Heying R., Cools B., Brown S., Louw J. Gewillig M.
Cardiology in the Young May 2012; 22; 1; S 78 (P-69)

Interventional closure of perimembranous ventricular septal defects: Experience using the Amplatzer Duct Occluder II.
Heying R., Cools B., Boshoff D., Eyskens B., Louw J., Gewillig M.
Cardiology in the Young May 2012; 22; 1; S 162 (P-298).

Treatment strategies for Pulmonary Sequestration in Childhood: resection, embolization, observation?
Brown S.C., De Laat M., Louw J.J., Proesmans M., De Boeck C, Van Raemdonck D, Heying R, Cools B, Eyskens B, Gewillig M.
Cardiology in the Young May 2012; 22; 1; S 112 (P-161).

March 2014: 48th Annual Meeting AEPC, Helsinki 2014.
Hybrid procedure to postpone successful biventricular repair in left heart obstructive lesions with borderline left ventricle.
Eyskens B., Cools B., Heying R., I. Denolf I., Louw J., Rega F., Meyns B., Gewillig M.

Follow-up after Melody revalvulation: "aggressive" prestenting has nearly abolished stent fractures; endocarditis is a concern.
Cools B., Boshoff D., Heying R., Jansen K., Louw J., Eyskens B, Frerich S, Troost E, Budts W, Gewillig M.

CONTINUING MEDICAL EDUCATION

- Basic Life Support (BLS) practical and theory course: 98%
- Advanced Trauma Life Support (ATLS®) practical and theory course: 83%
- European Paediatric Life Support (EPLS®) : 8-10 February 2010: passed with distinction
- European Echocardiography Course on Congenital Heart Disease: 6 -10 October 2010, London, UK
- Univentricular Heart – an ongoing challenge: 22-23 January 2011, Berlin
- The Children's Hospital of Philadelphia15th Annual Update on Pediatric and Congenital Cardiovascular Disease: 2-6 February 2011, Scottsdale, Arizona
- National Heart and Lung Institute, Imperial College London Hands-on Cardiac Morphology Course: 28th February – 2nd March 2011, London, UK
- 45th Annual Meeting of the Association of European Paediatric Cardiology: 18-21 May 2011, Granada, Spain
- European Society of Human Genetics Conference: 28-31 May 2011, Amsterdam, Netherlands
- Fetal and Pediatric Cardiology Seminar: 9-10 December 2011, Paris, France
- Training in Echocardiography Department: 6-17 February 2012, Great Ormond Street Hospital; London, United Kingdom
- Cardiology in the Young: 2-4 April 2012, Great Ormond Street Hospital; London, United Kingdom
- 46th Annual Meeting of the Association of European Paediatric Cardiology: 23-26 May 2012, Istanbul
- AEPC Arrhythmia Course, 19-20 April 2013, Amsterdam
- 47th Annual Meeting of the Association of European Paediatric Cardiology: 22-25 May 2013, London
- 5th Junior Training Course in Cardiac Catheterization and Interventions: 13-15 March 2014, Linz, Austria
- 25th European Dysmorphology Meeting, 10-12 September 2014, Strasbourg, France
- Pedirhythm 6: Pediatric and Congenital Rhythm Congress, 17-20 September 2014, Istanbul, Turkey
- Belgian Society Of Human Genetics Congress, 6 March 2015, Gosselies, Belgium
- Cardiogenetica symposium, 2015, Leuven, Belgium
- CEH Symposium "(R)Evolutie in de Cardiogentica", 7 March 2015, Leuven, Belgium
- Echocardiography course 25-26 September 2015, Aalst, Belgium
- Belgian Society Of Human Genetics Congress, 6 March 2016, Leuven, Belgium
- Cardiology in the Young, 21-23 March 2016, London, United Kingdom
- 50th Annual Meeting of the Association of European Paediatric Cardiology: 1-4 June 2016, Rome

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en tog nie die kos kan gaarkry.

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- C.J. Langenhoven, *Aan stille waters*, 11 Julie 1932