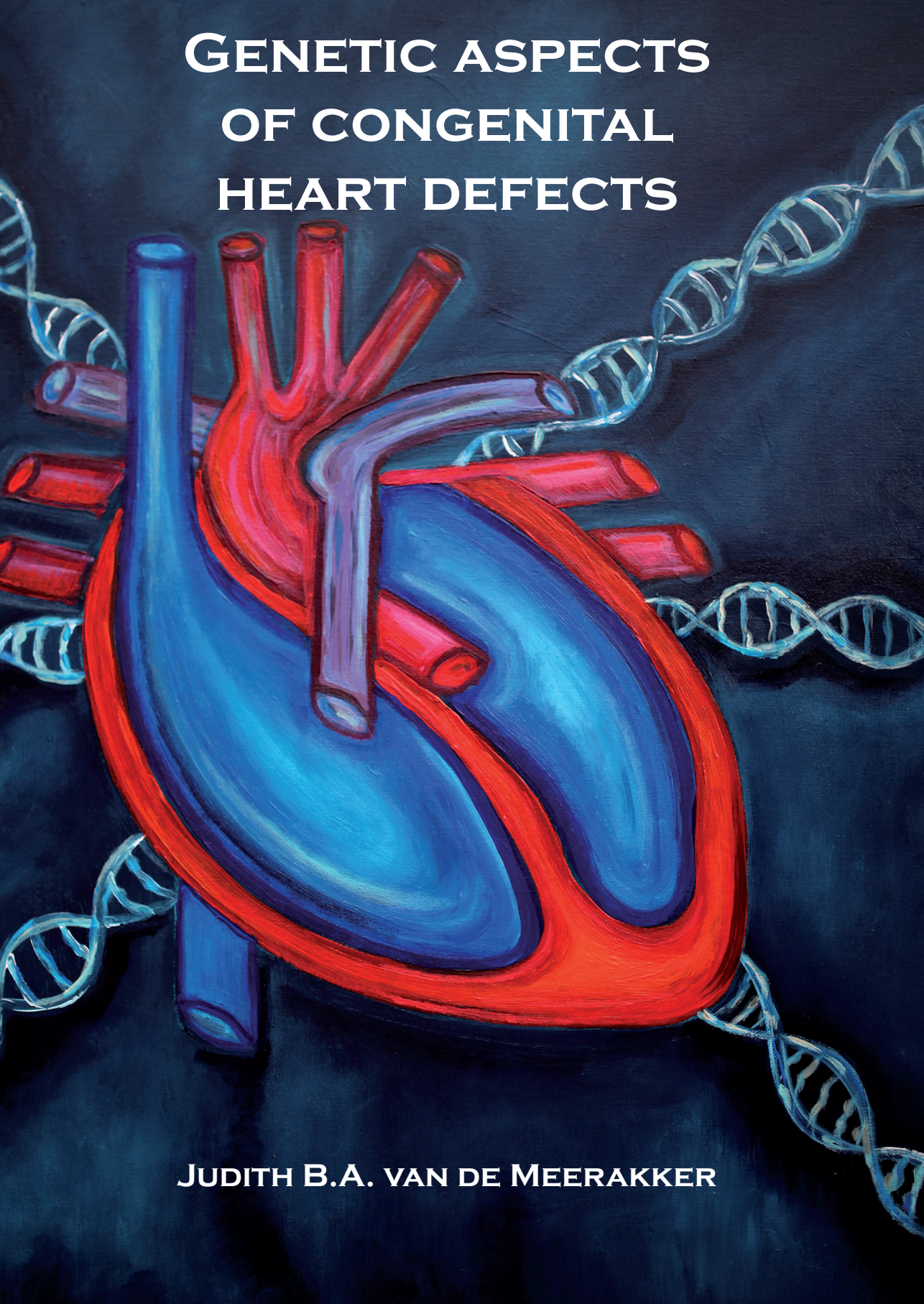
Genetic aspects of congenital heart defects



Judith B.A. van de Meerakker

Genetic Aspects of Congenital Heart Defects

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Genetic Aspects of Congenital Heart Defects

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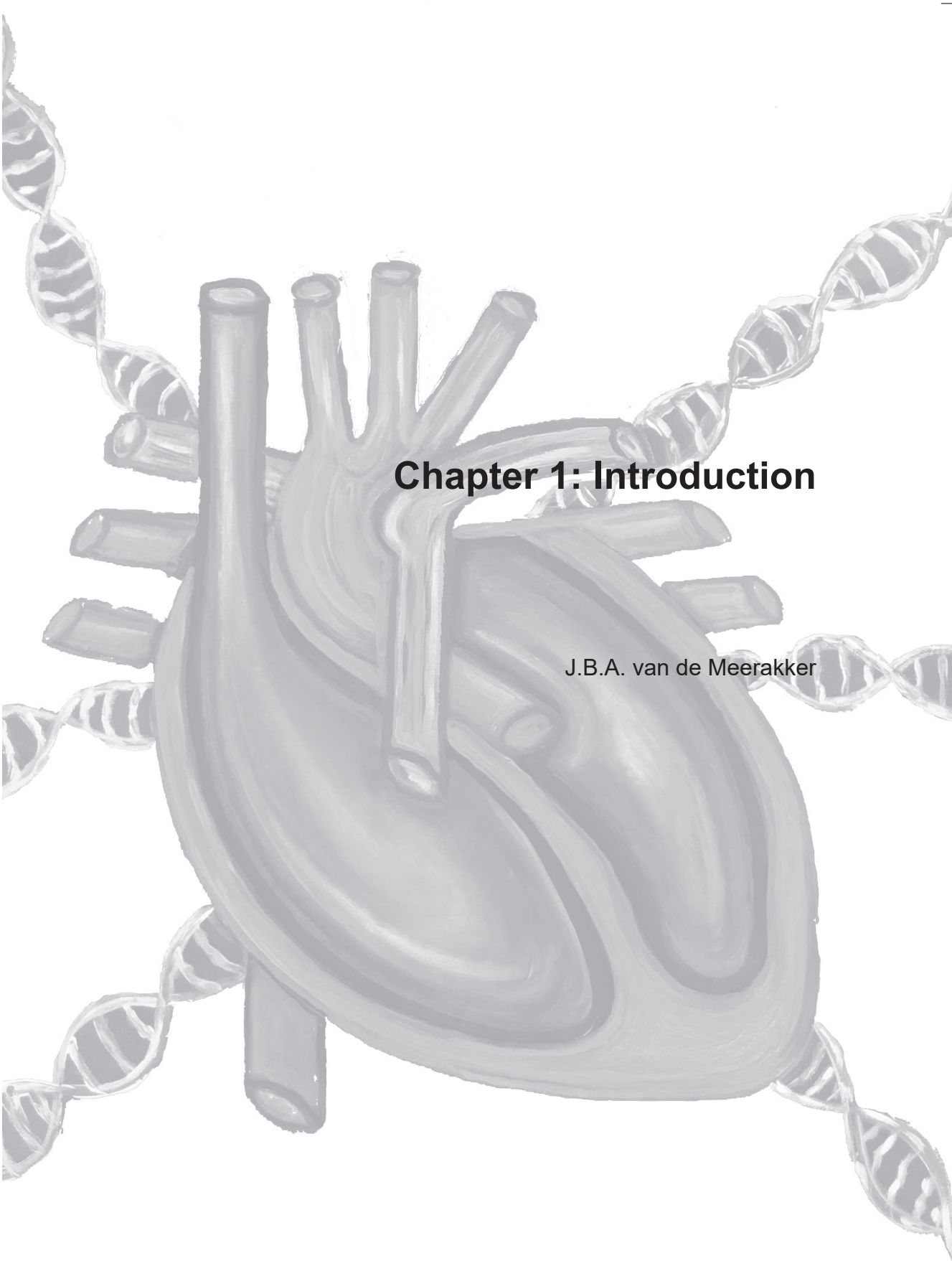

Chapter 1: Introduction

J.B.A. van de Meerakker

1

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List of Abbreviations

AS	aortic stenosis
AV	atrioventricular
AVC	atrioventricular canal
AVCD	atrioventricular canal defect
ASD	atrial septal defect
AVSD	atrioventricular septal defect
BAV	bicuspid aortic valve
CoA	coarctation of the aorta
CHD	congenital heart disease
CNV	copy number variation
DCM	dilated cardiomyopathy
DILV	double inlet left ventricle
DOV	double-outlet ventricle
DORV	double outlet right ventricle
EA	Ebstein anomaly
ECM	extracellular matrix
EMT	epithelia to mesenchymal transition
FHF	first heart field
HCM	hypertrophic cardiomyopathy
HRF	heart forming field
LVNC	left ventricular non-compaction
OFT	outflow tract
NCCM	Non-compaction cardiomyopathy
PDA	patent ductus arteriosus
PFO	patent foramen ovale
PS	pulmonic stenosis
PVS	pulmonary valve stenosis
SHF	second heart field
TA	truncus arteriosus
TGA	transposition of the great arteries
TOF	tetralogy of Fallot
VSD	ventricular septal defect

1. Heart Development

The cardiovascular system is the first system to develop and function in the vertebrate embryo. In humans, its formation starts in the middle of the third week of gestation. At this time point nutritional requirements cannot be satisfied by diffusion alone anymore. Numerous cellular activities such as cell growth, cell proliferation and apoptosis, are needed to form a fully developed four-chambered heart, consisting of ventricular and atrial chambers with inflow (venous) and outflow (arterial) regions and valves for a unidirectional blood flow. The development of the heart is highly conserved throughout evolution and follows the same general pattern in all vertebrates.[1-4]

Development of the heart can be subdivided into several distinct phases that partially overlap, i.e. formation of the linear heart tube, cardiac looping, chamber formation, septation, formation of the cardiac valves and maturation of the heart (see figure 1).

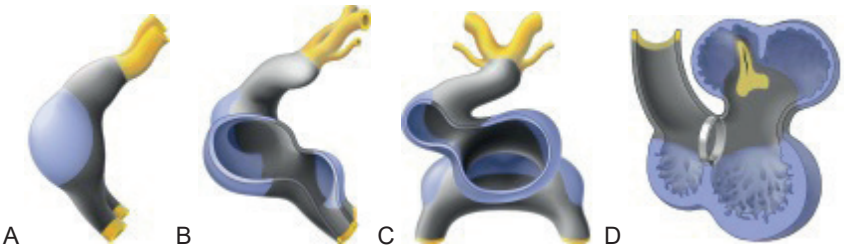

Figure 1. Development of the heart
A. linear heart tube, B looping of the heart tube C. formation of the cardiac chambers, D formation of the cardiac septa
(courtesy of the department of Anatomy, Embryology & Physiology)

This chapter discusses these phases, the pathophysiology of congenital heart disease (CHD) and CHD causative genes. As the studies presented in this thesis focus on structural heart defects, the development of the cardiac conduction system will not be discussed. An overview can be found in Bakker et al.[5]

1.1 Formation of the heart tube

The development of the heart starts with the formation of the heart tube. Subsequent to gastrulation, the embryo is a disk, consisting of three germinal layers, endoderm, facing the yolk sac, ectoderm, facing the amniotic cavity and mesoderm in between. The mesoderm will give rise to the cardiovascular system, among other things. Cardiac specification and differentiation takes place by a combination of positive, intracellular signalling events, such as bone morphogenetic proteins (BMPs) and fibroblast

growth factors (FGFs), and negative ones such as the β-catenin/WNT signaling.[6-8] The splanchnic mesoderm gives rise to two heart-forming regions (HFRs) on both sides of the midline,[9] which form the heart tube during folding of the embryo. The lateral limits of the HFRs fuse at the ventral midline of the embryo forming the linear heart tube, while the medial parts of the HFRs remain to be added to the forming heart tube at those sites where the heart tube is attached to the pericardial back wall. Together with the formation of the linear heart tube, the head of the embryo folds, positioning the heart in what will become the thoracic region. The tube is now positioned centrally in the embryo and has a bilaterally symmetrical inverted Y-shape.[3] The stem of the Y itself is the ventricular component of the tube connected to the body at the arterial pole, whereas the arms form the atrial component to the body at the venous pole (figure 1A). The early heart grows by the gradual addition of cells from the heart-forming region in the dorsal pericardial wall. Two sources of cells contribute to the growing heart.[9, 10] Cells from the so-called first heart field (FHF) produce the early heart tube, and eventually form the left ventricle. The rest of the heart is derived from cells of the second heart field (SHF), which are added at the arterial and venous poles. The epithelial sheet of splanchnic mesodermal cells that first differentiates and forms the primitive heart tube, makes up the first heart field. The adjacent pool of cardiogenic cells, located centrally and dorsally to the primary heart field makes up the secondary heart field, which differentiates at later developmental stages. Although the primary and secondary heart fields share a common origin.[11] The cells are contributed to the developing heart in a temporal- and spatial-specific manner.[9, 12]
SHF cells express the transcription factors islet1 (ISL1), T-box transcription factor-1 (TBX1), and fibroblast growth factors (FGFs) FGF8 and FGF10.[10] ISL1 along with TBX1regulates signalling pathways required to coordinate SHF deployment by regulating proliferation and differentiation of the cardiac precursor cells in the SHF and is thus essential for the elongation of the heart tube and the formation of the outflow tract (OFT).[13-17] TBX1 might also be important for the development of the right ventricle as it develops from the SHF. This is underlined by a recent study showing that a mutation in TBX1 can cause double outlet right ventricle (DORV) and ventricular septal defect (VSD) in humans.[18]

1.2.Looping of the heart tube

In the second stage of cardiac development the heart bends to the rig ht by a process called "cardiac looping", a process associated with the development of laterality of the embryo, in which the nodal signalling pathway plays an important role (figure 1B). Only the ventricular component and part of the atrioventricular has been formed initially.[19, 20] By the continuous addition of cells from the SHF, the heart tube elongates considerably and the initial linear heart tube will bend ventrally and rightwards. The linear heart tube initially is attached to the dorsal wall of the pericardial cavity by

the dorsal mesocardium. It breaks, freeing itself from the dorsal wall during cardiac looping at later stages.[3, 21] The heart tube acquires an S-shape form and remains attached to the coelomic wall at its arterial and venous poles. This sets the stage for the progress of the "looping" process in which the primary heart tube can be divided into atrial and ventricular components along with an outflow tract.[3] The signalling pathways that control the looping process are already established during the stage of gastrulation. Disturbances in this process lead to severe malformations, ranging from double inlet left ventricle (DILV), DORV and improper connections between the different cardiac components

1.3.Formation of the cardiac chambers

Subsequent to cardiac looping the cardiac chambers are formed by regional expansion, differentiation and re-initiation of cell proliferation of the primary heart tube.[22, 23] At the ventral side of the stem of the inverted "Y" the ventricular chambers form and at the dorso-caudal side of the inverted "Y" the atrial chambers (figure 1C). Proliferation rate is high in the forming working myocardium at the outer ventricular and atrial curvatures, but remains low in the primary myocardium of the OFT, the inner curvature and atrioventricular canal (AVC).[24] These proliferative differences are regulated by complex overlapping patterns of T-box containing transcription factors.[25]

1.3.1 Formation of the atria

The primary atrium is formed by dorso-lateral expansion of the caudal inlet part of the cardiac tube during looping.[21] While the right and left ventricle form in series, the left and right atrial chambers form in a symmetric fashion. Therefore, differences between the atria are a reflection of right-left signalling, which is not the case for the ventricles. Consequently, atrial isomerism does occur, but ventricular isomerism not. Although the left and right atria have different shapes, they have the same basic elements: a venous component, an appendage, and a vestibule, separated by a septum.[26] The appendages balloon out from the primary atrial component of the linear heart tube up to the right and left side of the outflow tract. The venous components of both chambers are then derived from different embryonic sources,[27] with a much larger contribution of the atrial part of the primary heart tube to the left atrium in comparison to the right atrium.[3] These atrial components can also be recognized based on molecular criteria, each having distinct left and right parts:[28] 1) the AVC-derived myocardium, comprising the smooth-walled lower rim of the atrial chambers, 2) the trabeculated atrial appendages, 3) the caval vein myocardium surrounding the caval veins and the smooth-walled myocardium, including the left and the right venous valve leaflets and 4) the mediastinal myocardium, comprising the pulmonary vein myocardium, primary and secondary atrial septa, and smooth-walled myocardium spanning from the point of entry of the pulmonary vein into the left atrium to the left venous valve leaflet.[28] These different domains emerge at different stages during development.

1.3.2 Formation of the ventricles

For a thorough review on the formation of the ventricles see Henderson et al.[29] In short, the ventricles are derived from the ventricular loop at the outer curvature. Subsequent to the bending of the tube the ventricular chambers start to expand concomitant with the formation of the ventricular septum. The region of the primary heart tube on top of the ventricular septum is called the primary interventricular foramen. In fact this is a misnomer, because this region is not interventricular, but forms the outlet of the left ventricle and the inlet of the right ventricle, or right ventricular junction.[3] This remodelling resulting into the proper connections between the different cardiac compartments is achieved during the 6th week of human development. Each ventricle possesses three developmental components: the inlet, the outlet, and the apical component.[29] Each apical part achieves its own inlet and outlet component by changes at the inner curvature,[3] and are formed in series, in contrast to the atria, by ballooning from the primary heart tube. First the apical part of the left ventricle balloons from the inlet of the ventricular loop, already visible before looping, and second the apical part of the definitive right ventricle from the outlet.[3, 29] As mentioned earlier, cells forming the left ventricle are derived from the first heart field and those forming the right ventricle are derived from the second heart field.[10]

1.4.Formation of the cardiac septa

For a thorough overview on the formation of the cardiac septa see Anderson et al.[30] and Sylva et al.[4] In short, septation of the heart occurs in humans between 4 and 7 weeks of development and can be subdivided into the septation of four different compartments, 1) the atria, 2) the ventricles, 3) the primary myocardium of the AVC and primary (or interventricular) foramen, and 4) the outflow tract. Different components contribute to the septation of the heart. The muscular septa divide the atrial and ventricular chambers, other components are also important in the septation of the heart such as the non-muscular endocardial atrioventricular cushions, outflow tract ridges and the dorsal mesenchymal protrusion (DMP), or vestibular spine.

1.4.1 Formation of the septa of the atria

Atrial septation occurs in phases to guarantee right-left atrial shunting of the blood during uterine life. The first sign of atrial septation is the formation of a primary septum. The muscular atrial septum primum grows from the atrial roof towards the AV-canal with a mesenchymal cap (a cushion-like structure) on its leading rim (figure 2A). At the same time two endocardial cushions (superior and inferior) are formed in the initially common atrioventricular (AV) junction (figure 2B). They will divide the AV canal and will eventually fuse with the mesenchymal cap, by which the primary atrial foramen, or "ostium primum", forming the space between the mesenchymal

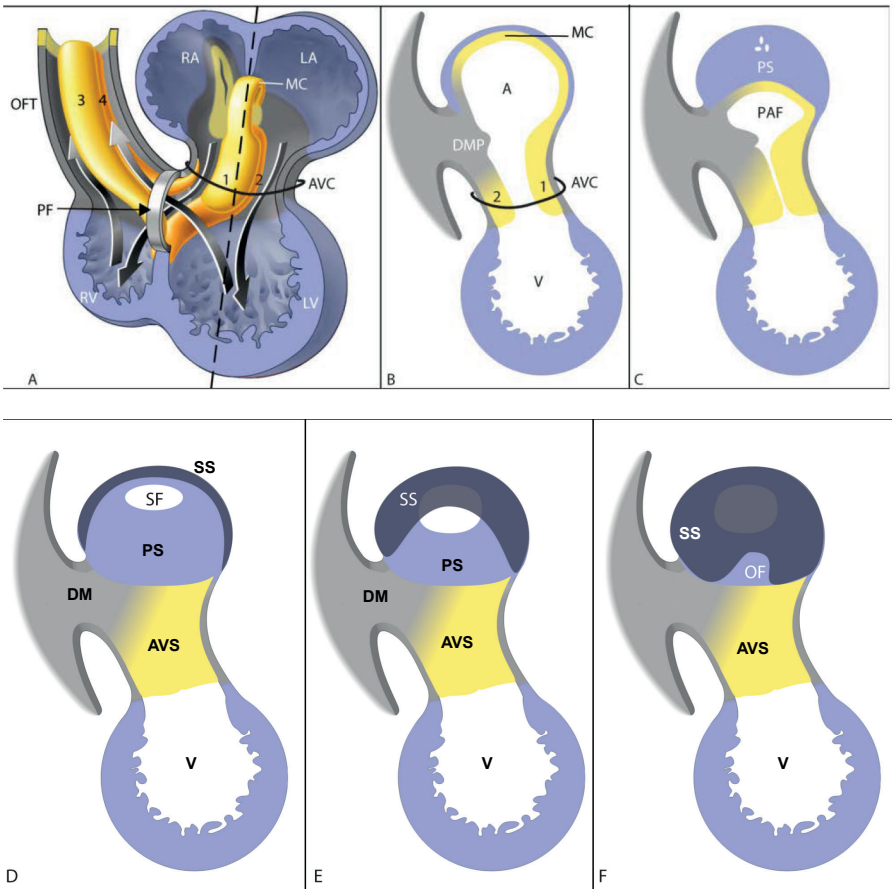

Figure 2. Septation of the atria and primary foramen.4
Abbreviations: 1-2 = ventral and dorsal endocardial cushions, 3-4 = septal and parietal outflow tract ridges, A = atrium, AVC = atrioventricular canal, DMP =dorsal mesenchymal protrusion, LA = left atrium, LV = left ventricle, MC = mesenchymal cap, PAF = primary atrial foramen, PF = primary foramen, PS = primary atrial septum, OF = oval fossa, OFT = outflow tract, , RA = right atrium, RV = right ventricle, SF = secondary foramen, SS = secondary septum, V = ventricle
A: schematic drawing of the chamber-forming heart. B-F: depict sagittal sections at the level of the dotted line in A.
B: the dorsal and ventral endocardial cushions are growing towards each other. C: the PAF is closing due to the in growing of the PS, with its MC, DMP and the endocardial cushions. C-D: in the primary septum small holes appear merging to form the SF. D-F: the SS grows to the right side of the primary septum covering the secondary foramen and the rest of the PAS, leaving at the right surface of the atrial septum only the OF uncovered.
(courtesy of the department of Anatomy, Embryology & Physiology)

cap and the AV-cushions closes (figure 2C). Concomitantly a secondary foramen or "ostium secundum" is formed by the development of small holes in the septum primum, mediated by apoptosis. This guarantees the maintenance of the right-left shunting of blood to bypass the pulmonary circulation. Then a secondary septum is formed by folding of the muscular wall of the right atrium and protrusion into the right atrial cavity, covering the secondary foramen (figure 2D-F). Only the oval fossa of the primitive septum is not covered by the secondary septum. Therefore blood can still flow from the right to the left because in the fetus blood pressure is higher in the right atrium than in the left atrium. This changes after birth closing the secondary foramen by squeezing the septums together, resulting in an isolated systemic and pulmonary circulation.

1.4.2. Formation of the septa of the ventricles

The septum between the ventricles in the formed heart has a myocardial and a membranous component. This last part is the remnant of the fused cushions of the AV-canal and the OFT. The myocardial component forms concomitant with the

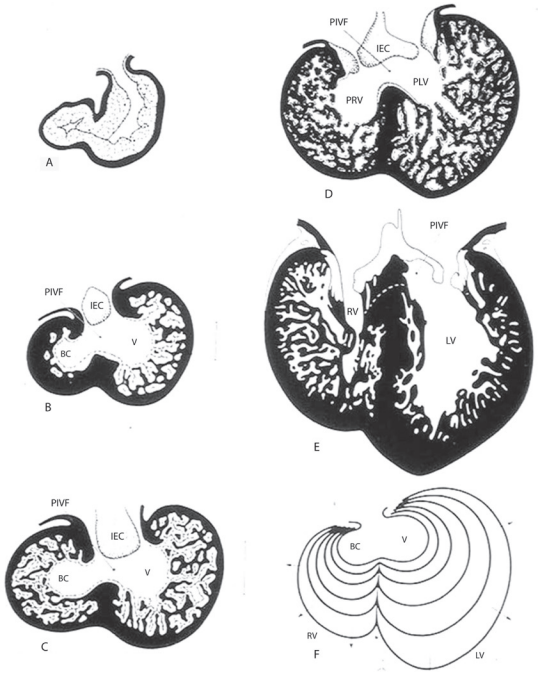

Figure 3[32]. Development of the ventricles
As the right and left ventricle differentiate and expand the muscular ventricular septum becomes visible. The septum is formed by ventricular cells opposed to the primary ventricular foramen

formation of the right and left ventricles by expansion of the ventral side of the primary heart tube. At the position of the so-called primary ventricular foramen the primary heart tube does not balloon out and ventricular cells are opposed to this region forming the muscular ventricular septum (figure 3). Therefore the top of the ventricular septum is the most primitive part and will form the atrioventricular bundle.[31]

1.5.Formation of the cardiac valves

In the last part of this section I want to discuss the formation of the cardiac valves. For more in-depth reviews see de Lange et al.[33], Wessels et al.[34] and Sylva et al.[4].
Heart valves are dynamic structures that open and close during the cardiac cycle to prevent backflow of blood. There are two sets of valves: inlet or AV valves (mitral and tricuspid valves which separate the atria from the ventricles) and outlet valves (aortic and pulmonic semilunar valves that separate the ventricles from the great arteries) (figure 4). Differences between these two sets of valves are: the atrioventricular valves are thin structures composed of endocardium and connective tissue while the semi-lunar valves are flaps of endocardium and connective tissue reinforced by fibers which prevent the valves from turning inside out. The AV valves have supporting chordae tendineae anchored to the walls of the ventricles while the seminlunar valves don't.[35] Moreover, the semilunar valves consist of 3 cusps, while the AV valves consist of two (mitral valve) or three (tricuspid valve) leaflets.[35]
Here I will only discuss the AV valves which, when definitive, consist of the fibrous

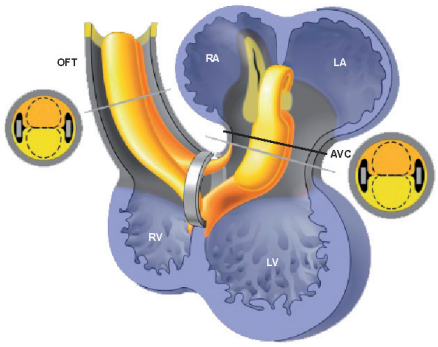

Figure 4. Development of the cardiac valves.
Within the remnants of the primary heart tube (grey) the extracellular matrix (also cardiac jelly) located between the myocardium and endocardium expands forming cushions (yellow). In both the atrioventricular canal (AVC) and outflow tract (OFT) two major cushions are found, the anterior and posterior atrioventricular cushions and the septal and parietal outflow tract cushions. These cushions not only contribute to the mitral, tricuspid and semilunar valves but also to the AV and outlet septum.
(courtesy of the department of Anatomy, Embryology & Physiology)

annulus, the leaflets, and the supporting tension apparatus. The latter made up of the tendinous cords and the papillary muscles.[36]
In the primary heart tube, without valves, blood is propulsed by waves of peristaltic contractions during which the cardiac lumen completely closes, thereby preventing blackflow of blood. This is facilitated by the extracellular matrix (ECM) or "cardiac jelly" between the endocardium and the myocardium.[4] When the chambers are formed this jelly is remodelled into two major cushions in the AV-canal. Later on, also minor cushions are formed, the so-called lateral cushions. The valves develop from both types of cushions, although the major cushions in the AVC are more important for the septation. The cushions are a-cellular at first but become populated with cells form different sources later on. The first cells are derived from the endocardium. Part of the endocardium overlaying the cushions undergoes epithelial to mesenchymal transition (EMT) which turns the epithelial cells into mesenchymal cells. Later on, at the mid-embryonic stage, the lateral cushions are a mix of cells from the endocardium as well as the epicardium, while mesenchyme of the major cushions are still cells predominantly from the endocardium.[34] During formation of the valves and the septum, the major cushions fuse and contribute to the formation of the aortic leaflet of the mitral valve and the septal leaflet of the tricuspid valve.[33] The lateral AV cushions do not fuse with each other or other mesenchymal tissues but contribute to the anterior and posterior (mural) leaflets of the tricuspid valve and the mural leaflet of the mitral valve. Also the cushions detach from the underlying myocardium and become thin valve leaflets which are attached to the papillary muscles with chordae tendineae.[33] The different valve leaflets develop in their own way. The septal leaflet of the tricuspid valve and the aortic leaflet of the mitral valve arise from the fused major cushions.[33] The aortic leaflet of the mitral valve is never supported by the myocardium, it protrudes from the beginning into the left ventricular lumen. Except for the cranial and caudal margins, which form the cords connecting to the papillary muscles.[33] Much later the septal leaflet of the tricuspid valve detaches by delamination of the septal myocardium caused by apoptosis.[33] The mural leaflets of the AV valves (anterior and posterior for the tricuspid valve) arise from the lateral AV cushions.[33] They are detached from the underlying myocardium by excavation or ventricularization: the ventricles grow behind the cushions creating space, liberating the valve leaflets. During subsequent development, apoptosis removes a small amount of remaining cardiomyocytes so the definitive valves do not contain cells from the myocardial lineage. The papillary muscles form the connection between the valves and the trabecular layer of the ventricle and are formed when the myocardium under the leaflets and cords disappears by apoptosis. The last stage of the AV valve is the maturation, the valve leaflets become slender by the remodelling of the ECM and organized three layers.

1.6. Summary of cardiac development

In conclusion, during gastrulation the mesoderm, which in turn gives rise to two heart forming regions (HFRs) from which the cardiac crescent arises. This gives rise to the linear heart tube during folding of the embryo. The linear heart tube grows by addition of cells from outside the heart at both the venous and arterial pole. Then the heart tube loops and chambers are formed by a process called "ballooning". Subsequent septation leads to the formation of the four-chambered heart. This requires several different morphogenetic processes: ballooning of the atria and ventricles and remodelling of the inner heart curvature, including trabeculation of the ventricles, chamber septation, and formation of the valves. Taken together, heart development is an extremely complex process and mistakes in the process leads to congenital heart defects which are detailed in the next section.

2. Congenital Heart Defects

Congenital heart disease is defined as "a gross structural abnormality of the heart or intrathoracic vessels that is actually or potentially of functional significance".[37] Congenital heart defects (CHDs) are the most common developmental errors in humans, affecting about 8 in 1000 newborns[38] and about 10% of stillbirths.[39] Although these rates are assumed to be similar across populations, studies show a variability between continents. This ranges from 1.9/1,000 live births in Africa to 6.9/1,000 in North America, to 8.2/1,000 in Europe to 9.3/1,000 in Asia.[40] The higher rates in Asia could be due to a greater amount of parental consanguinity.[41] CHDs can be divided into three classes of severity according to the frequency at which they should be seen: (1) CHD of great complexity, i.e. double-outlet ventricle (DOV), single ventricle, (2) CHD of moderate severity i.e. atrioventricular canal defect (AVCD), Ebstein anomaly (EA), tetralogy of Fallot (TOF) and (3) the minor forms of CHD which do not need specialized cardiologic care such as tiny ventricular septal defects (VSDs), atrial septal defects (ASDs) or small patent ductus arteriosus (PDAs), which close spontaneously or do not cause medical problems.[42, 43] The birth prevalence of both the severe and moderately severe CHDs are about 1.5 cases per 1.000 live births.[44] Of this prevalence, the general incidence of the various forms of CHDs is as follows: VSD: 30.7%, ASD: 11.6%, atrioventricular septal defect (AVSD): 2.0%, TOF: 7.4%, transposition of the great arteries (TGA): 2.4%, coarctation of the aorta (CoA): 1.4%, aortic stenosis (AS): 2.4% and pulmonic stenosis (PS): 7.5%.[45] Despite dramatic improvement of clinical diagnosis and treatment of CHD, it still causes a considerable portion of paediatric morbidity and mortality.[44] An important factor is the possible development of secondary heart disease later in life, as a result of corrective surgery

during infancy,[46] e.g. the development of heart failure after closure of a septal defect.[43] Fortunately, nowadays more than 75% of CHD children who survive the first year of life, including those with complex malformations, will live into adulthood.[47, 48] The prevalence of CHD in adults is therefore approximately 3/1.000.[49] There are sex differences in the outcomes of CHDs, women are at higher risk for pulmonary hypertension and at lower risk for endocarditis and aortic events and have lower mortality rates.[47, 50-53] While the majority of CHDs occur as sporadic isolated malformations, neurodevelopmental disorders (NDDs) can also emerge which occur in 10% of all children with CHD and in 50% with severe CHD.[54] Possible explanations for the occurrence of NDDs and extra-cardiac congenital anomalies are embryonic circulatory deficits and stresses associated with postnatal therapeutic interventions.[55] There is also a familial clustering of CHDs. Individuals who have a family history of CHDs have a 3- to 80-fold increased risk of CHD themselves compared to individuals without a family history of CHDs. This depends on the kind of heart defect and is higher when similar heart defects are involved compared to discordant heart defects.[56] Also there is a ratio for affected offspring born to affected mothers versus those born to affected fathers of 2.23.[57] In general, CHDs arise from abnormal heart development which is induced either by environmental influences, by an altered gene dosage or function, by stochastic factors or by combinations of these. Human genetic studies have implicated mutations of numerous genes and chromosomal regions in isolated or syndromic CHD some of which will be discussed in section 3.
Clinically CHDs can be roughly divided into three categories, septal defects, cyanotic heart disease and left-sided obstructions. Below I will discuss some of these in detail such as septal defects (ASD, VSD, AVSD) and cyanotic heart disease, (TOF and EA) and various cardiomyopathies (hypertrophic cardiomyopathy, dilated cardiomyopathy and left ventricular non-compaction).

2.1 Septal defects

Failure of atrial or ventricular septation accounts for nearly 50% of congenital cardiac diseases, when bicuspid aortic valve (BAV) is not included.[58] Familial recurrence is reported to exceed 5%.[57] Septation defects can affect septation of the atria (atrial septal defects (ASDs)), septation of the ventricles (ventricular septal defects (VSDs)) or formation of the structures in the central part of the heart (atrioventricular septal defects (AVSDs)). Of the total number of CHDs, 11.6% are ASDs, 30.7% are VSDs and 2% are AVSDs.[45] Cardiac septal defects, if not repaired, can cause pulmonary overcirculation, leading to pulmonary vascular disease, atrial enlargement predisposing to atrial arrhythmias, ventricular dilatation and ultimately decreased life expectancy.

2.1.1.ASD

Atrial septal defects (ASD) [MIM108800] can be divided into ASD type I, ostium primum defect, type II, ostium secundum defect, type III, sinus venosus defect and

sinus coronarius defect. It affects 1/1500 live births of which ~10% are familial.[59] Many patients have a patent foramen ovale with a tiny left-to-right shunt through it, and inclusion of these will inflate the incidence of ASD type I.[44] The incidence in childhood is often underestimated because an ASD is usually asymptomatic.[44] There is a female preponderance among children with ASD, especially for the secundum ASDs of which 65%-75% are females while for the ostium primum and sinus venosus ASDs the gender distribution is equal.[60] Men with an ASDII have a reduced survival rate.[61] ASDII is characterized by a defective or absent interatrial septum which causes oxygen –rich blood to mix with oxygen-poor blood. ASDs are thought to be the result of abnormal resorption of the primary septum, possibly caused by excessive apoptosis.[21] The septal defects display a variable range of phenotypic effects depending on the size of the shunt and may result in pulmonary hypertension, right heart volume overload and premature heart failure.[45, 62] ASDs can occur isolated or as part of a syndrome such as the Holt-Oram syndrome (HOS), caused by mutations in TBX5.[63] Another syndrome with cardiac septal defects and skeletal deformities is the Ellis-van Creveld syndrome,[64] in which the ostium primum variant is present.[60] In patients with isolated ASD, heterozygous mutations in several transcription factors expressed in the heart, such as NKX2-5, TBX5 and GATA4, have been reported.[65-67] Mutations in NKX2-5 were initially found to cause secundum ASD with progressive atrioventricular block.[67] Several families also included persons affected by diverse cardiovascular malformations other than ASDII, such as VSD and TOF.[67] Not only mutations in transcription factors can cause ASD, but also mutations in sarcomeric genes. One such example are families with isolated ASD in which mutations in the α-myosin heavy chain gene (MYH6) are found. Interestingly this cardiac specific gene is under control of TBX5.[68] Furthermore, mutations in other sarcomeric proteins, such as α-cardiac actin (ACTC1) are also found in patients with ASD.[58] Overall, more than 10 genes that cause ASD have been identified and they can be classified into three groups: 1) transcription factors, GATA4/6, TBX5/20, NKX2.5, ZIC3 and CITED2, 2) Ligands-receptors ALK2, CRELD1 and GJA1, 3) Structural proteins such as sarcomeric MYH6, MYH7 and ACTC.[69-72] Besides genetic factors, maternal exposure to environmental risk factors is also noted to cause ASDII.[73, 74] This will be further discussed in chapter 3.5.

2.1.2. VSD

Ventricular septal defects (VSDs) [MIM614429] are, in the isolated form, by far the most common CHD. As about 85%-90% of these defects close spontaneously by one year of age,[75-79] the reported incidence is dependent on the examination of the newborns. VSDs probably arise from abnormalities in cardiomyocyte proliferation and survival,[44] and can also occur as part of a syndrome e.g. HOS, caused by mutations in TBX5.[63] VSD associated mutations have been found in GATA4,[65] NKX2-5,[67] gene promoters of TBX2[80] and TBX18,[81] as well as a deletion in CITED2[82].

2.1.3 AVSD

Atrioventricular septal defect (AVSD) [MIM606215], also known as common atrioventricular (AV) canal or endocardial cushion defect, is a clinically significant heart malformation. It is a spectrum of malformations with deficiencies in the structures that normally separate the atrial- and ventricular-chambers. It is caused by incomplete development of the inferior portion of the atrial septum, the inflow portion of the ventricular septum and atrioventricular valves.[83] Isolated AVSDs occur at a rate of 0.98-1.32 per 10.000 births,[84] and have an increased recurrence rate of CHD among their offspring compared to patients with other types of CHDs,[57] which is around 10%.[60] Overall, AVSD is found most often in children with Down Syndrome (DS, trisomy 21), who account for approximately 65% of all AVSD cases.[85] Therefore the overall incidence varies with the age of the involved mothers, as DS is much more common in mothers >34 years old.[44] However, linkage studies of familial non-syndromic AVSD have not identified a causative interval on chromosome 21, which is shared with DS.[86-88] Nonetheless, studies of subchromosomal duplications of chromosome 21 suggests 21q22.3 as the causative interval for AVSD associated with DS.[89, 90] Other studies of AVSD and copy number variations show an association with 3p- syndrome and recurrent 8p23.1 deletions.[91, 92] Genomic variations in 1p36.6, 5p15.31, 8q22.3 and 17q22 show association with AVSD in DS.[90] Besides DS, AVSD can also be associated with HOS,[93] heterotaxy[94] and the Ellis-van Creveld syndrome[95]. All in all there are multiple AVSD risk loci scattered throughout the genome. On the other hand the gene most frequently associated with AVSD to date is CRELD1. Missense mutations in CRELD1 were originally identified in a small cohort of simplex AVSD cases,[96] with subsequent additional mutations in individuals with DS-associated AVSD.[97] It is estimated that approximately 5% of individuals with nonsyndromic AVSD carry a CRELD1 missense mutation.[98] Missense mutations in GATA4 have also been shown to cause cardiac septal defects in humans, including AVSD in families with autosomal dominant inheritance of CHD.[65, 99] Recently, exome sequencing has been performed in parent-offspring trios and unrelated individuals with AVSD in which rare and private variants in NR2F2 have been found probably contributing to AVSDs.[100]

2.2.Cyanotic heart disease

Infants with cyanotic heart disease appear blue as a result of the mixing of oxygenated and deoxygenated blood. Cyanotic heart disease include tetralogy of Fallot (TOF) and Ebstein's anomaly (EA).[46]

2.2.1 Tetralogy of Fallot

Tetralogy of Fallot (TOF) [MIM187500] consists of an unrestricted large anterior

malalignment subaortic VSD, a right ventricular outflow tract obstruction, a right ventricular hypertrophy and an overriding aorta.[101] Accompanying features can be additional muscular VSDs, AVSDs, anomalous coronary arteries, a right-sides aortic arch and patent ductus arteriosus (PDA).[101] TOF is probably caused by defects in the septation process itself or an altered proliferation, migration and/or differentiation of the progenitor cells of the secondary heart field.[102] It is the most common form of cyanotic CHD and occurs in 3 of every 10 000 live births.[103] TOF can occur in the context of a genetic syndrome as approximately 15% of TOF cases show a deletion of chromosome 22q11.2 (DiGeorge syndrome),[104] while 7% have trisomy 21 (Down syndrome)[105]. The most common large copy number variation (CNVs) after the 22q11.2 deletion is the 1q21.1 duplication.[106] Other syndromes that can include TOF are VACTERL association,[107] CHARGE syndrome[108] and Alagille syndrome, the latter caused by mutations in JAG1[109, 110]. TOF can also be caused by mutations in single genes such as GATA4,[111, 112] NKX2-5,[113] ALDHIA2 (RALDH2),[114] ZFPM2 (FOG2),[115] GATA6,[116] TBX5[117] and GDF1[118].

2.2.2 Ebstein's anomaly

Ebstein anomaly (EA) [MIM224700] is characterized by apical displacement and adherence of the septal and posterior leaflets of the tricuspid valve to the underlying myocardium, thereby displacing the functional tricuspid orifice apically and dividing the right ventricle into two portions.[119] It is rare, affecting about 1-5 in 200.000 live births, so it accounts for <1% of all CHDs.[101] EA can be caused by genetic, environmental as well as reproductive factors.[120] Although the genetic aetiology of EA is largely unknown, rare mutations in the transcription factor gene NKX2.5 have been found in patients with EA,[121] as well as chromosomal abnormalities.[122, 123] EA is more common in patients with a family history of CHD.[120] Accompanying features can be an interatrial communication (secundum atrial septal defect (ASDII) or patent foramen ovale(PFO)), bicuspid aortic valve (BAV), ventricular septal defect (VSD), and pulmonary valve stenosis (PVS).[124, 125] Another associated abnormality can be left ventricular non-compaction (LVNC)[126] and recently mutations in the sarcomeric protein gene β-myosin heavy chain (MYH7) were found in patients with EA and LVNC.[127, 128] In 2007 Budde et al investigated a family with LVNC and/or ASD and/or EA and discovered a mutation in MYH7.[127] This was later repeated by Postma et al in patients with EA and LVNC where mutations in MYH7 were only found in patients with EA as well as LVNC.[128] This article is also a chapter in this thesis.

2.3.Cardiomyopathies

Cardiomyopathies are a heterogeneous group of diseases of the myocardium associated with mechanical and/or electrical dysfunction that usually exhibit inappropriate ventricular hypertrophy or dilatation and often lead to cardiovascular death or

progressive heart failure-related disability.[129] Cardiomyopathies are a major cause of morbidity and mortality in both children and adults and therefore a frequent indication for cardiac transplantation. They occur in isolated forms (primary cardiomyopathy, restricted to the heart) or as part of generalized systemic disorders (secondary cardiomyopathies). Primary cardiomyopathies were recently reclassified into three distinct forms: 1) genetic etiology (e.g. hypertrophic cardiomyopathy (HCM)), 2) mixed genetic/nongenetic etiology (e.g. dilated cardiomyopathy (DCM)) and 3) acquired etiology (e.g. myocarditis).[129] Alterations in over 50 genes have been found as a cause of genetic cardiomyopathies that lack a clear genotype-phenotype correlation.[130-132] The main function of the heart is the generation of contractile force (by the sarcomere) and its transmission to the extracellular matrix (ECM).[133] Mutations have primarily been found in genes encoding sarcomeric proteins, which were first described to be involved in hypertrophic and dilated cardiomyopathies and later also in Non-Compaction CardioMyopathy/Left Ventricular NonCompaction (NCCM/LVNC).[134]
The cardiomyopathies that will be described here are HCM, DCM, and LVNC.

2.3.1. Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) [MIM 192600] is a relatively common disease with a frequency of 1/500 in the general adult population.[135] It is characterised by primarily left ventricular hypertrophy with an unknown cause (resulting from increased cell size or volume), increased systolic function and decreased diastolic function and at the histological level, hypertrophy and disarray of cardiomyocytes and interstitial fibrosis.[136] Although HCM normally has a more benign course than DCM, it can still lead to the heart failure syndrome and lethal arrhythmias,[137] notably, it is the most common cause of sudden cardiac arrest in young athletes.[138] HCM has predominantly a genetic etiology. Alterations are mostly found in genes encoding sarcomeric or desmosomal proteins.[139] Mutations in two genes, MYBPC3-encoded myosin-binding protein C and MYH7-encoded β-myosin heavy chain, account for 40-45% of HCM.[139] Besides mutations in genes encoding for myofilament proteins, mutations have also been described in glycogen storage disease-associated genes,[140, 141] genes encoding Z-disc proteins,[142, 143] and genes encoding calcium handling proteins.[144] HCM can also occur as a part of a syndrome. For instance, in Leopard syndrome up to 80% of the patients with the disorder have HCM.[137]
Since more and more genes are known to be involved in HCM (50+), the use of next generation sequencing in diagnostics is mandatory.[145]

2.3.2. Dilated Cardiomyopathy

Dilated cardiomyopathy (DCM) [MIM 115200] has an estimated prevalence of 1:2500.[146, 147] In contrast to HCM, idiopathic DCM is characterized by ventricular

dilatation and contractile dysfunction in the left and/or right ventricle(s) which leads to a decreased systolic function, in the absence of secondary causes for the observed myocardial abnormality.[129] This usually leads to heart failure and sometimes the development of lethal arrhythmias.[137] The origin of DCM is heterogeneous but the genetic etiology accounts for up to 50% in diverse populations.[146-148] With multiple modes of inheritance, including autosomal dominant (~60%), autosomal recessive (~16%), X-linked (~10%) and mitochondrial (~8%).[149] DCM is mainly caused by mutations in sarcomeric proteins,[150] encoding cytoskeletal and costameric proteins as well as cardiac contractile proteins. But it can also be caused by transcription factors; Z-disc proteins and channel proteins, including NA^{+-}, Ca^{2+-}, and K^{+}-channels.[139] Although more than 30 genes have been reported to be involved, most single genes account only for a small fraction.[129, 139, 151-153] With the exception of titin (TTN). Because the most common genetic cause of DCM are truncating mutations in TTN, occurring in approximately 25% of familial cases and 18% of sporadic cases.[153] Another important gene, especially in combination with progressive conduction system disease is Lamin A/C,[154] encoding a structural protein of the inner nuclear membrane. The same combination of symptoms can also be caused by mutations of the sodium channel voltage gated type V α subunit (SCN5A).[155] A lot of genes, for instance α-tropomyosin-1 (TPM1), involved in DCM have first been discovered in HCM.[134] As mutations in genes encoding sarcomeric proteins also can induce HCM, it apparently depends on the location of the alteration which of the two cardiomyopathies is induced. Mutations in regions directly involved in force transmission cause HCM and regions involved in force transmission from the sarcomere to the cytoskeleton cause DCM.[150] DCM can also occur with non-compaction cardiomyopathy (NCCM, see below).[134, 156] There is considerable heterogeneity in the clinical features and age of onset of DCM also in families with a single gene mutation.[129, 134] Most genetic DCM cases become apparent during the 4th or 5th decade of life, but first signs can also be noticed in young children and the severity of symptoms and survival, even in affected members of the same family can vary considerably.[134, 157]

2.3.3. Left ventricular noncompaction

Left ventricular non-compaction (LVNC) [MIM 604169] has an estimated prevalence 1/200 to 3/1250 per year.[158] It is characterised by a thickened two-layered myocardium with a thin, compact epicardial layer and a severely thickened endocardial layer with a "spongy" appearance due to prominent trabeculations and intertrabecular recesses.[159] This can lead to variable clinical features which include heart failure, arrhythmias and thromboembolism, but patients can also be asymptomatic.[160, 161] LVNC is thought to result from altered regulation in cell proliferation, differentiation and maturation during ventricular wall formation,[162] and an arrest in directional growth may play a

role in the association of EA and LVNC.[33, 163] Up to 64% of isolated LVNC has been reported to be familial, mostly consistent with an autosomal dominant mode of inheritance.[161] Most LVNC mutations have been found in the MYH7 gene, in the absence of CHD.[161, 164] But other sarcomeric proteins can also be involved, such as cardiac myosin-binding protein C (MYBPC3), α-cardiac actin (ACTC1), cardiac troponin T (TNNT2), α-tropomyosin (TPM1) and cardiac troponin I (TNNI3).[161, 164-166] Other reported gene mutations include α-dystrobrevin,[167] Cypher/ZASP,[168] lamin A/C,[169] and LIM domain-binding 3 genes.[170] Apart from the isolated form, LVNC can also be part of a syndrome. For example the Barth syndrome associated with mutations of the taffazin (G4.5) gene, can also include DCM.[167] Similarly, 17% of patients with Wolff-Parkinson-White syndrome (WPW), which is frequently seen in children, but not adults, have LVNC.[171] In 2007 Budde et al investigated a family with patients who had EA and/or ASD in addition to LVNC and found a mutation in MYH7.[127] This was later confirmed by Postma et al in patients with EA who also showed signs of LVNC, no MYH7 mutations were found in EA patients without signs of LVNC.[128] Besides the clinical presentation, the age of onset can also vary highly in LVNC.[172-174] Moreover, LVNC can occur in patients with an initial diagnosis of HCM or DCM,[175] and LVNC and DCM can co-occur in the same family.[134, 156, 171, 175] Van de Meerakker et al described a family in which there were patients with DCM as well as LVNC who had a mutation in TPM1.[134] The age of onset was highly variable in this family with the earliest diagnosis of DCM/LVNC at the age of about five months.[134] LVNC in children is commonly regarded as a relatively rare paediatric entity but accounts for about 9% of all forms of primary childhood cardiomyopathies.[171, 176]

Taken together, as most cardiomyopathies show a variable age of onset, mostly in adulthood, and a variable penetrance, clinical screening of first-degree relatives of adults diagnosed with cardiomyopathy is recommended.[177]

3.Causes of Congenital Heart defects

The genetic architecture of congenital heart defects (CHDs) is still incompletely understood, but it has been known for a long time that CHDs, often in combination with extracardiac abnormalities can be caused by chromosomal as well as single gene defects. Familial CHD can occur as autosomal dominant, autosomal recessive or X-linked traits with a variable to incomplete penetrance making CHDs clinically heterogeneous. CHDs can be associated with chromosomal defects such as Down syndrome (DS) caused by a trisomy 21 characterized by atrioventricular canal defects (AVCD) and Turner syndrome (45, X) associated with coarctation of the aorta (CoA). A well-known example of a partial chromosomal defect is the 22q11 deletion syndrome, in which patients can have interrupted aortic arch type B (IAA), truncus

arteriosus (TA) and tetralogy of Fallot (TOF).[104] Single gene defects in PTPN11 and TBX5 can cause Noonan syndrome and Holt-Oram syndrome respectively whereas mutations in GATA4 and NKX2-5 cause isolated CHDs. Still, the currently established genetic causes of CHD account for less than 20% of all CHD.[178] Mutations in different genes can cause a specific heart defect as well as mutations in a single gene can cause different heart defects. Individuals with a family history of CHD have a 2- to 4-fold increased risk of having children with CHDs compared to individuals without a family history of CHDs, there is no excessive clustering of specific CHD phenotypes among first-degree relatives.[179]
Overall, causes of CHD can be divided into four categories: 1) teratogenic factors, 2) chromosomal anomalies, 3) single gene defects and 4) multifactorial inheritance.
Here I will discuss first the chromosomal anomalies then the single mutations in transcription factors and the sarcomere genes, followed by inherited and noninherited risk factors.

3.1.Chromosomal anomalies and congenital heart defects

Large chromosome aberrations account for approximately 10-12% of all CHDs in live born infants[180, 181] and include among others trisomy 13, 18 and 21, cri-du-chat and Turner syndrome.[181] An overview of developmental syndromes with prominent CHD phenotypes can be found in Fahed et al.[69]

3.1.1. Trisomy 21

One of the most well-known chromosomal aberrations is trisomy 21 or Down syndrome (DS), it is one of the most common genetic cause of CHDs,[44] and associated with AVSD, mostly seen in DS compared to the normal population.[85, 182] The prevalence of CHD in DS ranges from 40-50%.[183-187] A lot of candidate genes have been identified for DS-CHD. With the exception of collagen Vi A1 and A2, none map to chromosome 21.[188] There have been several reports determining the chromosomal region critical for DS-related CHDs. Investigations revealed that the region can be narrowed to ~5.27 Mb between markers D21S3 and PFKL,[188] which can be further narrowed down to 1.77 Mb between DSCAM and ZBTB21 when looking only at endocardial cushion defects in DS.[189] Several of the genes included in the DS-CHD interval[188, 189] are known to be expressed in the heart and among this group a subset encode extracellular proteins or proteins interacting with them: SH3BGR, DSCAM, COL6A1, COL6A2 and COL18.[190] Besides trisomy 21, mutations in CRELD1 have also been found in individuals with DS and complete AVSD, suggesting that single mutations also contribute to the DS phenotype.[97]

3.1.2. Submicroscopic rearrangements (CNVs)

The development of methodologies to define subchromosomal changes in genome structure (copy number variants (CNVs)) exposed more subtle chromosomal anoma-

lies linked to CHD. CNVs describe all quantitative variation in the genome,[191] i.e. gains or losses of DNA ranging in size from 1 Kb to several Mbs, affecting roughly 10% of the human genome.[192] It is known that pathologic CNVs contribute significantly to the pathogenesis of CHD.[193] Studies have shown that rare and de novo copy number variation in the human genome contribute 5-10% of the population risk of sporadic CHD.[106, 194-196] The burden of functionally relevant CNVs ascertained as microdeletions and microduplications is reported between 3% and 20% in CHD cases, depending on the assertion of non-syndromic or syndromic classification respectively.[197-199] To date there are over 40 clinically delineated deletion and duplication syndromes with specific association to CHD, with an overrepresentation of microdeletions.[200] Submicroscopic rearrangements can result in genomic disorders, such as the microdeletion 22q11.2 (DiGeorge) syndrome, Williams-Beuren syndrome, and Smith-Magenis syndrome,[201] which can also give a clue about the genetic origin of isolated CHDs. Changes can roughly be grouped in three categories: de novo aberrations, inherited changes that are not known as frequent CNVs and commonly encountered CNVs.[201] Recent studies have also shown that rare de novo and inherited copy number variants (CNVs) occur in 5-10% of probands with CHD, classified in a variety of ways,[106, 194, 195, 197, 201-203] in both syndromic[199, 204-206] and non-syndromic[201] CHD patients. The frequency of causal CNVs in non-syndromic CHD populations is lower than that in syndromic CHD population (3.6% vs 19%), moreover, causal CNVs in non-syndromic CHD mostly involve imbalances with a moderate effect size and reduced penetrance whereas the majority of causal imbalances in syndromic CHD consistently affects human development and significantly reduces reproductive fitness.[197] Studies of CNVs suggests that genetic associations with CHD exhibit a considerable degree of phenotypic specificity.[194, 207] Causal CNVs in non-syndromic CHD mostly involve chromosomal regions 22q11 and 1q21.[197] The 22q11.2 deletion is classically associated with TOF but also frequently found in patients with interrupted arch, isolated VSDs, truncus arteriosus, and various other type of CHD.[208] After 22q11.2 deletions, the most common large CNV was the recurrent 1q21.1 duplication with a prevalence of about 1.18%.[106, 194, 207] CHDs that have been reported with 1q21.1 duplication include TOF, VSD, univentricular heart and complex cardiac disease.[106, 194, 207, 209, 210] A lot of CNV research has been done in patients with TOF. For instance, CNVs at 4 loci (1q21.1, 3p25.1, 7p21.3 and 22q11.2) were found in at least 2 individuals with TOF.[194] Many CNVs associated with TOF appear to disrupt gene pathways that control cell migration and vasculature development, both potentially important in cardiac development.[211] Also in patients with heterotaxy syndrome CNVs have been found,[212] as well as in patients with left-sided CHDs,[203] both revealing new candidate genes. An overview of CNVs that are associated with recurrent cases of nonsyndromic CHD can be found in Fahed et al.[69] Although most CNVs concentrate on protein coding regions, non-coding regions may also contribute to the pathogenesis of CHD, because

they may contain for example miRNAs. One study shows that there are CNVs in CHD patients that contain miRNAs targeting genes that show differential expression between DS patients with and without CHD or genes that are already known to be related to CHD.[213] Besides the fact that CNVs are causative, they can also attribute to the risk of CHDs. Overall, 13.6% of the CHD cases had CNVs>400 kb with <1% frequency.[195] In general, the more genes spanned by a CNV, the greater its potential to be pathogenic. Besides CNVs, single nucleotide polymorphisms (SNPs) can also contribute to the risk of CHD. This can be investigated by performing genome wide association studies (GWAS), which is also suitable to detect CNVs. This will be discussed in paragraph 3.4.

Overall, as discussed, large deletions and duplications of the genome, including whole chromosomes, can cause CHD. In contrast, a single nucleotide change in a gene can also cause CHDs. Such mutations are mostly found in transcription factors (next paragraph) and the sarcomere (paragraph 3.3).

3.2.Transcription factors involved in congenital heart disease

The heart is the first organ to form during embryogenesis and its development involves the expression of numerous genes. Defects in single genes are estimated to account for 3-5% of all CHD cases,[49] but can be higher in severe CHD cases.[214] For an overview of genes involved in syndromic and non-syndromic CHD in humans see Lalani et al.[200] Mutations in genes that cause syndromic CHD can occasionally also produce isolated heart malformations.[215-217] Most of these genes that cause CHD are highly conserved across species and encode transcription factors such as Gata4, Nkx2-5 and Tbx5,[65-67, 218] which regulate specific events in heart development such as ventricular septation, or outflow tract (OFT) morphogenesis.[46] Human genetic studies showed that dose-dependent mutation or misexpression of these genes can cause CHDs.[46]

Because single gene defects in NKX2-5, GATA4 and TBX5 have been shown to induce CHD in humans and are the most studied,[219] they will be further discussed below.

3.2.1. NKX2-5

NKX2-5 [MIM 600584] (or NK2 homeobox 5 or CSX1) is a homeodomain-containing transcription factor which maps to 5q34 and binds to the 5'-CAAGTG-3'motif in target promoters.[22] It plays a crucial role in heart development and function,[221] and is the vertebrate homolog of the Drosophila homeobox gene tinman.[133] NKX2-5 is one of the earliest markers of mesoderm[133] and required for its dorsal vessel development.[222-224] When Nkx2.5 is deleted in fly, cardiac development is

lost.[225] In the mouse however, targeted disruption of Nkx2.5 (Nkx2.5$^{-/-}$) causes embryonic lethality due to the arrested looping morphogenesis of the heart tube and growth retardation.[226, 227] In humans, heterozygous loss of function mutations cause a mild conduction delay and atrial septal dysmorphogenesis, leading to an increased frequency of patent foramen ovale (PFO), atrial septal aneurysm and atrial septal defect (ASD) (see below).[228] Overall, NKX2-5 is required for establishing heart function in the developing mouse embryo and later in adults for the maintenance of it.[229] Multiple human dominant NKX2.5 mutations have been identified that cause primarily structural malformations (ASDs and ventricular septal defects (VSDs)) accompanied by atrioventricular conduction delay and cardiac hypertrophic remodelling.[67] Mutations in NKX2-5 first have been found in families with inherited autosomal dominant ASD and atrioventricular (AV) conduction block.[67, 121, 230] As well as in families with ASD without AV conduction block.[231] Other congenital anomalies have been observed at low penetrance in these families, including VSD, Ebstein anomaly (EA), tetralogy of Fallot (TOF), subvalvular aortic stenosis,tricuspid valve abnormality, hypertrophic left heart syndrome (HLHS).[67, 121, 231]

3.2.2. TBX5

TBX5 [MIM142900] (T-box transcription factor 5) is a member of the T-box transcription factor family that regulates a wide variety of developmental processes in vertebrates and invertebrates, including specification of the mesoderm and development of the heart, vasculature and limbs.[232] The T-box genes are related to the founder member Brachyury (T) and to each other, by a conserved protein domain. This is T-box, which encodes a DNA binding motif, the T-domain,[233, 234] which recognizes a specific DNA element and the T-half site, that occurs singly, or in multiples with different orientation and spacing in promoters of target genes, and mediates transcriptional activation and/or repression.[235] In humans, 14 different T-box genes have been identified so far.[236] TBX5 is expressed among others in the forelimbs and embryonic heart,[232] preferentially in derivates of the first heart field (FHF), such as the inflow region, the atria and the left ventricle.[237] Later in heart development the function of TBX5 changes from chamber differentiation to chamber septation and conduction system development.[238] In the heart, TBX5 regulates transcription of downstream genes such as the atrial natriuretic factor (NPPA) and fibroblast growth factor (FGF10) by binding to T-box binding elements (TBEs),[239] often in combination with NKX2-5.[240] Overall putative targets for TBX5 include HOX genes, other T-box genes and members of the BMP, WNT and FGF gene families.[241, 242] In mice, the deletion of one Tbx5 allele causes the development of ASDs, VSDs and a variety of complex cardiac malformations, whereas homozygous deletion of Tbx5 is lethal because heart development is arrested.[243] On the other hand, heart-wide overexpression in mice resulted in looping defects, expansion of the left ventricular identity

and loss of the ventricular septum and AV cushions.[244] While mice homozygous for Tbx5 null mutations establish a linear heart tube, components derived from the FHF become hypoplastic.[243] In humans, TBX5 mutations contribute to the Holt-Oram syndrome (HOS), characterized by upper limb and cardiac deformities. These cardiac deformities encompass VSD, ASD, HLHS, total anomalous pulmonary venous return (TAPVR), TOF, double outlet right ventricle (DORV),[200] as well as conduction system anomalies, such as paroxysmal atrial fibrillation.[63] HOS is commonly caused by loss-of-function mutations in TBX5,[245] although a gain of function mutation has also been described.[63]

3.2.3. GATA4

GATA4 [MIM 600576] is localized to 8p23.1,[246, 247] and is a cardiac specific member of the GATA family of zinc finger transcription factors, which consists of six members.[248, 249] The C-terminal zinc finger is essential for DNA-binding and interaction with co-factors.[250] The GATA family recognizes the characterizing consensus DNA sequence 'GATA' motif,[251] (5'-(A/C/T)GATA(A/G)-3') in the promoters of many genes involved in embryogenesis and myocardial differentiation.[248, 249] Therefore, it is considered as a master regulator of developmental processes of the heart, such as cardiomyocyte proliferation, differentiation and survival.[252-257] GATA4 plays an especially critical role during several stages of cardiogenesis, from formation of the primitive heart tube to maturation of the four-chambered heart.[258] And is expressed during fetal development in yolk sac endoderm and cells involved in heart formation.[246] In mice with a defect in GATA4 the fusion of the bilateral cardiac primordial is inhibited and the linear heart tube cannot be formed.[259]

GATA4 was first suspected to be implicated in CHD when a deletion and a duplication of the chromosomal segment containing GATA4 were discovered in patients with cardiac malformations[260, 261]. Mutations were first identified in patients with inherited septation defects.[65] Loss of function mutations typically cause ASDs, however a subset of these mutations disrupts GATA4-SMAD4 interactions, critical for valve development, causing atrioventricular canal defects.[262] Nowadays a whole spectrum of CHDS can be caused by mutations in GATA4, these include ASD, VSD, AVSD, TOF, pulmonary valve stenosis (PVS), hypoplastic right ventricle (HRV) and total anomalous pulmonary venous return (TAPVR).[65, 99, 112, 263-266] Besides structural defects, individuals with GATA4 mutations can also develop dilated cardiomyopathy (DCM) later in life.[65] The same CHD phenotype may be caused by different GATA4 mutations, and the same GATA4 mutation may result in different phenotypes in different individuals, even individuals belonging to the same family.[65] Hence, to date over 100 non-synonymous mutations have been found in the GATA4 protein.[267] And most of these mutations occur in the segments of the gene encoding the zinc-finger motifs.[258] From mouse models it was concluded that TBX5 and NKX2-5 interact physically and

synergistically to activate their downstream targets,[240, 243] for instance to promote cardiomyocyte differentiation, whereby the N-terminal domain and N-terminal part of the T-box of Tbx5 and the homeodomain of Nkx2-5 are necessary for their interaction. Besides TBX5 and NKX2-5 also GATA4 and NKX2-5 physically interact,[268, 269] as well as GATA4 and TBX5.[65] Mutations in GATA4, that underlie CHDs might cause defective interactions between GATA4 and NKX2-5 and GATA4 and TBX5.[46]
In this paragraph, single mutations in the transcription factors NKX2-5, TBX5 and GATA4 that cause CHDs have been described. Such mutations have also been found in sarcomere genes, this will be discussed in the next paragraph.

3.3.Sarcomere genes and structural heart defects

Mutations in sarcomere genes were initially discovered in cardiomyopathies.[134] However, subsequently sarcomeric mutations were also found in patients with congenital heart defects (CHDs), indicating sarcomeric structures are likely to be involved in normal cardiac development. This was confirmed when reports showed that actin polymerization is important in the early looping of the avian heart.[270, 271] The first link between mutations in sarcomeric proteins and CHDs was provided by Ching et al who reported mutations of α-myosin heavy chain (MYH6) in patients with atrial septal defect (ASD) with or without cardiomyopathy.[68] More reports followed showing mutations in sarcomeric genes in families with CHD with or without cardiomyopathies.[58, 127, 166] I will discuss a few examples below.

3.3.1. ACTA2

α-Smooth muscle actin (ACTA2) encodes a sarcomeric protein of smooth muscle cells (SMCs) which is crucial in the structure and contractile force of the aorta, namely actin. In the mouse, Acta2 is the first to be expressed in the very early stages of in vivo cardiogenesis.[272] It is highly expressed in the ventricular conduction system,[273] as well as in the aorta.[274] Because Acta2 is crucial for the maturation of the cardiomyocytes (differentiation),[273] Acta2^{+} cardiomyocytes show mainly characteristics of ventricular cardiomyocytes.[274] Acta2 is later on in development replaced by Acta1 and Actc1,[275, 276] and in the rat it disappears shortly after birth.[277]
Recently mutations of the ACTA2 gene were found in patients with thoracic aortic aneurysm and/or dissection (TAAD), patent ductus arteriosus (PDA) and bicuspid aortic valves (BAVs).[278-280] ACTA2 is the most frequently mutated gene causing familial thoracic aortic aneurysms and dissection and is responsible for 12-21% of them without features of connective tissue disorders.[278, 280-282] The mutations have possibly an effect on the TGFβ-signalling pathway.[280]

3.3.2. ACTC1

α-Cardiac-actin (ACTC1) is essentially the only actin present in the embryonic heart muscle.[283, 284] A morpholino (MO) knockdown in chick embryos gives shorter, less-

developed atrial septa and a delayed s-looping compared to wild-type embryos. Reduced levels of ACTC1 may lead to failure of the atrial septum to close.[58] Mice lacking cardiac actin do not survive >2 weeks.[277]
Mutations in ACTC1 were first found in individuals with dilated cardiomyopathy (DCM).[285] Later mutations have also been found in a family with isolated atrial septal defect (ASD),[286] a family with ASD/ventricular septal defects (VSD) in combination with HCM/noncompaction cardiomyopathy (NCCM)[166] and in two families with isolated ASD/VSD, who harboured the same mutation as well as a 17 bp deletion in a patient with ASD type II (ASDII).[58] More recently, a mutation has been found in a family with various congenital heart defects, mainly ASD.[287] These days, as whole genome sequencing is becoming more common practise, a recent study already using it revealed another family with isolated ASD and a mutation in ACTC1.[288] For an overview of mutations in ACTC1 see Augière et al.[287]

3.3.3. MYBPC3

Cardiac myosin binding protein C (MYBPC) binds myosin heavy chain in thick filaments and titin in elastic filaments. Animal models for MYBPC3 mainly focus on cardiomyopathy, cardiac hypertrophy or cardiac dysfunction and till now no literature on animal models showing congenital heart disease has been published.
In a large cohort of Amish children, homozygous truncating mutations in MYBPC3 have been found in patients with severe lethal HCM and several of these patients also had a VSD.[286] In three unrelated newborns, compound heterozygosity for two truncating mutations in MYBPC3 caused severe lethal NCCM with ASD or VSD.[289, 290] Also in an indexpatient with ASDII, a MYBPC3 variant was found but whether this is a pathogenic mutation or a variant is not clear yet, no other causative mutation was found.[291]

3.3.4. MYH6

In the human heart two conventional myosin heavy chain (MYH) proteins are expressed, α-myosin heavy chain (MYH6) and β-myosin heavy chain (MYH7). MYH6 is exclusively expressed in the atria.[292] A MO knockdown of MYH6 in chick embryos results in disrupted atrial septation.[70]
Recent studies have reported mutations in MYH6 in patients with various forms of CHD,[293] with the most observed malformations being atrioventricular septal defect (AVSD) and ASDII.[291] Several families with ASD(II) have been described with MYH6 mutations.[68, 291]
MYH6 is a downstream transcriptional target of GATA4 and TBX5.[294] The activation of the MYH6 promoter is dependent on the T-box binding site,[295] so MYH6 expression is activated by TBX5.[68] However, mutations in GATA4 are also known to affect the activation of MYH6 expression.[65]

3.3.5. MYH7

β-myosin heavy chain (MYH7) is, in contrast to MYH6, exclusively expressed in the human ventricles[296]. There is also an isoform (MYH7b) that is highly expressed in the myocardium of the mature human heart.[297]

Budde et al. described a family with members who showed NCCM as well as ASD and Ebstein anomaly (EA) combined or with solely ASD.[127] As EA and NCCM is a frequent association,[126, 298] MYH7 was screened in 141 unrelated probands with EA and MYH7 mutations were found in 8 probands, who also all showed LVNC, while family members of these probands showed various CHDs as well as LVNC.[128] More recently another family was described with a mutation in MYH7 with EA, LVNC and VSD.[299]

3.3.6 MYH11

Smooth muscle myosin heavy chain 11 (MYH11) encodes just like ACTA2 sarcomeric proteins of SMCs in the aorta, specifically βMHC (MYH7).

So it's of no surprise that mutations in the MYH11 were found in patients with TAAD associated with PDA, although they are not found in families with only TAAD.[280, 300, 301] And the reported mutations have a dominant negative effect.[280, 301] This has possibly an effect on the TGFβ-signalling pathway.[280]

There are also 2 Dutch families reported with a variant in MYH11, but the variant did not segregate with the phenotype in these families.[302] So maybe other (genetic) factors also play a role.

Overall, in the early days, mutations in sarcomeric proteins were only found in patients with several different types of cardiomyopathy. Nowadays, mutations are also found in patients with congenital heart disease, such as ASD, VSD, EA, PDA and TAAD. These defects can occur with or without cardiomyopathy in the same patient or in the same family. Since nowadays more and more whole exome sequencing is used to find mutations causative for CHDs, the prediction is that more mutations will also be found in sarcomeric proteins. Recently the miRNA 208 was found to be encoded in MYH6 and it regulates the expression of MYH7. Because mutations in the genes encoding for these proteins are now recognized to cause CHDs, maybe also miRNAs can be involved in the development of CHDs.

Single gene mutations, deletions and duplications are causative for CHDs. But there are also risk factors that elevate the risk of developing a CHD. These can be divided into inherited (next paragraph) and noninherited (paragraph 3.5) risk factors.

3.4.Inherited risk factors and congenital heart defects

Single nucleotide polymorphisms (SNPs) are rare variants, usually present in less than 1% of the normal population but overrepresented in CHD patients. Initially a lot of

SNP investigations in relation to CHD were focussed on the suspicion that folate deficiency could be a CHD risk factor, but the overall evidence remains inconclusive.[303] Homozygosity for the thymidine allele (T/T genotype) of the MTHFR gene on C/G677 position causes lower levels of plasma folate, thereby probably increasing the risk of developing CHD.[304] Previous studies on the association of the C677T genotype and CHD risk were small and when these studies were combined, the results were inconclusive.[304] Also, a new study reports on large numbers of CHD cases and controls in combination with a meta-analysis of already available data and found no significant effect of MTHFR C677T on CHD risk.[304] So most probably the MTHFR risk allele C677T has no profound effect on the development of CHD in humans.
Larger genome wide association studies (GWAS) are performed nowadays to look for risk factors for CHD in the whole genome. Large amounts of patients need to be pooled to achieve adequate power in order to detect and replicate a number of genetic effects. In one study there was an association between three common SNPs at 4p16 (adjacent to the MSX1 and STX18 genes) and the risk of ASD; the association accounts for around 9% of the population-attributable risk.[305] This risk was absent when taken all CHDs together and diminishes when AVSD cases were added to the ASD cases, so the risk is specific to secundum ASD.[305] This was replicable in the Han Chinese populations were there was also only an association with ASD and not with other septum defects however only with two out of three SNPs published before and with a population attributable risk of 7% instead of 9%.[306] Another study showed an association between SNPs on chromosome 12q24 and a variety of complex genetic conditions, including TOF.[307] In this region the strongest candidate gene is the (PTPN11), because an association between a SNP in PTPN11 and TOF was previously demonstrated.[308] Other regions such as 13q32, as well as regions on chromosome 10, 15 and 16 have also been associated with TOF.[307] As this study was performed with European cases and controls, the study was repeated in Chinese of Najing. Here only an association with 10p11 and TOF was found.[309] In a Chinese population with ASD and VSD no association was found.[309] Another study in the Han Chinese population, showed association of two different loci (on 1p12 and 4q31.1) total CHD.[310] Although few GWA studies have been performed so far, some risk attributable loci have been found for CHD.
Overall, the finding of inherited risk factors gives hope for the future to further understand the etiology of CHD.

Besides inherited risk factors there are also noninherited risk factors involved in the etiology of CHDs, these will be briefly discussed in the next paragraph.

3.5.Noninherited risk factors and congenital heart defects

The development of congenital heart defects (CHDs) can have several causes. We have discussed the genetic and inherited risk factors earlier but there are also noninherited risk factors which can solely or in combination with the genetic factors cause CHDs. As the critical period for cardiac development is between 2 and 7 weeks of gestational age, only the parental conditions during the periconceptional period, which is defined as the 3 months before pregnancy through the third month (first trimester) of pregnancy, will probably be of influence and therefore are the only ones discussed here. Below I only discuss maternal pregestational diabetes, maternal sociodemographic characteristics, paternal age and teratogen exposure as risk factors. For a total overview of the noninherited risk factors for CHDs, see Jenkins et al.[73] and Patel et al.[311]

3.5.1. Maternal pregestational diabetes

Maternal diseases, such as phenylketonuria, febrile illness, rubella, HIV infection and maternal pregestational diabetes,[73] can cause up to 1% of cases of CHDs.[311, 312] A lot of research has been done on CHDs in infants of mothers with pregestational diabetes. They have a 3-5 fold increased risk of having a child with a CHD.[313-315] Several types of CHDs have been described such as atrial septal defect (ASD), atrioventricular septal defect (AVSD), CoA, double-outlet right ventricle, pulmonary atresia, total anomalous pulmonary venous return, transposition of the great arteries (TGA), TOF, truncus arteriosus (TA) and VSD.[316-319] It is striking that the CHDs often occur in structures that develop early in cardiogenesis (before the seventh week of gestation) or primary cardiogenesis.[319-321] One possible explanation can be that vascular endothelial growth factor (VEGF) variations have been associated with CHDs[322, 323] as well as diabetes-induced vascular proliferation.[324-326] The vascular complications of diabetes can be induced by individual variations in glucose metabolism and/or protein glycosylation, possibly of genetic origin,[327] and serve as a common pathway for cardiovascular maldevelopment. There is evidence that fluctuating glucose concentrations may be more harmful to the embryo than consistently elevated levels, which may lead to switching to a glycolytic, low-energy pattern of metabolism.[320] This can cause yolk sac impairment which may lead to the diabetic embryopathy.[320] Another explanation can be the altered, possible teratogenic, intrauterine environment in early gestation.[319, 328, 329] The precise mechanism behind this is unknown, but hyperglycemia, ketoacidosis and associated biochemical and vascular abnormalities have been implicated in several studies.[330] Hyperglycemia seems to be the main risk factor,[316, 331] because the prevalence of birth defects among diabetic women with good glycemic control is similar to that in the general population.[332] Some studies state that there is an increased risk for fetal malformation at even slightly raised HbA_{1c} (glycosylated haemoglobin) values during early pregnancy, while women with type 1 diabetes who have a normal HbA_{1c} show

a rate similar to the general population.[331] This is debated in another study as glucose variability was more of a risk factor than the HbA_{1c}, because they found that even with normal and almost normal HbA_{1c} values, the incidence of fetal malformations is still higher than in the general population.[333] So they state that although HbA_{1c} values are a good indicator of glucose levels, they apparently aren't the same and glucose levels are directly linked to CHDs.

3.5.2 Maternal sociodemographic characteristics

Another class of non-inherited risk factors encompass the maternal sociodemographic characteristics, such as race/ethnicity, reproductive history, maternal stress and age. There is evidence that white infants have an increased prevalence of Ebstein anomaly (EA), aortic stenosis, AVSD, ASDs, pulmonary atresia, CoA, TA, TGA, TOF and HLHS,[334-338] but a decreased prevalence of PDA, PS and heterotaxia[335, 336] compared to black infants. Another factor, history of reproductive problems, has been associated with an increased risk of CHDs. One study states that previous miscarriage is associated with an increased risk of TOF and EA, previous stillbirth with nonchromosomal AVSD, and previous preterm birth with ASDs.[317] However, a recent Polish study could not confirm these results; they only found an increased risk of isolated CHDs among women who have had previous pregnancies irrespective of the outcome of these pregnancies, with the highest risk was among women with 3 or more previous pregnancies,[339] which is also found in earlier studies in Sweden and China.[340-342] A third characteristic is maternal stress. A case-control study in China showed that having a mother who reported stressful life events during the periconceptional period, had a 3-fold greater risk of having an infant with CHD.[343] The heart defects were mainly conotruncal defects,[334] TGA to be more specific.[344] A final factor can be maternal age. A maternal age ≥30 years is associated with an increased risk of TGA and EA while a maternal age of > 34 years is associated with an increased risk of bicuspid aortic valve and ASDs. On the other hand, a young maternal age (<20 years) is associated with an increased risk of tricuspid atresia.[317]

3.5.3 Paternal age

Not only maternal age is important but paternal age is also associated with an increased risk for offspring with CHD.[73] While an increasing paternal age is associated with an increased risk for ASDs, VSDs, and PDA[345] even after adjustment for maternal characteristics,[346] a young paternal age (<20 years) gives a higher risk for VSDs and possibly ASDs.[345]

3.5.4. Teratogen exposure

Another important class of noninherited risk factors are the environmental teratogens, e.g. thalidomide, alcohol, smoking, and anti-epileptic drugs.[73] Exposure of

these teratogens during pregnancy increase the risk of having a fetus with a CHD. A lot has been published about the fetal alcohol syndrome since the first description in 1973 by Jones and Smith.[347] Several studies have shown a wide range of teratogenic effects of alcohol consumption during pregnancy (periconceptional period) , including cardiac defects.[348] However, conflicting results have been reported regarding children with CHD, as some studies report a significant association between maternal alcohol use and CHDs, but the majority of the studies don't.[311] One study used the Pregnancy Risk Assessment Monitoring System database and found a threefold risk for CHDs among mothers who reported periconceptional alcohol use.[349] While the BWIS study found an increased risk for only small muscular VSDs and heavy consumption of alcohol (5 drinks on a single occasion) during the periconceptional period, but no trend in an association of a risk of any cardiac defect with increased exposure.[317] Regarding paternal alcohol consumption, only one study identified a fourfold increase in risk for VSDs among fathers who reported alcohol use,[350] no other reports found a significant association for any type of CHD and alcohol consumption. Besides alcohol, also maternal smoking is a teratogen that has been investigated with conflicting results.[311] Analyses of several databases did not identify a significant association between maternal periconceptional cigarette smoking and the development of CHDs as a group, only when divided into subtypes,[351-353] other studies did find an increased risk for CHDs as a group.[354-357] When divided into subtypes, there is an increased risk for pulmonary valve stenosis (PVS) when mothers were ≥34 years and smoked more than 1 pack of cigarettes per day,[317] and a possible dose-response relationship between the number of cigarettes smoked and non-syndromic AVSDs.[351] While other data showed an increased risk for ASD, AVSD, pulmonary valve stenosis and VSD with active smoking and AVSDs with passive maternal smoke exposure.[357] Overall, mothers with infants with septal heart defects (both ASDs and VSDs) were more likely to have smoked in the periconceptional period through the end of the first trimester than control mothers with an increasing relative risk with the number of cigarettes smoked.[357] Another teratogen can be anti-epileptic drugs. And although early studies state that the prevalence of CHDs in the offspring of epileptic mothers is the same as in the normal population,[358, 359] nowadays many large studies do show a difference, but whether the malformations are due to epilepsy itself or the anticonvulsant therapy is still not clear.[73] CHDs can be caused by all the major antiepileptic drugs[360, 361] with CHDs being more frequent with polytherapy compared to monotherapy.[362] There is especially a strong association between the risk of single VSD and maternal epilepsy with antiepileptic drug treatments.[363] One mechanism is that of the antiepileptic drug Valproate, which alters the epigenome of expesed cells by inhibiting histone deacetylases (HDACs) and inducing DNA methylation.[364-367] The association of CHDs and the use of antiepileptic drugs is widely recognized,[368] but the possibility

that epilepsy itself is associated with an increased risk of birth defects is an ongoing discussion. A meta-analysis showed that untreated women with epilepsy were not at an increased risk of having infants with birth defects compared to healthy women.[369] Another study shows that there is no association between CHDs and epilepsy syndrome, seizure frequency and folate use.[362] Folic acid shows a protective effect in the development of CHDs in general.[73, 370] Apparently, the increased risk for mothers with epilepsy of having a child with a CHD is independent of maternal epilepsy characteristics but caused by other external or environmental factors including exposure to antiepileptic drugs.[362]

Here I have discussed several noninherited risk factors such as maternal pregestational diabetes, maternal sociodemographics, paternal age and teratogenic exposure. As stated in this paragraph CHDs do not only develop under the influence of maternal factors but also of paternal factors. In the end the development of a CHD is a combination between genetic, nongenetic and epigenetic factors.

4.Conclusion

Heart development is a complex process taking place at the early stages of embryonic development. Insight in the general development of the heart has given, in turn, insight in the development of congenital heart defects (CHDs) and also vice versa. As patients with CHDs live longer nowadays, due to the vast improvements of surgical techniques, they now reach reproductive age. This has led to families with CHDs in which involved genes can be detected, contributing to insight in the genetics of congenital heart defects. Coupled with the development of new techniques such as Next Generation Sequencing (NGS), this can contribute to the discovery of many more genetic causes of CHD. Furthermore, these novel techniques also allow the investigation of the epigenome and promoters and enhancers. However, besides genetics, environmental factors are also an important cause for CHD.
In the end the causes of CHD will likely be a combination of (novel) genetic and environmental factors. Perhaps someday we will be able to predict the outcome of a CHD in a patient based on his (epi)genome and the environmental factors.

5.Scope/Outline of the Thesis

The introduction in **chapter 1** gives an overview of the normal structural development of the heart and describes congenital heart defects (CHDs) and their causes. **Chapter 2** describes a family clinically characterized by atypical Holt-Oram features

and paroxysmal atrial fibrillation in which a gain-of-function mutation was found in the TBX5 gene. **Chapter 3** consists of a paper describing mutations in the MYH7 gene in patients with Ebstein's anomaly. **Chapter 4** describes a family with congenital heart defects and low atrial rhythm, which we suspect to be left atrial isomerism, in which we have found a causative region on chromosome 9q through linkage analysis. **Chapter 5** describes a novel mutation in alpha-tropomyosin found in a family with DCM and non-compaction cardiomyopathy which diminishes the binding to actin. And in **chapter 6**, patients with DCM were screened for HCM/DCM genes by targeted sequence capture and GS-FLX Titanium sequencing in order to validate the test so it could be implemented into diagnostics. While finally **chapter 7** comprehends the discussion and summary of the results of the previous chapters.

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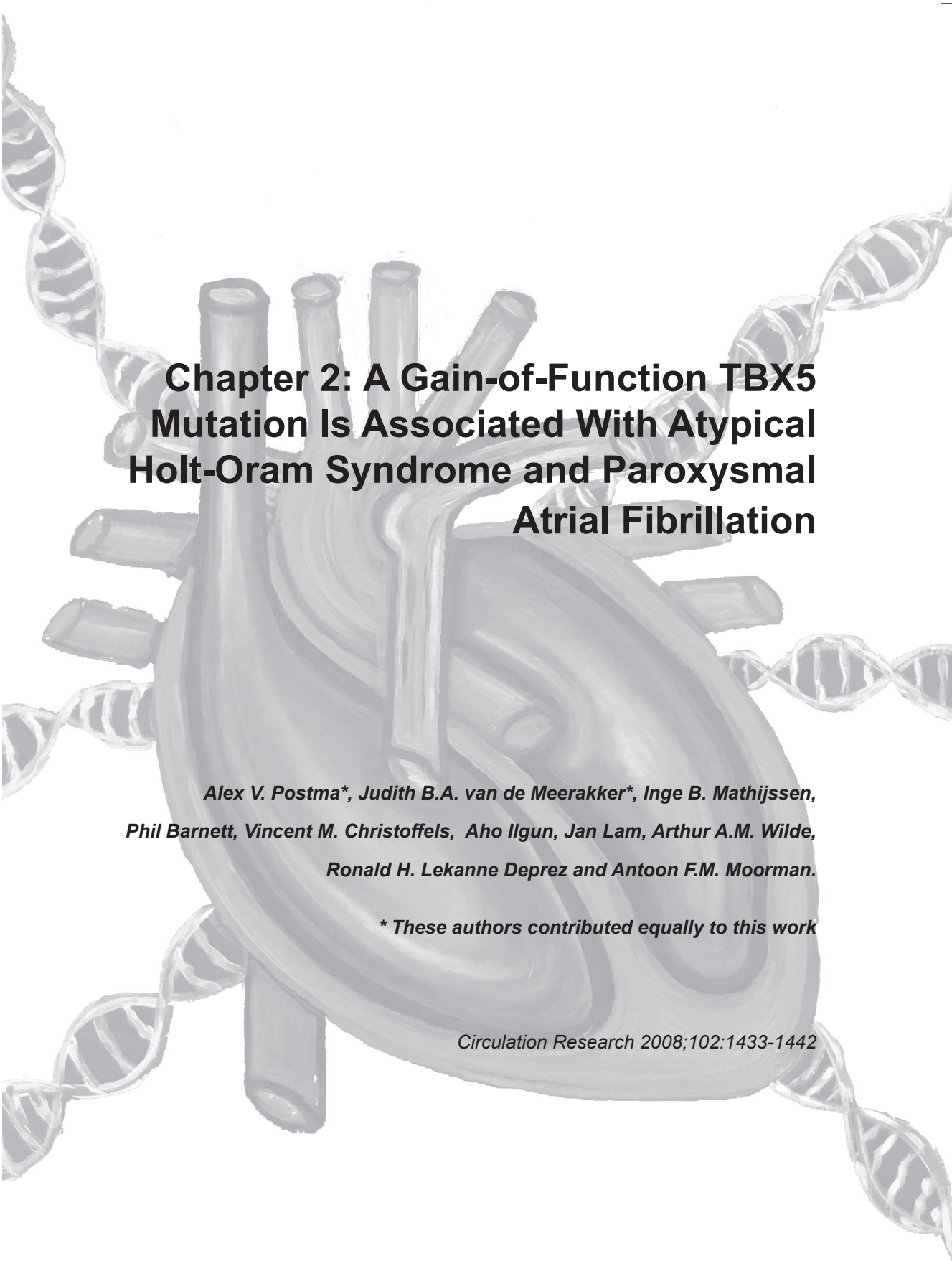

Chapter 2: A Gain-of-Function TBX5 Mutation Is Associated With Atypical Holt-Oram Syndrome and Paroxysmal Atrial Fibrillation

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Abstract

Holt–Oram syndrome (HOS) is a heart/hand syndrome clinically characterized by upper limb and cardiac malformations. Mutations in T-box transcription factor 5 (*TBX5*) underlie this syndrome. Here, we describe a large atypical HOS family in which affected patients have mild skeletal deformations and paroxysmal atrial fibrillation, but few have congenital heart disease. Sequencing of TBX5 revealed a novel mutation, c.373G>A, resulting in the missense mutation p.Gly125Arg, in all investigated affected family members, cosegregating with the disease. We demonstrate that the mutation results in normal Nkx2-5 interaction, is correctly targeted to the nucleus, has significantly enhanced DNA binding and activation of both the *Nppa*(*Anf*) and *Cx40* promoter, and significantly augments expression of *Nppa*, *Cx40*, *Kcnj2*, and *Tbx3* in comparison with wild-type TBX5. Thus, contrary to previously published HOS mutations, the p.G125R TBX5 mutation results in a gain-of-function. We speculate that the gain-of-function mechanism underlies the mild skeletal phenotype and paroxysmal atrial fibrillation and suggest a possible role of TBX5 in the development of (paroxysmal) atrial fibrillation based on a gain-of-function either through a direct stimulation of target genes via TBX5 or indirectly via TBX5 stimulated TBX3. These findings may warrant a renewed look at the phenotypes of families and individuals hitherto not classified as HOS or as atypical but presenting with paroxysmal atrial fibrillation, because these may possibly be the result of additional TBX5 gain-of-function mutations.

Congenital heart defects are among the most common congenital defects in children, occurring in 1% to 2% of live births and in ≈5% of stillbirths.[1] Congenital heart defects can either appear as a spontaneous defect or as part of a syndrome. One such syndrome is the Holt–Oram Syndrome (HOS) (Online Mendelian Inheritance in Man [OMIM] no. 142900),[2] appearing in 1 of 100 000 live births[3] and segregating in an autosomal dominant fashion. It is characterized by bilateral forelimb deformities and congenital heart defects. Clinically, there are 3 variations of HOS: affected individuals may have only skeletal anomalies (27.4%), only cardiac defects (3.9%), or both (68.7%).[4] The limb and heart malformations can vary from mild to severe, even within families, and no correlation exists between the severity of the cardiac and skeletal abnormalities of the patient.[5] The congenital heart malformations are generally secundum atrial septal defects (ASD II) or ventricular septal defects, but others, such as mitral valve defects and cardiac conduction defects, most notably atrioventricular block, have also been reported.[3, 6] Single-gene mutations were identified in the T-box transcription factor 5 (TBX5) in multiple HOS patients.[7] TBX5 is a member of the T-box transcription factor family that regulates a wide variety of developmental processes in vertebrates and invertebrates, including specification of the mesoderm and development of the heart, vasculature, and limbs.[8] Mutations in several other

T-box genes have been associated with malformations such as septal defects and dilated cardiomyopathy (*TBX20*),[9] cleft palate (*TBX22*), and syndromes such as DiGeorge syndrome (*TBX1*) and Ulnar– Mammary syndrome (*TBX3*).[10] TBX5 is expressed, among others, in the embryonic heart and forelimbs.[8] In the heart, it regulates transcription of downstream genes such as the atrial natriuretic factor (*NPPA*) and fibroblast growth factor 10 (*FGF10*) by the binding to T-box binding elements (TBEs)[11], often in combination with the NKX2-5 transcription factor.[12] Seventy percent of the identified TBX5 mutations lead to a premature stop codon and, in these patients HOS, is presumably caused by haploinsufficiency. Although the HOS-associated mutations are distributed across all exons of TBX5, the majority are found within the T-box DNA binding domain.[13] However, there is no evidence that either the type of mutation or the location of a mutation is predictive for the severity of the heart or limb malformations in HOS patients.[14] Here, we describe a family with an atypical form of HOS, previously reported by Van Bever et al in 1996.[15] Besides some characteristics of HOS, 6 of 9 affected alive patients also experience paroxysmal atrial fibrillation (AF). We identified a novel TBX5 mutation (p.G125R) that which cosegregates with the disease and, in contrast to previously reported TBX5 mutations, causes a gain-of-function. We speculate that this novel TBX5 mutation results in the atypical HOS seen in this family.

Patients and methods

Clinical Details

The clinical findings in this family have been previously described by van Bever et al.[15] Additional family members were investigated, new symptoms developed in several persons, and (affected) family members were born (see Results). The study was performed according to a protocol approved by the local ethics committees. Informed consent was obtained from all patients.

Linkage Analysis, Mutation Screen, and Confirmation

TBX5 haplotype analysis in the family was carried out using microsatellite markers D12S129, D12S821, D12S354 and D12S369 flanking the gene (Table II in the data supplement). Mutation screening is detailed in the data supplement; in short, whole exons were PCR amplified using intronic primers (supplemental Table I) from peripheral blood genomic DNA and sequenced.

Plasmid Constructs and Reporter Gene Assays

Expression plasmids (FLAG-TBX5, full-length mouse FLAG- Nkx2-5) and reporter plasmids (pGL3-Nppa-luciferase, pGL3-Cx40- luciferase) and introduction of mutations in these are detailed in the data supplement. Neonatal rat heart myocytes,

immortalized with a temperature-sensitive SV40 T antigen (H10 cells[16]) and an embryonic heart tissue cell line (H9C2 cells[17]), were used according to standard methods as detailed in the data supplement. After transfection, the luciferase activity of the reporter constructs was measured and corrected against an internal control (renilla). The experiments were repeated at least 3 times in duplicate or triplicate. Data are listed as means±SEM.

Electromobility-Shift Assays and Western Blot Analyses

Nuclear extracts were prepared from HEK cells and transfected with the appropriate expression constructs. Electromobility-shift assay (EMSA) was performed as described previously.[18] The oligonucleotide corresponds to the TBE–Nkx binding element site (NKE) site at −250 in the Nppa promoter. See the data supplement for details. Western blot analysis of nuclear extracts was performed according to standard procedures. The complexes were visualized using the ECL detection kit (Amersham).

In Vitro Yeast Two-Hybrid X-Gal Assay and Binding Assays

All procedures concerning the yeast 2-hybrid system were carried out as described previously.[19] In short bait (pcDNA3.1-mouse NKX2-5 carrying the GAL4 DBD fusion construct) and prey (pcDNA3.1-human TBX5wt/Gly125Arg carrying the GAL4 AD fusion construct) were cotransformed into *Saccharomyces cerevisiae* AH109 (Clontech), and the ability to grow on minimal media was examined using β-galactosidase filter assays. Binding assays were set up as described previously.[20] Basically, equivalent amounts of myelin basic protein (MBP)/MBP fusion protein were loaded onto PBS-Triton columns equilibrated with amylose resin (New England Biolabs). After washing, the purified glutathione *S*-transferase (GST)/GST fusion was passed over. After washing and elution, the eluate was used for SDS-PAGE and Western blot analysis. Further details are found in the data supplement.

Immunocytochemistry

COS7 cells were seeded on cover slips; after transfection, fixation, and permeabilization, they were incubated with the primary anti- FLAG antibody. The next day, the secondary antibody, Alexa488- conjugated goat anti-rabbit IgG (Molecular Probes), was added. Further details are available in the data supplement.

RNA Isolation, cDNA Synthesis, and Quantitative PCR

H10 and H9C2 cells were used for quantitative PCR as detailed in the data supplement. For calculation of PCR efficiency, the program LinReg was used.[21] Measurements per RNA sample were in triplicate and were averaged. The experiments were repeated at least 3 times. Results are expressed as means±SEM, as indicated in the legends. To remove variations from multisession experiments attributable to day-to-

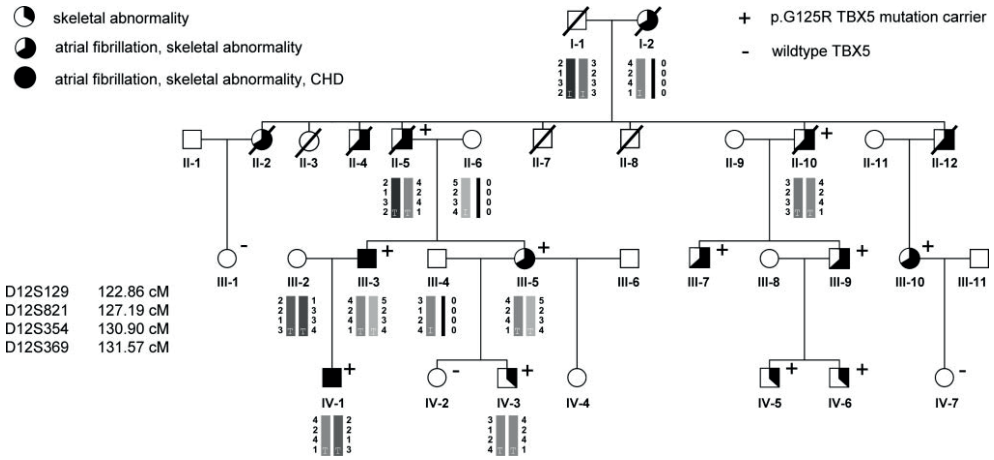

Figure 1. Pedigree of the family with atypical HOS and paroxysmal AF, including linkage with microsatellite markers surrounding *TBX5* and the resultant 4-2-4-1 haplotype cosegregating with the phenotype. Carriers of the p.G125R TBX5 mutation are noted with +; individuals with wild-type TBX5 are noted with –.

day differences in transfection efficiency and expression levels, a factor correction[22] was applied. The corrected data set was used for statistical analysis. Comparisons between 2 groups was performed using Student's *t* tests. $P<0.05$ was considered statistically significant.

Results

Clinical Details

The family presented with an atypical form of HOS (Figure 1); the major clinical details are summarized in the Table. The skeletal findings include: (sub)luxation of the radial head in all examined affected patients (Figure 2A), carpal synostosis in the majority, and scoliosis and scapular dysplasia in some. Careful examination showed slight thenar muscular atrophy, limited opposition of the thumbs, and limited flexion of the distal interphalangeal joints in several family members (Figure 2B).

Only 2 of 15 family members (13%) with skeletal findings also had a congenital heart malformation, 1 ASD II and 1 (small) ventricular septal defect. Interestingly, the majority (78%) of the affected members developed clinically documented paroxysmal AF at an early age, with the onset between 9 and 38 years, in the absence of congenital heart disease. Finally, the 2 youngest members did not develop paroxysmal AF, to date, and have no congenital heart defect but do have skeletal problems (Table).

Table Summary of clinical features of the atypical HOS family

Family Member	CHD	Echo Parameters				ECG		Arrhythmia, Age of Onset (Years)	Radial Luxation	Carpal Synostosis	Scoliosis	Other Anomalies
		LVED	LVSD	SF	LA	PQ Time	Axis					
I-2	(—)	NA	NA	NA	NA			(+)	(+)	?	?	
II-2	(—)	NA	NA	NA	NA			(+)	(+)	?	(—)	
II-4	—	NA	NA	NA	NA			AF	+	+	+	Scapular Dysplasia
II-5	—	60	40	33	50	0.12		pAF (5-15), iRBBB	+	+	+	Scapular Dysplasia
II-10	—	Normal	Normal	Normal	50		Left horizontal	AF (38) pacemaker	+	+	+	Scapular Dysplasia
II-12	(—)	NA	NA	NA				pAF	(+)	?	(—)	
III-3	ASD II	47	35	27#	—	0.16	Left horizontal	pAF (28), iRBBB	+	+	—	
III-5	—	48	28	41	—		Intermediate	pAF (28)	+	+	+	
III-7	—	51	30	40	39	0.16	Left horizontal	pAF (21), preceded by AT	+	?	—	
III-9	—	54	35	35	32	0.16	Left horizontal	(+) (20)	+	+	—	
III-10	—	50	32	37	—		Left horizontal	pAF (28)	+	?	—	
IV-1	VSD	51	32	36	—	0.12	Left horizontal	pAF (9)	+	?	—	
IV-3	—	52	35	33	—	0.2	Intermediate	suspected pAF	+	—	+	
IV-5	—	Normal*	Normal*	Normal*	—	0.16	Left horizontal	sinus rhythm	+	?	—	
IV-6	—	Normal*	Normal*	Normal*	—	0.16	Left horizontal	sinus rhythm	+	?	—	

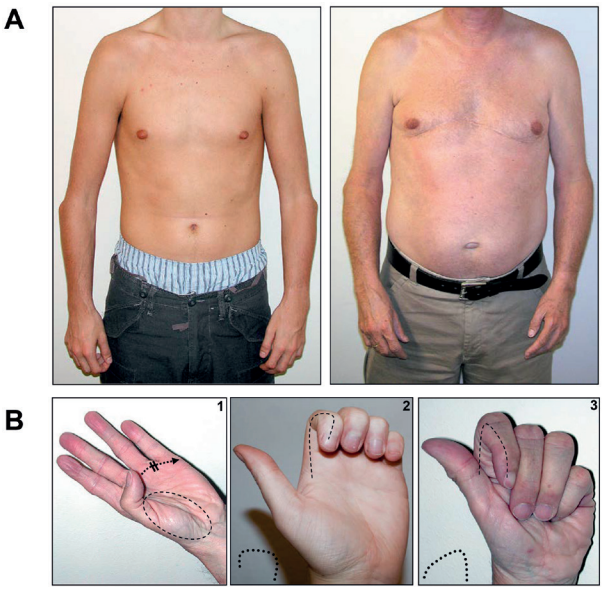

Figure 2. A, Photographs of the upper torso of IV-1 and III-3 depicting the (sub) luxation of the radial head. B1, Hands of patient III-3 indicating thenar muscular atrophy (striped circle) and limited opposition of the thumbs (blocked arrow). Normally, the thumb is able to reach the end of the arrow. B2 shows normal flexion of distal interphalangeal joints in an unaffected person (2X90° angle), indicated by line drawing, whereas B3 shows that patient III-3 has a limited flexion of the distal interphalangeal joints, because the patient cannot make a similar movement as in B2.

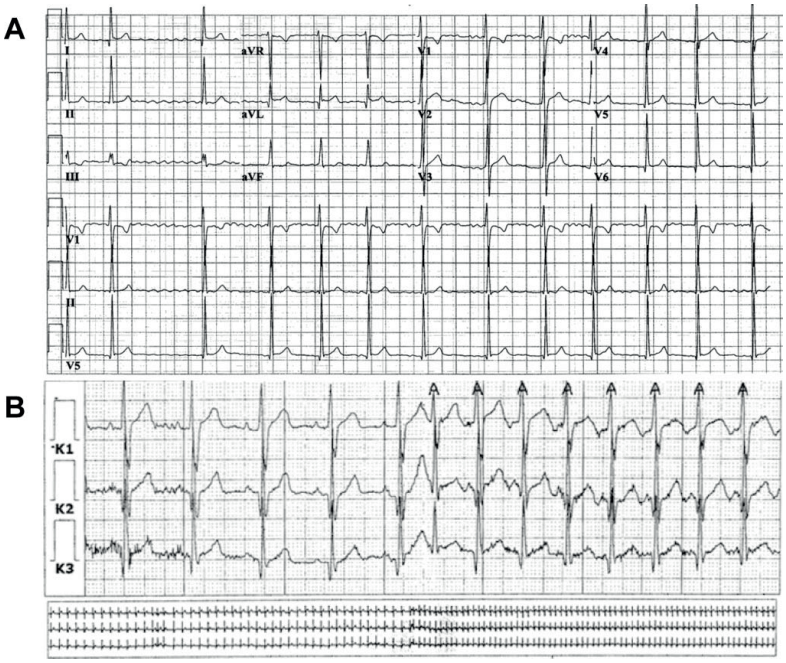

Figure 3. A, Twelve-lead ECG of patient IV-1 showing AF. B, ECG of patient III-7 with recorded supraventricular, presumably atrial, tachycardia.

Available ECG parameters of family members are within normal limits, as well as the echo parameters, with the exception of III-3 who retained abnormal septal wall motion after closure of his ASD II. II-5 and II-10 show signs of an enlarged left atrium, which could be caused by long-term paroxysmal AF in this older generation (Table). ECGs of patient IV-1 with AF and patient III-7 with a supraventricular tachycardia (presumably atrial) that preceded development of his paroxysmal AF are depicted in Figure 3. Natriuretic peptide levels were unavailable for the patients.

Linkage Analysis and Mutation Screen

Because TBX5 mutations are associated with HOS, we performed linkage analysis with 4 microsatellite markers surrounding the *TBX5* gene in 6 affected family members and 1 nonaffected relative related by marriage (1 branch, II-5 and further, and his brother II-10, the other haplotypes are implied). Linkage to the *TBX5* gene was found (with a multipoint logarithm of the odds score of 0.98), because all investigated affected members share the same 4-2-4-1 haplotype (Figure 1). Inclusion of the G125R mutation increases the logarithm of the odds score to 1.51. Subsequent sequencing of *TBX5* revealed a heterozygous single–base pair substitution at nucleotide 373 (Figure 4A), resulting in

a G>C substitution shared by all the affected patients (c.373G>C, DNA was unavailable of I-2, II-2, II-4, and II-12). This nucleotide change results in the substitution of an evolutionary conserved glycine for an arginine, p.G125R (Figure 4A and 4B; reference sequence NM_000192.3). Family members without skeletal findings, heart anomalies, or the risk haplotype do not carry the *TBX5* mutation. The p.G125R mutation is absent from 600 chromosomes of control individuals. According to a TBX5 homology model, the introduction of such a positively charged arginine at position 125 could lead to a favorable charge– charge interaction with the negatively charged DNA, because the mutant arginine side chain is in close proximity to the DNA (7Å) (Figure 4C).

No mutations were found in either connexin (Cx)40 or TBX3, nor were Cx40 haplotypes associated with AF. See the data supplement for details.

G125R TBX5 Protein Displays Enhanced DNA Binding and Normal Nkx2-5 Interaction

The primary function of TBX5 proteins is the regulation of gene transcription by binding to DNA target sequences, often in cooperation with protein partners. One of the best studied examples of this is the interaction with the NKX2-5 transcription factor.[12] Initially, we assessed the DNA binding of the G125R TBX5 mutant protein in HEK-293 cells transfected with FLAG-tagged TBX5 expression plasmids. In addition, G80R, a well-described *TBX5* mutation was included,[23] as was G125E, to study the effect of a negatively charged side chain in comparison with the positively charged arginine at position 125. All of the wild-type and mutant proteins were successfully expressed (data not shown). Subsequently, using the nuclear extracts of the transfected HEK-293 cells, EMSAs were performed using a probe corresponding to the TBE site at position −250 in the *Nppa* promoter, previously shown to be required for T-box factor–mediated *Nppa* gene regulation in vivo.[18] This element also contains a NKE, required for Nkx2-5 binding. In Figure 5, we show the effect of TBX5 missense mutations on *Nppa*-DNA binding activity. Both wild-type TBX5 and the G125R and G125E mutants bind to the probe, whereas G80R[23] mutant is unable to bind (Figure 5A). To investigate the DNA binding strength, we varied the amounts of wild-type and mutant G125 proteins. As can be appreciated from Figure 5B, mutant TBX5 G125R protein shows a strong and significantly enhanced DNA binding, up to 6 times, in comparison with wild-type TBX5 or G125E TBX5 at all protein amounts. G125E shows a slightly attenuated DNA binding, which is in line with the effect of a negative charge on this position, although this was not significant (P=0.07).

The interaction between the TBX5 mutants and Nkx2-5 and the result on DNA was investigated using EMSAs (Figure 5A), an in vitro yeast 2-hybrid X-gal assay (Figure 6A) and a pull-down assay (Figure 6B). There was no difference in the strength of the

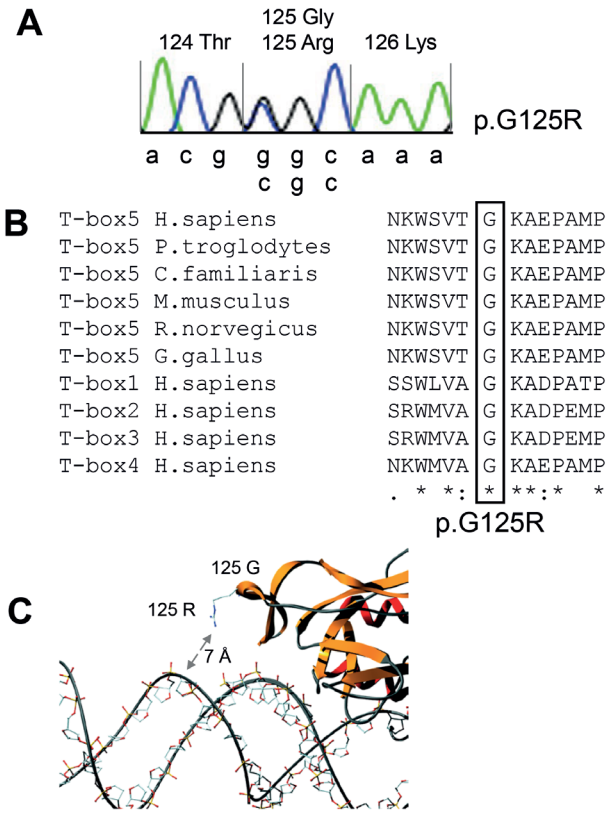

Figure 4. A, Electropherogram depicting the nucleotide change and consequent amino acid change of the p.G125R mutation in the proband. B, Alignment of parts of the TBX5 protein with various species and human TBX1-4 proteins. C, TBX5 homology model of the p.G125R mutation showing that the introduced positively charged arginine is in close proximity (7 Å) to the negatively charged DNA.

interaction of wild-type or mutant TBX5 with Nkx2-5; this is further explained in the data supplement.

G125R TBX5 (Over)stimulates the Nppa and the Cx40 Promoter

Subsequently, we investigated the effect of the G125R and G80R TBX5 mutants on transcriptional activation of the *Nppa* and *Cx40* (Figure 7A). In accordance with previously published work,[18] wild-type TBX5 gives higher relative expression in

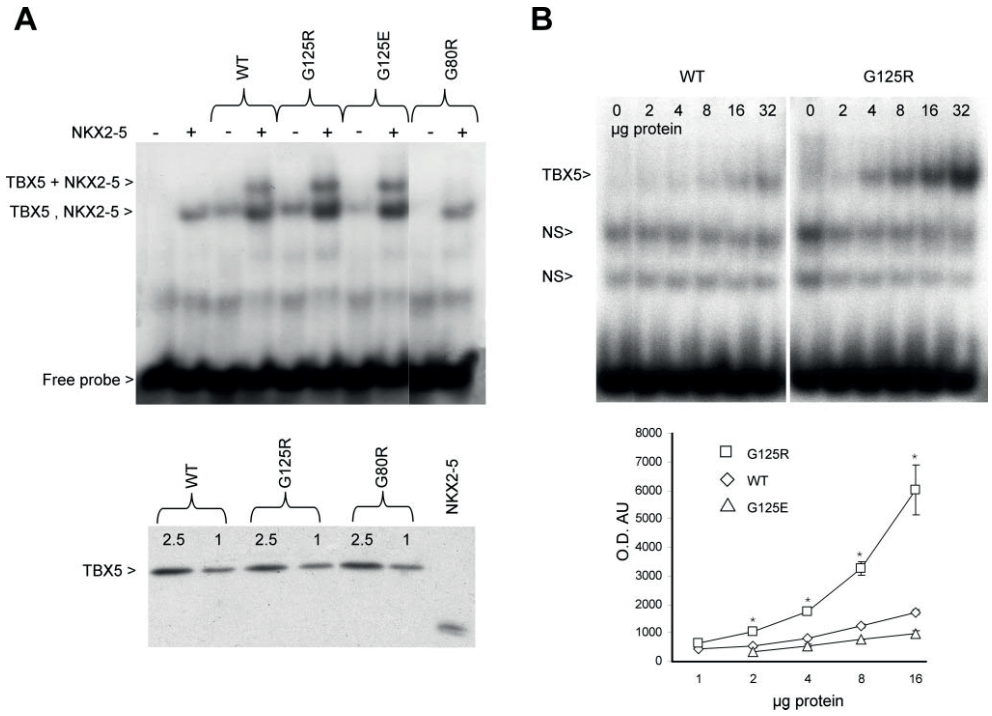

Figure 5. A, The effect of missense mutations of *TBX5* on DNA binding activity in the presence and absence of Nkx2-5. Note that p.G125R retains DNA binding ability and Nkx2-5 interaction. The bottom blot shows a Western blot indicating that equal amounts of all proteins used in the EMSA were loaded. B, Blot demonstrating the binding strength between DNA and various amounts of wild-type, p.G125E, and p.G125R TBX5 protein; nonspecific bands are marked with NS. Bottom, Quantification of DNA binding strength according to optical density using densitometric quantification of DNA (n=3, SD). *Statistically significant differences (Student's *t* tests, $P<0.05$) between G125R and wild-type or G125E TBX5.

the presence of Nkx2-5, whereas the G80R TBX5 mutation displays significantly lower *Nppa* activation with or without Nkx2-5 compared with wild-type TBX5.

Strikingly, the G125R TBX5 mutant displays a significantly higher activation of the *Nppa* promoter in the presence of Nkx2-5 in contrast to wild-type Tbx5 (mean difference 3 [95% confidence interval 0.7 to 5.3]). In absence of Nkx2-5 (0.70 [−0.13 to 1.53]), a similar trend can be identified although this difference is not significant ($P=0.09$) (Figure 7A). This effect is dose-independent, because the transactivation remains equal for both wild-type and G125R TBX5 regardless of the dosage of TBX5 (data not shown). Similar experiments were performed on the *Cx40* promoter (Figure 7B), again the G125R TBX5 mutation shows a significantly higher activation of the promoter than wild-type TBX5 (0.21 [0.04 to 0.39]), whereas the G80R mutant does not show any activation.

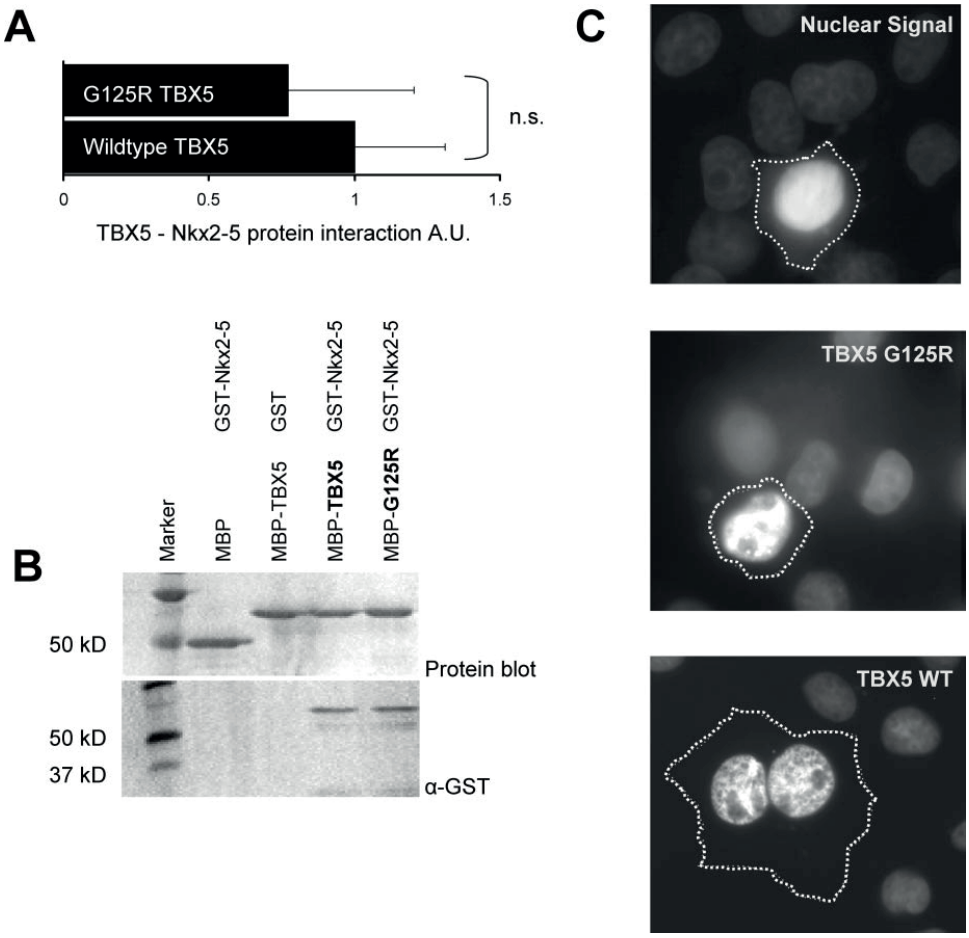

Figure 6. A, In vitro yeast 2-hybrid X-gal assay with p.G125R and wild-type TBX5 as prey and Nkx2-5 as bait, quantified with β-galactosidase filter assay; mean values±SD of 4 measurements on independent transformations (Student's *t* tests, $P<0.05$). B, Binding assays of wild-type and mutant TBX5 MBP/MBP fusion proteins. C, Trafficking of both wild-type and p.G125R TBX5 in COS7 cells. Transfected cells overexpressing TBX5 are immunostained with anti-FLAG for TBX5 and the nucleus is stained with 4',6-diamidino-2-phenylindole.

Trafficking of G125R TBX5 to the Nucleus Is Normal

To investigate whether the G125R mutant is correctly targeted to the nucleus, we transfected COS7 cells with both G125R mutant and wild-type TBX5. Using either an anti- FLAG antibody (Figure 6C) or a TBX5–yellow fluorescent fusion protein (data not shown). The G125R TBX5 protein is located in the nucleus, similar to wild-type TBX5 protein, indicating that the mutant is correctly transported to the nucleus.

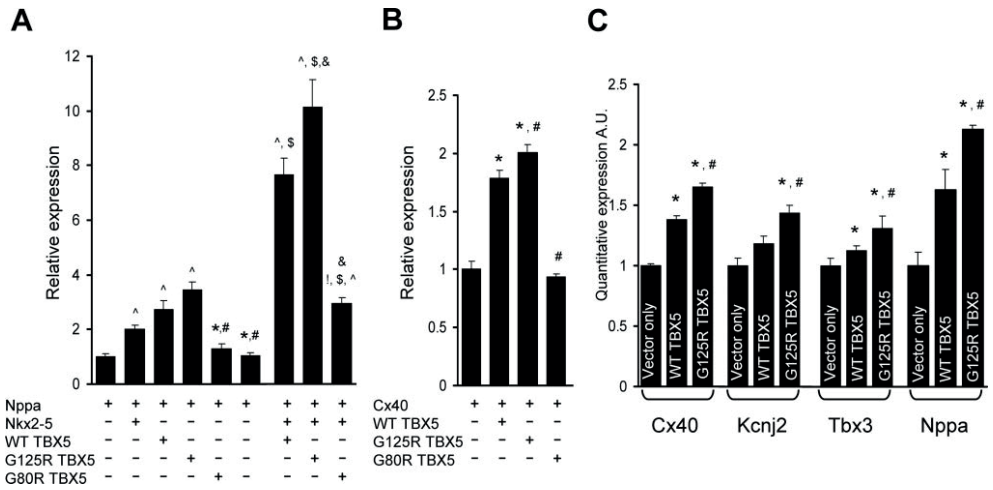

Figure 7. A, Transcriptional activation of Nppa promoter luciferase construct by wild-type and mutant TBX5 proteins with or without Nkx2-5 in H10 cells. Results are normalized to the Nppa construct. Statistically significant difference with Nppa construct alone; *statistically significant difference vs G125R TBX5 without Nkx2-5; #statistically significant difference vs wild-type TBX5 without Nkx2-5; &statistically significant difference vs wild-type TBX5 with Nkx2-5; !statistically significant difference vs G125R TBX5 with Nkx2-5; $statistically significant difference vs wild-type or mutant TBX5 with Nkx2-5 compared without Nkx2-5 (Student's *t* tests, $P<0.05$). Values are expressed as average±SEM, representing 3 independent transfections, each done in triplicate (n=9). B, Transcriptional activation of Cx40 promoter luciferase construct by wild-type and mutant TBX5 proteins in H10 cells. Values are expressed as average±SEM, rep resenting 3 independent transfections, each done in triplicate (n=9). *Statistically significant difference vs wild-type TBX5; #statistically significant difference vs G125R TBX5 (Student's *t* tests, $P<0.05$). C, Quantitative expression of Cx40, Kcnj2, Tbx3, and Nppa on transfection of H10 cells with vector only, wild-type TBX5, or G125R TBX5. Values are normalized on vector-only construct, expressed as average±SEM, representing 3 independent transfections, each done in triplicate (n=9). *Statistically significant differences between the wild-type or G125R TBX5 and vector only; #statistically significant differences between G125R TBX5 and wild-type TBX5 (Student's *t* tests, $P<0.05$).

TBX5 G125R Stimulates Gene Expression

To assay the effect of the mutant G125R protein on gene transcription, we transfected neonatal rat heart myocytes, immortalized with a temperature-sensitive SV40 T antigen (H10 cells[16]), with both the wild-type and mutant TBX5 protein. Endogenous levels of TBX5 in H10 are negligible (data not shown). Subsequently, using quantitative PCR, the transcription levels of various genes reported to act down-stream of

TBX5 (*Tbx3*, *Cx40*, *Nppa*, *Myh6*) or genes involved in or associated with AF (*Kcnj2*, *Cx40*, *Cx43*, *Hcn4*, *Kcne5*, *Kcna5*, *Kcne1*, *Kcne3*, *Kcnq1*) were measured. Consistent with the promoter analysis, transcripts of both *Nppa* and *Cx40* were significantly upregulated in H10 cells when transfected with the G125R mutant protein in comparison with wild-type TBX5 and vector control (Figure 7C). Interestingly, besides *Cx40*, transcripts of *Tbx3* and *Kcnj2* were also significantly upregulated on transfection with G125R TBX5 in comparison with vector control or wild-type TBX5 (Figure 7C). In contrast, although the expression levels of *Cx43*, *Hcn4*, *Scn5a*, and *Kcnh2* were detectable by quantitative PCR, these genes did not demonstrate a significant up- or downregulation in the presence of TBX5 G125R, in comparison with wild-type TBX5 (data not shown). Unfortunately, although transcript levels of *Myh6* are well known to respond to TBX5 levels, endogenous levels of this gene in H10 cells are not detectable by quantitative PCR, so the effect of G125R TBX5 on transcript levels could not be determined. Similarly, the transcripts levels of *Kcne5*, *Kcna5*, *Kcne1*, *Kcne3*, and *Kcnq1* were undetectable in H10 cells with or without wild-type or G125R TBX5. This was corroborated in a second cell line (supplemental Figure I) derived from embryonic heart tissue (H9C2 cells)[17]; this is further detailed in the data supplement.

Discussion

We have identified a novel missense mutation in *TBX5*; the p.G125R mutation is present in a large family with an atypical form of HOS, in which the majority of affected patients have both mild skeletal deformations and AF and few have congenital heart disease. The mutation results in normal Nkx2-5 interaction, enhanced DNA binding, and activation of the *Nppa* (*Anf*) and *Cx40* promoter, and upregulation of various genes.

Van Bever et al in 1996[15] already described the initial phenotype of the family and compared it with symptoms found in similar entities, such as HOS and heart-hand syndrome type II. It was concluded that the phenotype did not satisfy all HOS characteristics, because thumb, pectoral, or clavicle anomalies were found in none of the family members. Renewed careful examination, however, showed slight thenar muscular atrophy and limited opposition of the thumbs. These symptoms, however, are much less severe than expected in patients with HOS. Furthermore, only 13% of the affected individuals have a congenital heart malformation compared with 75% in the regular HOS population. However, among the most striking features is the occurrence of paroxysmal AF in the majority of the affected members, especially because most developed it at an unusually young age and in the absence of congenital heart disease. AF has occasionally been described in patients with HOS,[2, 3] although principally in the setting of congenital heart disease and as a result of hemodynamic effects. However, the question remains whether the detection of a TBX5 mutation

justifies the diagnosis of HOS. To date, no TBX5 mutations have been found in individuals with atypical phenotypes of HOS who did not meet the strict phenotypic criteria for HOS.[24] We suggest that the specific phenotype in this family with atypical signs of HOS relates to an underlying pathological mechanism different from other HOS patients, i.e., a gain-of- function (see below). Likewise, the phenotypes of other families or individuals hitherto not classified as HOS or as atypical may similarly be the result of a gain-of-function mutation.

TBX5 functions by DNA binding and activating target genes such as *Nppa* and *Cx40*, with or without a synergistic interaction with NKX2-5.[12, 25] It has been established that the N-terminal part of the protein, including the T-box, is essential for DNA binding, and that removal of the C-terminal 281 amino acids significantly enhanced DNA binding affinity.[11] In contrast to all currently known HOS TBX5 (missense) mutations, the G125R mutation exhibits normal Nkx2-5 interaction and transactivation, has a significantly increased DNA binding affinity, displays increased transcriptional activity on the *Nppa* and *Cx40* promoters, and significantly stimulates transcription of various endogenous target genes in comparison with wild-type TBX5. The location of the G125R mutation in the tertiary structure of the T-box supports an increase in DNA binding activity, because it is juxtaposed in close proximity to the bound DNA. A glycine-to-arginine substitution at this position could generate extra favorable charge–charge interaction with the DNA, resulting in enhanced DNA binding. Although the R279X TBX5 mutation is also reported to have an increased DNA binding, this was not quantified, nor was it shown that this mutant protein is actually present in the cell. Such a large deletion could lead in vivo to nonsense-mediated decay and consequently haploinsufficiency, as was also noted by the authors.[11] For TBX1 mutations, however, 3 gain-of-function mutations were recently reported. The authors concluded that this was likely the result of enhanced stabilization of the protein dimer DNA complex.[26] In contrast, to the atypical HOS phenotype caused by the TBX5 gain-of-function mutation reported in this study, the phenotype of the gain-of- function TBX1 mutation patients was the same as those with loss-of-function or deletions.[26] An extended discussion on this topic can be found in the data supplement.

The initial formation of limbs is initiated by the expression of FGF10 in the limb field mesenchyme and this expression is directly regulated by TBX5.[27] The subsequent patterning of the digits, wrist bones, and sternum occurs via Cx40-containing gap junctions, again regulated by TBX5.[28] Because both *FGF10* and *Cx40* are highly sensitive to TBX5 dosage, it is not surprising that the skeletal phenotypes of the family reported here reflects the underlying mutation, namely a mild skeletal phenotype as a result of a gain-of-function mutation, in which the core functionality of TBX5 is retained, in contrast with the more severe phenotypes caused by the common loss-of-function TBX5 mutations.[13]

We also investigated whether the prevalent paroxysmal AF in this family could be the

consequence of a change in expression levels of genes that are known to be associated with AF. Several studies indicate that genetic variations in the *Cx43* and *Cx40* may predispose to arrhythmia vulnerability in humans[29] There was no association between promoter *Cx40* polymorphisms and AF, but G125R TBX5 leads to a significant enhanced transcription of endogenous *Cx40*. However, these results are difficult to interpret because G125R TBX5 also enhances expression of *TBX3* (see below), which is known to decrease Cx40 levels. Recently, a familial KCNJ2 mutation cosegregating with AF was found.[30] Interestingly, the G125R mutant significantly enhanced the transcription of KCNJ2 in comparison with wild-type TBX5. Furthermore, because overexpression of KCNJ2 in mice induces AF,[31] and acquired AF in human is associated with increased expression of the *I*K1 channel,[32] increased expression of KCNJ2 under the influence of G125R TBX5 could potentially contribute to or underlie the observed AF.

Recent publications demonstrated that TBX3 is highly sensitive to TBX5 dosage[33] and that it controls the sinoatrial node gene program.[34] TBX3 is selectively expressed in the entire central conduction system.[35] Strikingly, ectopic expression of TBX3 in mice induces pacemaker genes and leads to ectopic pacemaker activity, which is a precursor for AF.[34] Thus, because we found significantly enhanced transcription of TBX3 in the presence of G125R TBX5, this could suggest that induced TBX3 expression may contribute to the paroxysmal AF phenotype in atypical HOS patients, analogous to the mouse model.

In conclusion, contrary to previously published HOS mutations, we identified a gain-of-function TBX5 mutant that associates with an atypical, mild form of HOS and paroxysmal AF. A possible role of TBX5 in the development of paroxysmal AF based on a gain-of-function is suggested either through a direct mechanism via TBX5 or indirectly via TBX5 stimulated TBX3, although other mechanisms cannot be ruled out. These findings might warrant a renewed look at the phenotypes of families and individuals hitherto not classified as HOS or as atypical presenting with paroxysmal AF, because these may possibly be the result of additional TBX5 gain-of-function mutations.

Limitations of the Study

We identified a cosegregating TBX5 missense mutation in a family with atypical HOS and paroxysmal AF, and we argue that this mutation underlies both phenotypes. Because we did not have myocytes of the proband or family members, and we are not able to get them, we lack a human molecular correlate and thus cannot proof in vivo

that this mutation leads to enhanced transcription of the various identified (AF) genes. One way around this is to test the functional consequence of the gain-of-function in a Tbx5 knock-in mouse model. Although feasible, it is likely that (because of the small size) these mice might not develop AF, limiting its usefulness, although they may exhibit electrophysiological changes in atrial conduction and excitability. We are currently exploring this possibility.

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Disclosures

None.

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Data supplement

Material and Methods

Linkage analysis, Mutation screen

Genomic DNA of family members was extracted from peripheral blood according to standard procedures. PCR amplification, labelling with Cy5dCTP (Amersham) and electrophoresis using automated laser fluorescence (ALF, Pharmacia Biotech) of these markers was performed according to standard methods. *TBX5* was PCR amplified using *TBX5* specific primers (table 2), located in flanking intronic sequences. PCR products were analyzed by direct sequencing, using the BigdyeTerminator v3.1 Kit on an ABI 3700 Genetic Analyzer (Applied Biosystems). Mutation analysis of *NKX2-5*, *CX40* and *TBX3* was also performed in family members III-7, III-9, IV-1 and IV-3. Primers were located in flanking intronic sequences (see table 2).

Plasmid constructs

The full-length human FLAG-TBX5 and full-length mouse FLAG-Nkx2-5 expression plasmids and the pGL3-Nppa-luciferase and pGL3-CX40-luciferase reporter plasmids have been described previously.[1, 2] The missense mutations p.Gly125Arg, p.Gly125Glu (control) and p.Gly80Arg[3] were introduced into the wild-type FLAG-TBX5 construct by PCR-based site-directed mutagenesis (QuickChange II Site-Directed Mutagenesis Kit, Stratagene) and verified by sequencing.

Reporter gene assays

Neonatal rat heart myocytes, immortalized with a temperature-sensitive SV40 T antigen (H10 cells[4]) and the embryonic heart tissue cell line(H9C2 cells[5]) were cultured in a 12-well plate at 33°C under standard conditions for 48h after transfection. One day before transfection, cells were plated at a density of 2×10^5 cells/well. Escort V (SIGMA) was used for transfection of H10 cells according to the manufacturer's instructions. Cells were transfected, per well, with 700ng of luciferase reporter plasmids and 100ng of each expression plasmid. The total amount of DNA used for transfection was kept constant at 700ng by addition of empty pcDNA3 vector. In every experiment 1-2.5ng of Renilla luciferase plasmid, phRL vector (Promega) was co-transfected as an internal control. Transfected cells were washed once the following day with medium before being refreshed with new medium. 48 hours after transfection, cells were lysed and luciferase activity was measured with a luminometer (Glomax 20/20, Promega) by using the Renilla reporter assay system (Promega).

Electromobility shift assays and Western blot analyses

Electromobility shift assays (EMSAs) are used to detect the interaction of DNA binding proteins with their DNA recognition sequences. Purified proteins or crude cell extracts are incubated with a radiolabelled DNA probe. The complexes are separated from the free probe by migration through a nondenaturing polyacrylamide gel, with the complexes migrating more slowly. Nuclear extracts were prepared from HEK cells cultured under standard conditions for 48h after transfection, in 10cm dishes (1×10^6 cells/dish) and transfected with 40μg of expression constructs. EMSA was performed as described previously.[1] Oligonucleotide used was: TN (corresponding to the TBE-NKE site at position −250 in the Nppa promoter, 5'-TCTGCTCTTCTCACACCTTTGAAGTGGGGGCCTCTTG). Western blot analysis of nuclear extracts was performed according to standard procedures. Primary antibody used was anti-FLAG rabbit IgG (1:700; ABR) and the secondary antibody was anti- rabbit IgG-HRP (1:5000; Amersham). The complexes were visualized using the ECL detection kit (Amersham).

In vitro Yeast two-hybrid X-gal assay

To obtain full-length TBX5 cDNA, we screened a gt11 human fetal heart cDNA library (Clontech). We generated the p.G125R TBX5 mutant by PCR and subcloned these into a pcDNA3 plasmid (Invitrogen). Bait (pcDNA3.1-mouse NKX2-5 carrying the GAL4 DBD fusion construct) and prey (pcDNA3.1-human TBX5wt/Gly125Arg carrying the GAL4 AD fusion construct) were co-transformed into Saccharomyces cerevisiae AH109 (Clontech), and then the ability to grow on minimal media was examined. The strength of protein interaction was quantitated using a β-galactosidase filter assay. To measure the interaction quantitatively, β-galactosidase liquid assays were carried out using four independent colonies. Chlorophenol red-β-D-galactopyranoside (CPRG) was used as substrate.

In vitro binding assays

Basically, equivalent amounts of MBP/MBP-fusion protein from cleared BL21 DE3 cell lysates, were loaded onto PBS-triton equilibrated with 300μl amylose resin (New England Biolabs) columns. The MBP/MBP-fusion loaded resin was subsequently washed with 1ml PBS-triton. A total volume of 1ml PBS-triton containing approximately 100μg of purified GST/GST-fusion was then passed over the MBP/MBP-fusion loaded amylose resin. The column was subsequently washed with 4ml PBS-triton before elution of the MBP/MBP- fusion with 0.5ml PBS-triton containing 20mM maltose. 10μl of eluate was used for SDS- PAGE and Western blot analysis.

Immunocytochemistry

COS7 cells were seeded on cover slips (18mm, Fisher Emergo) in a standard 12-well plate at a density of 1×10^5 cells and cultured under standard conditions for 24-48h after transfection. Cells were then fixed in 2% paraformaldehyde, washed in PBS, permeabilized in 0.3% Triton X-100, washed in PBS and incubated with the primary antibody anti-FLAG rabbit IgG (1:1000; ABR) in PBS/1% BSA at 4°C overnight. The next day the secondary antibody, Alexa488-conjugated goat anti-rabbit IgG (Molecular Probes), was added and incubated at room temperature for 1h. Slides were mounted using anti-fading vectashield with DAPI and cells were viewed under a Zeiss Axiophot fluorescence microscope equipped with a Leica DFC 320 camera.

RNA isolation, cDNA synthesis and qPCR

H10 cells were plated at a density of 4×10^5 cells per well on a 6-well plate. Escort V (SIGMA) was used for transfection according to the manufacturer's instructions. Cells were transfected, per well, with 1.25µg of each expression plasmid in the presence of DMEM 1x (GIBCO). Transfected cells were washed the following day once with medium before refreshing them. 48h after transfection, total RNA was prepared using the Nucleospin RNA II kit (Clontech) following the manufacturer's recommendations. Isolated RNA was reverse transcribed and amplified using oligo(dT) primer and the Superscript II RT-PCR kit (Invitrogen) following the manufacturer's protocol.
For qPCR, briefly, a master mix was aliquoted to a 384-well plate (ROCHE). Each well contained 0.3-5.0µl (depending on the gene of interest) cDNA, 5µl of 2x SybrGreen I mix (ROCHE), 0.5µl of primer mix (10 pmol/µl of each primer, see table 2) and supplemented with H20 up to 10µl. Real-time PCR protocol was as follows: preincubation 95°C 5 min, followed by 45 cycles of: 95°C 10 sec, 58 or 60°C (depending on the gene of interest) for 20 sec and 72°C for 20 sec. Readings were carried out on an ROCHE LC 480 Lightcycler System. The average/mean PCR efficiency was subsequently used for calculating the average mRNA copy number. HPRT expression levels were used to correct for variations in RNA input. qPCR levels were normalized on vector only.

Statistical analysis

Results are expressed as mean±SD or SEM as indicated in the legends. To remove variations from multi-session experiments due to day-to-day differences in transfection efficiency and expression levels, a *factor correction* (calculated as previously described[6]) was applied. The corrected data set was used for statistical analysis. Comparisons amongst two groups was performed using Student's t tests. $P<0.05$ was considered statistically significance.

Results

TBX3 and CX40 genetic screen

Polymorphisms in the promoter region of *CX40*, an atrial specific gap junction protein, are implicated in atrial fibrillation.[7] Haplotype analysis did not reveal linkage between the SNPs in the *CX40* promoter region and atrial fibrillation (data not shown), nor were mutations present in the coding region of *CX40*. As overexpression of *TBX3* has recently been shown to lead to ectopic pacemaker activity[8](see discussion), and because it lies in very close proximity to *TBX5* and is present in the linking chromosomal region, it was also screened. No mutations were identified.

TBX5, NKX2-5 Interaction

Co-transfection of Nkx2-5 expression plasmids allowed us to investigate the interaction between TBX5 mutants and Nkx2-5 and the result on DNA binding. With the addition of Nkx2-5, complexes of TBX5-Nkx2-5 can be seen in the wild-type as well as the G125R and G125E mutants (0A), but these complexes are absent in the presence of the G80R mutant. Moreover, using an *in vitro* yeast two-hybrid X-gal assay, we determined that there was no difference in the strength of the interaction of wild-type TBX5 or G125R TBX5 with Nkx2-5 (figure 6A), indicating that the mutation does not disturb protein-protein interaction. This was further confirmed by use of a pull down assay, as both wild-type and G125R TBX5 protein can be pulled down using Nkx2-5 protein (figure 6B), indicating that the TBX5 G125R mutant binds DNA and associates with Nkx2-5 in a manner comparable to wild-type TBX5 protein.

TBX5 G125R also stimulates gene expression in *H9C2* cells

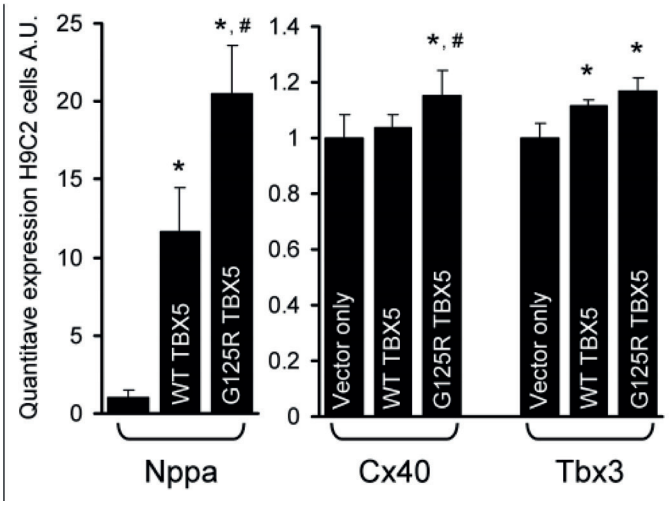
We performed similar experiments in a second cell line derived from embryonic heart tissue (H9C2 cells).[5] Correspondingly to the response of G125R TBX5 in H10 cells, transcripts of both *Nppa* and *Cx40* were significantly up-regulated in H9C2 cells transfected with G125R mutant protein in comparison to wild-type TBX5 and vector control (supplemental figure 1). Although *Tbx3* levels showed a comparable trend, this was not statistically significant between wildtype and mutant TBX5, but *Tbx3* was significantly up-regulated for both groups in comparison to vector only. Unfortunately, endogenous levels of *Kcnj2* were not detectable by qPCR in these cells.

Discussion

TBX1 vs *TBX5* gain-of-function phenotypical discussion

In contrast, to the a-typical HOS phenotype caused by the TBX5 gain-of-function mutation reported in this study, the phenotype of the gain-of-function TBX1 mutation

patients was the same as those with loss-of-function or deletions.[9] This was in line with the fact that mice that over- or under-express Tbx1 have identical phenotypes,[10] supporting the idea that both loss- of-function and gain-of-function mutations lead to the same phenotype for TBX1 mutations. In contrast, the gain-of-function TBX5 mutant reported here results in a phenotype distinctly different from loss-of-function TBX5 mutations, indicating a difference in dosage sensitivity in the heart and limbs for TBX5. Although Tbx5 overexpression has been studied in mouse models with a cardiac-specific promoter,[11] the level of over-expression is relatively high, i.e. much higher than what would be expected in case of a gain of function mutation that we describe. The general result is a thinned and hypoproliferative ventricular myocardium, which is roughly reminiscent of what is published about Tbx5 knockout mice, but less dramatic.[12] However, unpublished data from our lab, using a similar overexpression model, suggest that heart development is arrested and the chambers fail to form, but, unlike the Tbx5 knock-out, activation of *Nppa* and *Cx40* is seen. Taken together, even high levels of over- expression of this very dose-sensitive transcription factor leads to a response distinct from deficiency.



Supplement Figure 1 Quantitative expression of Nppa, Cx40, and Tbx3 upon transfection of H9C2 cells with vector only, wild-type TBX5 or G125R TBX5. Values are normalized on vector only construct, expressed as average ± SEM, representing three independent transfections, each done in triplicate, n=9. * indicates statistically significant differences between the wild-type or G125R TBX5 and vector only, and # between G125R TBX5 and wild-type TBX5 (Student's t tests, $P<0.05$).

Supplement table 1 Primer pairs for amplification of human TBX5 and rat qPCR primers

Exon	Forward Primer 5'-3'	Reverse Primer 5'-3'	Amplicon (bp)	Tm (ºC)
5'UTR	GCG CCT CGG TAT TCA TTT	AGG GCC AAG TGC AAA GA	760	65-55
1	TGC TCC CTC CTG TCA CTA GA	CGT TGG GTT CGT TTT GG	405	65-55
2	GGG AAG GAG GAG CAG TCT CT	TCT TCA CCT CTC CCA CAA TTT	251	65-55
3	CCC GGG ATG GAT CTT G	GGA CAG ACG CCT TTA GCA	401	65-55
4	GGG AGA GCC TCC AGA TTA TTC	AAT GGA GGA AAG AAA AGG AGA A	403	65-55
5	TGT GTG CCT TGG GTA GAT TC	GCA GAT TCA TGC AAA AGA AAG	452	65-55
6	ACG TGA CTG GCT TAA TTT GCT	GGT TGC TGC TGG CTT ACC T	301	65-55
7	TCA GCC ACT CAG GAA ATC TG	GGA ACA TGT CAA GGG AAC TTT	481	65-55
8+3'UTR	GCC AAA TAA CTG TCT CCA CTT TT	GCA TCC AGC GAC CTT GAG	803	65-55
Gene				
rat Nppa	ATT TCA AGA ACC TGC TAG ACC A	CAA GAG GGC AGA TCT ATC GG	217	60
rat Cx40	AAG AAT CCA GAC CCT CTG GC	TGA CCT TGC TTT GCT GCT G	189	58
rat Cx43	ATG GGT GAC TGG AGT GCC TTG	GAA GCG CAC GTG AGA GAT GG	231	58
rat Tbx3	CTG CGT TAC AGC CCG TAT TC	AGC GGC TAT TCA GTT CCG AC	175	58
rat Kcnj2	TCC ATT GAG ACC CAG ACA AAC	CCA TGA AAA CAG CAA TCG GG	76	58
mouse Scn5a*	GGG ACT CAT TGC CTA CAT GAT GA	GCA CTG GGA GGT TAT CAC TG	137	58
rat Myh6	ATT TCA AGA ACC TGC TAG ACC A	CAA GAG GGC AGA TCT ATC GG	218	58
rat HPRT	GGT CCA TTC CTA TGA ACT GTA GAT TTT	CAA TCA AGA ACG TTC TTT CCA GTT	125	60
rat Kcnh2	CTTACTGCCCTCTACTTCATCTC	GAATCATGTTGGTATCTCGAAGG	279	58
rat Kcnq1	GTT TCG TGT ACC ACT TCA CC	ACA AGG ACA ATC TCC ATC CAG	126	58
rat Hcn4	ATG TTC GGC AGC CAG AAA GC	GAC TTG GTC CTC AAC TTC CGC	249	58
rat Kcne1	GCA GAG GTT TTG CTC CAC ATC AG	AAG GCA GAA CAG TTG TGG AAT TG	77	58
rat Kcne2	AAA TCT TCG TCC AAT CGT	GCG TCA AGT TGG CTA AAG	108	58
rat Kcne3	CAG AGT CAG ATC ACA TTC CAG CTC	GGT CTC AGT CCC GTT GGA AG	76	58
rat Kcna5	TCT GCA GTC GCT TGG AGT GG	GCC ATC CTC AGG GTG ATC C	92	58
rat Kcne5	TTT ATA TCT GGG CCT GGC TCC	TCA TCA TCT TCT GGG CCT CTG	76	58

*primers work on rat cDNA

Supplement table 2 location of microsatellites and genes

Name	centiMorgan	Basepair	Heterozygosity
D12S129	122.86 cM	111,359,715	1
D12S821	127.10 cM	113,049,753	0.818
D12S354	130.90 cM	113,511,973	0.74
D12S369	131.57 cM	113,995,727	0.8
TBX5	-	113,276,119	-
TBX3	-	113,592,442	-

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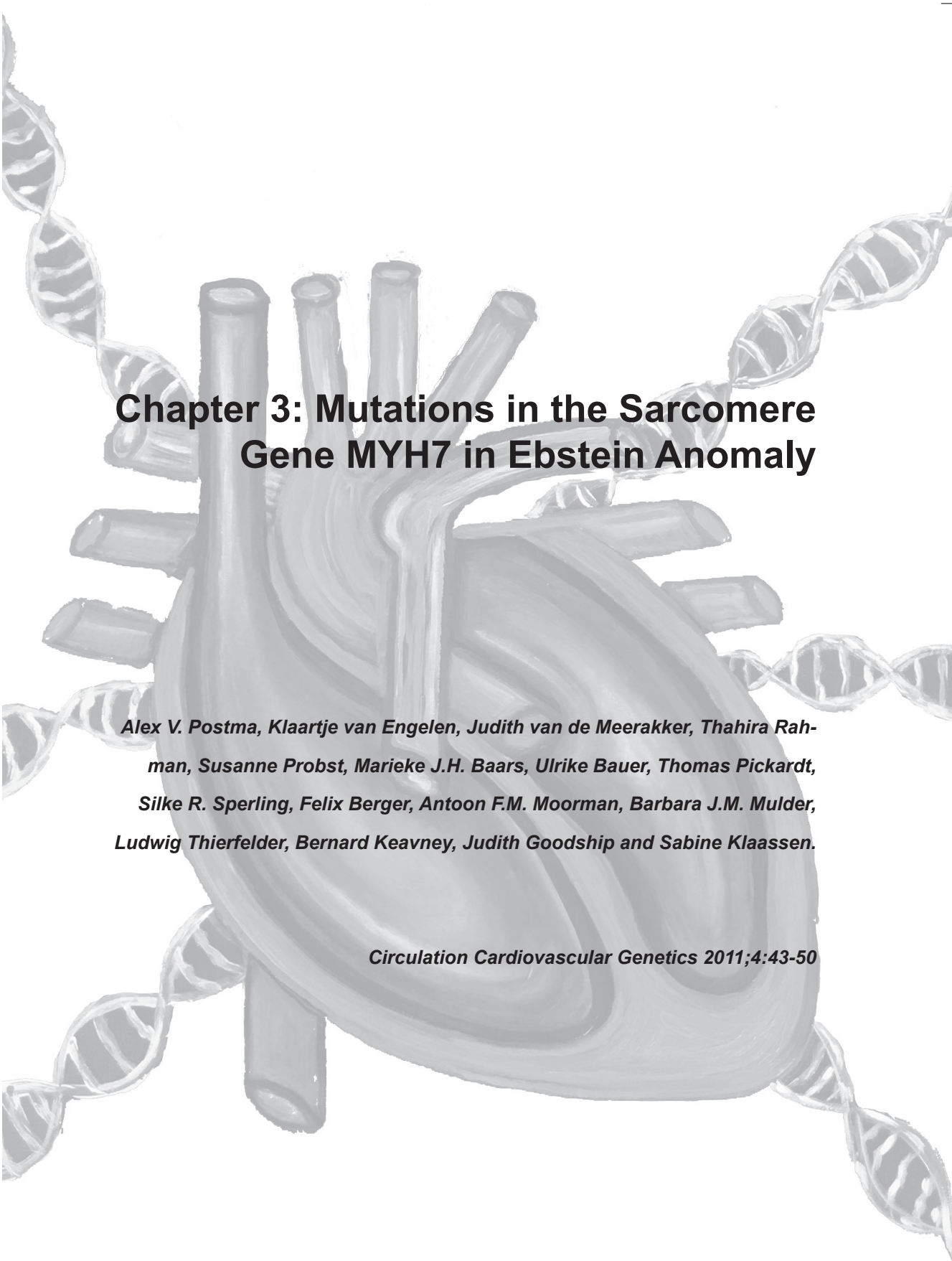

Chapter 3: Mutations in the Sarcomere Gene MYH7 in Ebstein Anomaly

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Background

Ebstein anomaly is a rare congenital heart malformation characterized by adherence of the septal and posterior leaflets of the tricuspid valve to the underlying myocardium. An association between Ebstein anomaly with left ventricular noncompaction (LVNC) and mutations in MYH7 encoding β-myosin heavy chain has been shown; in this report, we have screened for MYH7 mutations in a cohort of probands with Ebstein anomaly in a large population-based study.

Methods and Results

Mutational analysis in a cohort of 141 unrelated probands with Ebstein anomaly was performed by next-generation sequencing and direct DNA sequencing of MYH7. Heterozygous mutations were identified in 8 of 141 samples (6%). Seven distinct mutations were found; 5 were novel and 2 were known to cause hypertrophic cardiomyopathy. All mutations except for 1 3-bp deletion were missense mutations; 1 was a de novo change. Mutation-positive probands and family members showed various congenital heart malformations as well as LVNC. Among 8 mutation-positive probands, 6 had LVNC, whereas among 133 mutation-negative probands, none had LVNC. The frequency of MYH7 mutations was significantly different between probands with and without LVNC accompanying Ebstein anomaly ($P<0.0001$). LVNC segregated with the MYH7 mutation in the pedigrees of 3 of the probands, 1 of which also included another individual with Ebstein anomaly.

Conclusions

Ebstein anomaly is a congenital heart malformation that is associated with mutations in MYH7. MYH7 mutations are predominantly found in Ebstein anomaly associated with LVNC and may warrant genetic testing and family evaluation in this subset of patients.

Ebstein anomaly is a rare congenital heart malformation affecting both the tricuspid valve and right ventricle.[1] The septal and posterior leaflets of the tricuspid valve are displaced apically and divide the right ventricle into two portions. The effective tricuspid orifice is displaced downward into the right ventricular cavity, at the junction of the inlet and apicotrabecular components of the right ventricle. Tricuspid valve incompetence is the main hemodynamic abnormality in Ebstein malformation. A secundum atrial septal defect (ASDII) is present in more than one-third of patients, and the majority of the remainder have a patent foramen ovale resulting in a right-to-left shunt.[2] Abnormalities of left ventricular morphology and function, as well as other left-sided heart lesions, also occur in Ebstein anomaly,[3, 4] in one study, 18% of patients had left ventricular dysplasia resembling left ventricular noncompaction (LVNC).[4] The genetic basis of Ebstein anomaly is largely unresolved. Although Ebstein anomaly is more common in patients with a family history of congenital heart disease,[5] most cases are sporadic and familial Ebstein anomaly is rare. Mutations in the cardiac transcription factor *NKX2.5* are responsible for a variety of cardiac structural anomalies including Ebstein anomaly and ASD.[6] In 1 LVNC family carrying a mutation in *MYH7* encoding the sarcomere gene β-myosin heavy chain (*MYH7*), 4 individuals had Ebstein anomaly.[7] Mutations in sarcomere genes are a major cause of cardiomyopathy. LVNC has recently been classified as a primary cardiomyopathy with a genetic etiology,[8] and is morphologically characterized by a severely thickened 2-layered myocardium, numerous prominent trabeculations, and deep inter-trabecular recesses.[9] Mutations in 6 sarcomere genes, *MYH7*, *a*-cardiac actin (*ACTC1*), cardiac Troponin T (*TNNT2*), α-tropomyosin (*TPM1),* cardiac Troponin I (*TNNI3*), and cardiac myosin-binding protein C (*MYBPC*3) have been described in familial or nonfamilial LVNC.[10-14] *MYH7* is the most frequent disease gene (13%) in adult patients with LVNC, in the absence of other congenital heart anomalies.[13] Interestingly, mutations in *ACTC1* have been associated with ASD and cardiomyopathy,[15, 16] and some individuals have both defects.[14] Because a possible association between Ebstein anomaly with LVNC and *MYH7* mutations previously was shown, this led us to test the association between Ebstein anomaly and *MYH7* mutations in a large cohort. We performed mutational analysis of *MYH7* in a cohort of 141 unrelated probands with Ebstein anomaly using both next generation sequencing and direct DNA sequencing. Mutations were identified in 8 of 141 probands (6%), the largest resequencing study of Ebstein anomaly so far. We provide further evidence for a link between structural proteins, cardiomyopathy, and congenital heart malformations.

Methods

Clinical Evaluation

Unrelated patients were recruited from 3 sources: (1) CONCOR (National Registry and DNA bank of congenital heart defects), The Netherlands (n=114); (2) National Registry for Congenital Heart Defects, Berlin, Germany (n=19); and (3) The Institute of Human Genetics, Newcastle University, United Kingdom (n=8). Informed consent was obtained from all participants according to established guidelines. Probands and available family members were evaluated by history taking, review of medical records, physical examination, 12-lead ECG, and transthoracic echocardiography. All probands had a physical examination for dysmorphic features, and patients with abnormalities pointing to syndromic features or neuromuscular involvement were excluded. Echocardiography in Ebstein anomaly shows apical displacement of the septal leaflet of the tricuspid valve from the insertion of the anterior leaflet of the mitral valve by at least 8 mm/m^2 body surface area.[3, 4] Marked enlargement of the right atrium and atrialized right ventricle may be present, as well as varying degrees of regurgitation of the tricuspid valve. The diagnosis of LVNC was made by echocardiography, based on the presence of the established criteria by Jenni et al.[9] In partially penetrant cases of LVNC, the ratio of noncompacted to compacted myocardium is <2. A diagnosis of LVNC was made irrespective of the presence of heart failure or left ventricular systolic dysfunction.[8] Echocardiographic studies were performed/reviewed by 2 independent observers.

Mutation Screening

Mutation screening was carried out with genomic DNA samples from 141 probands. Fusion primers were designed using Primer3 and IDT primer design portal to amplify *MYH7* (Genbank accession No. NM_000257) coding and 5'/3' untranslated regions; 20 ng of genomic DNA was amplified using FastStart HighFidelity enzyme in a total reaction volume of 50 μl. Amplification was performed by initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at the recommended temperatures for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 2 minutes. Amplicons were purified using Solid-Phase Reversible Immobilization beads (Beckman Coulter Genomics, High Wycombe, Buckinghamshire, United Kingdom). Amplicon quality was assessed using the DNA 1000 LabChip on an Agilent Bioanalyzer and quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, United Kingdom). Fifty PCR amplicons from 141 patients were pooled at equimolar ratios and sequenced on 3 GS FLX LR70 PicoTitrePlates. Library immobilization and emulsion PCR were performed using genome sequencer (GS) emPCR kits II and III (Roche, Welwyn Garden City, Hertfordshire, United Kingdom). After DNA bead recovery,

bead enrichment and sequencing-primer annealing, the DNA beads, packing beads, and enzyme beads were deposited on a GS FLX PicoTiter Plate and sequenced using GS LR70 Sequencing kit (Roche). GS reference mapper was used to map sequence reads obtained with reference sequences from the Human genome hg18 assembly (NCBI build 36.1). The average read length was 244 bp, and average fold coverage was 45X per allele. Putative variants detected by GS Amplicon Variant Analyzer software (Roche) that were supported by both forward and reverse reads or with a variant frequency of >1.0% on either the forward or reverse reads were selected for further analyses by MassARRAY MALDI-TOF (Sequenom, San Diego, CA) to validate changes and, as the sequencing had been carried out in pooled samples, to identify in which samples they were present. After this, each change was confirmed by Sanger sequencing as previously published.[13] When a putative mutation was identified, at least 490 ethnically matched controls (980 chromosomes) were screened for the absence of the sequence variation (P<0.0001). The microsatellite marker D14S990 was used to rule out a founder mutation for *MYH7*.[13]

The Fisher exact test was used to analyze noncontinuous data; probability values <0.05 were considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

A cohort of 141 unrelated white individuals of western European descent (61 men and 80 women; 138 adults and 3 children; mean age, 46 years; range, 4 months to 77 years) with the diagnosis of Ebstein anomaly were investigated. Sixty-four probands had no associated cardiac anomalies, the most common associated cardiac malformation being ASDII (48 probands) and cardiomyopathy (7 probands) (Table 1). Heterozygous mutations were identified in 8 of 141 samples (6%). Seven distinct mutationsere found; 5 were novel and 2 were known to cause hypertrophic cardiomyopathy. Two probands had the same mutation. All mutations except for 1 3-bp deletion are missense mutations. In 6 of 8 probands with *MYH7* mutations LVNC was identified in addition to Ebstein anomaly. One of 8 mutation-positive probands had partially penetrant LVNC (kindred 109.787, III-2, Figure 1), and in 1 other proband (AD) LVNC was uncertain. *MYH7* mutations were not reported in the subcohort of 133 probands with Ebstein anomaly without evidence of LVNC. The frequency of w*MYH7* mutations between those Ebstein patients with LVNC and those without LVNC was significant (P=2 e-07, P<0.0001). Clinical phenotypes were assessed in all available family members of the 8 probands with mutations, and familial congenital heart malformations or cardiomyopathy was found in 3 of them. In these kindreds,

Table 1 Cardiovascular anomalies of probands

Phenotype of probands	N
Ebstein only	64
ASD II	42
PFO	7
Left ventricular noncompaction (with or without ASD, VSD, PFO, pulmonary artery hypoplasia)	6
CCTGA (with or without ASD, VSD, LVOT obstruction)	5
ASD, VSD	3
Pulmonary valve stenosis	2
VSD	1
VSD, PFO	2
Coarctation of the aorta	2
ASD II, PFO	1
Aneurysm of membraneous septum	1
Pulmonary valve stenosis and VSD	1
Aortic valve stenosis	1
Aortic valve abnormality	1
Partial anomalous pulmonary venous connections, sinus venosus ASD, PFO	1
Hypertrophic cardiomyopathy, LVOT obstruction,	1
Total	141

ASDII, secundum atrial septal defect; VSD, ventricular septal defect; PFO, patent foramen ovale; CCTGA, congenitally corrected transposition of the great arteries; LVOT, left ventricular outflow tract

MYH7 mutations segregated with LVNC, and there was an additional individual with Ebstein anomaly (110.647). The clinical characteristics of all family members with mutations are presented in Table 2 and described below.

Familial Cases Kindred 110.647

We identified a missense mutation at nucleotide 933 in exon 10, which replaces tyrosine with aspartic acid at residue 283 (designated Tyr283Asp) in the proband. She had been (III-6) diagnosed with Ebstein anomaly and ASDII at 29 years of age,

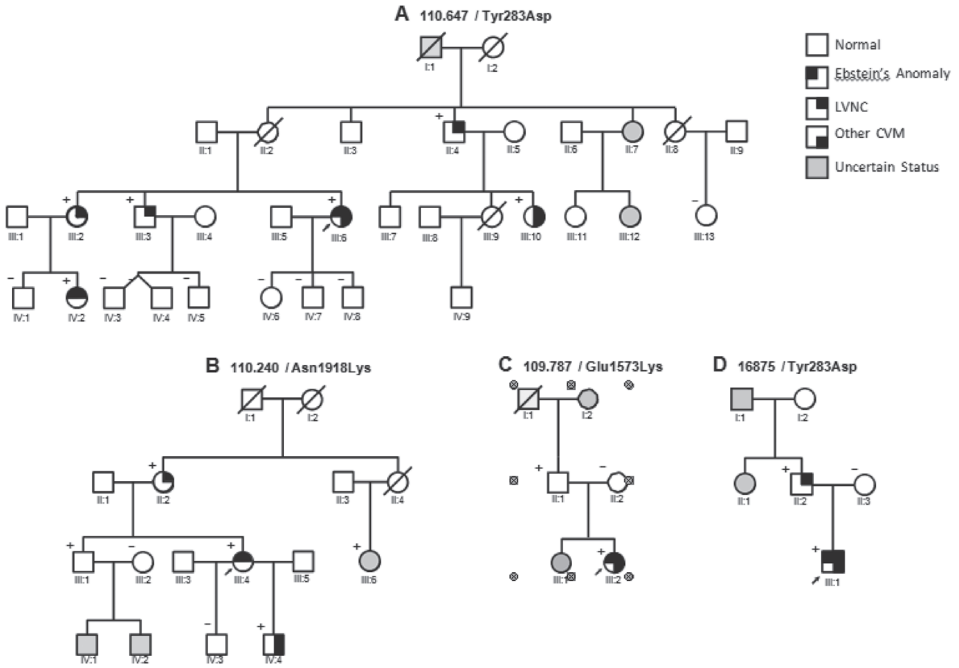

Figure 1. Pedigrees of kindreds with *MYH7* mutations. A, 110.647 (Tyr283Asp); B, 110.240 (Asn1918Lys); C, 109.787 (Glu1573Lys); and D, 16875 (Tyr283Asp). Filled symbols indicate cardiovascular phenotype by a box in the left rectangle/circle for Ebstein anomaly, in the upper right quadrant for LVNC, and in the right lower quadrant for other cardiovascular malformation (CVM). Open symbols indicate normal cardiovascular phenotype; shaded symbols, uncertain clinical status. Plus signs indicate the presence of a mutation; minus signs, absence of a mutation

which were surgically corrected. At age 49 years, echocardiography revealed LVNC with abnormal LV diastolic function. Family screening identified Ebstein anomaly and LVNC in the proband's 24-year-old niece (IV-2) (Figure 2A and 2B). Individuals III-2 and III-3 showed only mild left ventricular apical hypertrabeculation (partially penetrant phenotype). III-10 had been diagnosed with a perimembranous ventricular septal defect at 18 years. MRI performed at age 59 years because of palpitations, and the unexplained sudden death in her 40-year-old sister (III-9) showed marked LVNC with mildly abnormal systolic function. A cardioverter- defibrillator was implanted. Her asymptomatic father (II-4) was subsequently diagnosed with LVNC (Figure 1A).

Kindred 110.240

In the proband (III-4), a missense mutation (Asn1918Lys) in exon 39 was found. Ebstein anomaly was established after evaluation of a cardiac murmur at 3 years of

Table 2. Clinical characteristics of family members with *MYH7* mutations

				Echocardiography					
Family	ID	Age(y) /Sex	Mutation	Site of LVNC*	RV†	LVEDZ-score	EF/FS (%)	Type CVM	Cardiovascular complications
110.647	II-4	78/M	p.Y283D	2	no	-2.8	47/NA	None	None
	III-2	54/F	p.Y283D	1§	no	-1.9	68/36	None	None
	III-3	50/M	p.Y283D	1§	no	-2.2	NA/43	None	None
	III-6	49/F	p.Y283D	2	yes	-3.8	53/35	Ebstein´s, ASDII	TV reconstruction, ASD closure, NSAT
	III-10	49/F	p.Y283D	2	no	-2.0	65/35	Perimembranous VSD	Palpitations, ICD
	IV-2	24/F	p.Y283D	2	yes	-1.2	61/36	Ebstein´s	Syncope
110.240	II-2	61/F	p.N1918K	2	no	0	NA/34	None	None
	III-1	43/M	p.N1918K	2	no	1.8	45/23	None	None
	III-4	39/F	p.N1918K	2	no	0.8	NA/30	Ebstein´s	None
	IV-4	5/M	p.N1918K	3	no	1.9	62/30	Coarctation of the aorta, BAV	Coarcectomy
109.787	II-1	66/M	p.E1573K	0	no	-3.0	78/39	None	None
	III-2	33/F	p.E1573K	1§	no	0.3	68/32	Ebstein´s, Perimembranous VSD	None
16875	II-2	42/M	p.Y283D	2	no	-1.4	55/32	None	None
	III-1	0.4/M	p.Y283D	2	no	1.5	47/23	Ebstein´s, pulmonary artery hypoplasia, ASDII	Surgery with aorto-pulmonary shunt, CHF
Sporadic	**AO**	35/M	p.1220 DelE	1	no	-2.2	55/38	Ebstein´s	None
Sporadic	**DB**	26/F	p.Y350N	2	yes	NA	65/NA	Ebstein´s	None
Sporadic	**BT**	59/M	p.L390P	2	no	-7.0	60/32	Ebstein´s, PFO	AF, CVI, TV reconstruction, PFO closure
Sporadic	**AD**	58/F	p.K1459N	0	no	-3.0	65/NA	Ebstein´s	NSAT, EPI/ ablation

* Noncompacted segments: None=0; Apex=1; Apex, midventricular wall=2; Apex, midventricular wall, basis=3; † right ventricular involvement; § partially penetrant phenotype; Inheritance of sporadic mutations: Not tested, AO, BT, and AD; de novo, DB; ID, probands are marked in bold;
LVED, left ventricular enddiastolic diameter; Z score, normal reference range -2 < +2; EF/FS, left ventricular ejection fraction/fractional shortening; NL, normal limits; NA, not available; AF, atrial fibrillation; NSAT, nonsustained atrial tachycardia; CHF, congestive heart failure; CVM, cardiovascular malformation; DCM, dilated cardiomyopathy; ICD, intracardiac defibrillator; EPI, electrophysiologic investigation; CVI, cerebrovascular insult; ASDII, secundum atrial septal defect; PFO, patent foramen ovale; VSD, ventricular septal defect; TV, tricuspid valve

age. She has always been asymptomatic despite significant tricuspid regurgitation from the age of 30 years. Marked LVNC was found at age 39 years (Figure 2E and 2F). Her youngest son (IV-4) had a bicuspid aortic valve and aortic coarctation, and echocardiography at age 5 years showed LVNC. The proband's asymptomatic brother (III-1) had LVNC and LV dilatation with LV dysfunction; her mother (II-2) was also found to have LVNC (Figure 2G and 2H). Echocardiography of III-6 could not rule out cardiomyopathy because of poor imaging quality (Figure 1B).

Kindred 109.787

A Glu1573Lys missense mutation in exon 34 was detected in the proband (III-2) and her asymptomatic father. Evaluation of a cardiac murmur in the proband's first year of life led to the diagnoses of Ebstein anomaly and a small doubly committed subarterial ventricular septal defect. Echocardiography at age 33 years showed mild hypertrabeculation of the apex, not fulfilling the criteria for LVNC (partially penetrant phenotype). The parents of the proband had normal echocardiography (Figure 1C).

Kindred 16875

The proband (III-1) and his father (II-2) carried the same Tyr283Asp missense mutation as in kindred 110.647. Haplotype analyses ruled out a founder mutation in these 2 families. The proband presented with Ebstein anomaly, LVNC, and pulmonary hypoplasia as a neonate and had an aortopulmonary shunt at the 2nd day of life (Figure 2C and 2D). By family screening, his asymptomatic father was diagnosed with LVNC. The paternal aunt (II-1) was reported to have heart failure and the paternal grandfather (I-1) had received an implantable cardioverter-defibrillator (Figure 1D).

Nonfamilial Cases

There were 4 sporadic cases with *MYH7* mutations. These included 1 de novo mutation; in 3 probands, the parental DNA or clinical information was unavailable.

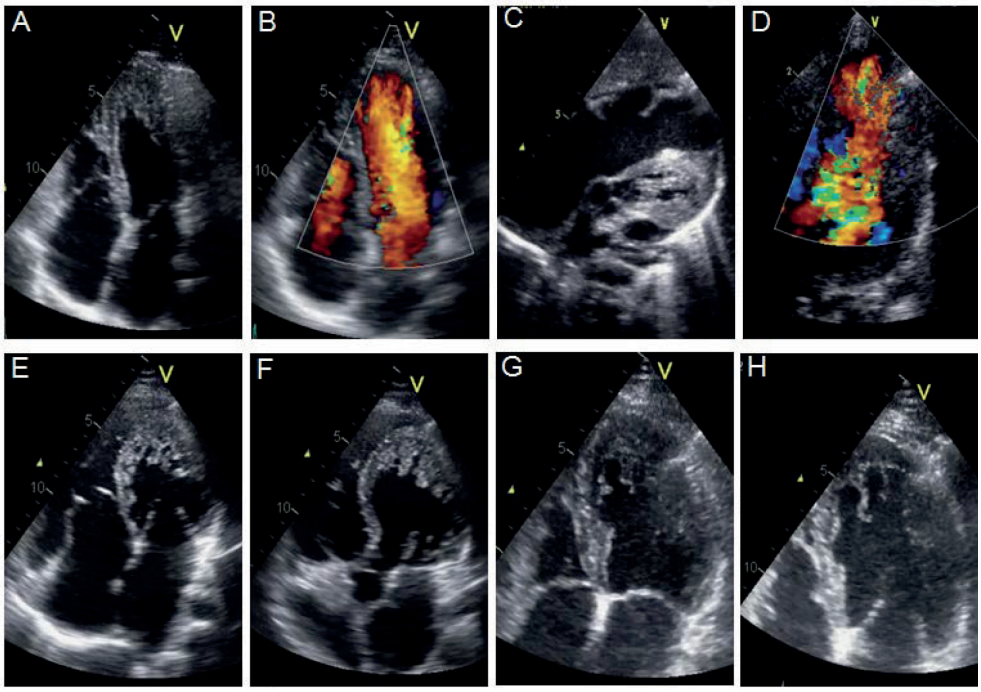

Figure 2. Echocardiographic images of affected individuals. A, Apical 4-chamber view, individual IV:2 of family 110.647with Ebstein anomaly and LVNC. B, The same patient as in A; color Doppler image showing deep recesses filled with blood from the left ventricular cavity at end-diastole. C, Modified 4-chamber view, patient III:1 of family 16875, demonstrates the atrialized portion of the right ventricle in between the enlarged right atrium and small right ventricle and the thickened left ventricle. D, The same patient as in C; this color Doppler apical 4-chamber view (at end-diastole) demonstrates the left ventricle with prominent trabeculations and deep recesses. E, End-diastolic, and F, end-systolic still frame, apical 4-chamber view, patient III:4 of family 110.240 with Ebstein anomaly and LVNC. G, End-systolic apical 4-chamber view, individual II:2 with LVNC without Ebstein anomaly of the same family 110.240. H, End-diastolic apical 2-chamber view, individual II:2 of family 110.240.

In proband AO, who presented with LVNC and LV diastolic dysfunction, a 3-bp in-frame deletion was detected, leading to the removal of a glutamic acid at residue 1220 in exon 27 (1220delGlu). In proband DB, a tyrosine was substituted by an asparagine at residue 350 in exon 12, which was not present in her unaffected parents and had occurred de novo (Tyr350Asp). This patient presented with biventricular non- compaction with preserved function. In proband BT, a missense mutation (Leu390Pro) was found in exon 13. Cardiac MRI was undertaken as echocardiography of left ventricular morphology was uninformative because of weight-related imaging difficulties and revealed extensive LVNC. In proband AD, a Lys1459Asn

substitution in exon 32 was present. Echocardiography of left ventricle morphology was also uninformative; this patient had not had an MRI.

Genetic and Clinical Evaluation of the Cohort With MYH7 Mutations
Three of the 7 distinct mutations reside within the genomic sequence of exon 10 to exon 13 of *MYH7,* which encode the head region of the molecule (Figure 3A). Four mutations are located throughout the rod domain of the β-myosin heavy chain molecule. All identified missense mutations affect amino acids with high degrees of conservation throughout evolution, underscoring the functional importance of these residues (Figure 3B). The Tyr350Asn substitution occurred de novo. Together with the observation that none of the mutations are present in more than 980 chromosomes from a control population of western European descent, our findings strongly support a disease-causing role for these mutations. Mutation-positive probands and family members showed various congenital heart disease including ASDII, ventricular septal defect, coarctation of the aorta, bicuspid aortic valve, and pulmonary artery stenosis/hypoplasia as well as cardiomyopathy including LVNC (Table 2). All individuals with LVNC, including the 3 partially penetrant cases (kindred 110.647, III-2, III-3; kindred 109.787, III-2), carried a *MYH7* mutation. There was only 1 mutation carrier with a normal echocardiogram (kindred 109.787, II-3). All individuals without an *MYH7* mutation that were part of kindreds with *MYH7*-positive individuals (Figure 1) had normal echocardiograms. Of the 18 individuals with *MYH7* mutations, 16 had LVNC, whereas 9 had Ebstein anomaly (Table 2).
The 8 probands with *MYH7* mutations were in New York Heart Association class I at the time of genetic evaluation. Except for the infant with additional pulmonary artery hypoplasia (16875, III-1), congestive heart failure had not been present at initial diagnosis. Severe regurgitation required surgical reconstruction of the tricuspid valve in 2 patients. LVNC was always observed in the left ventricular apex, and 2 of 8 probands had biventricular involvement. Left ventricular end-diastolic diameters were not enlarged, and left systolic function was preserved in 7 of 8 probands (Table 2). Sustained ventricular tachycardias or sudden cardiac deaths were not seen in probands; however, in kindred 110.647, there had been 1 sudden cardiac death. In 2 probands, nonsustained atrial tachyarrhythmias were present.

Discussion

Comprehensive genetic analyses of 141 unrelated probands with Ebstein anomaly identified 8 disease-associated mutations in the gene encoding β-myosin heavy chain. Mutation- positive probands and family members showed various congenital heart malformations as well as LVNC. Significant pleiotropy and reduced penetrance were characteristic of *MYH7* mutation-positive congenital heart malformations. The LVNC

phenotype had a higher penetrance, with only 1 mutation carrier having a normal echocardiogram. Mutations in *MYH7* can cause hypertrophic cardiomyopathy, dilated cardiomyopathy, restrictive cardiomyopathy, and LVNC.[17] This study further broadens the spectrum of phenotypes associated with a defect in a structural protein from cardiomyopathies to congenital heart malformations.

The main hemodynamic abnormality in Ebstein anomaly producing symptoms is tricuspid regurgitation.[1] When the tricuspid dysplasia or right ventricular myocardial hypoplasia is severe or associations with other cardiac lesions are present clinical symptoms occur in infancy as seen in the proband III:1 of kindred 16875. In contrast, Ebstein anomaly may be asymptomatic in adolescents and adults,[1] as in most of the mutation-positive probands in the present cohort. In such cases, supraventricular arrhythmias may be the main clinical problem as in probands BT and AD.

The frequency of *MYH7* mutations between those patients with LVNC and those without LVNC was significantly different. All 8 *MYH7* mutations were found in 8 probands with LVNC or with LVNC being partially penetrant or uncertain. There were no *MYH7* mutations in 133 probands without LVNC. Because there was no family screening by echocardiography in the 133 mutation-negative probands, the true prevalence of cardiomyopathy or congenital heart malformations in the families of the 133 mutation-negative probands remained unknown. In mutation-positive probands, several family members were shown to have congenital heart malformations as well as LVNC, of which some were not known before family screening. In Ebstein anomaly associated with mutations in *NKX2.5* mutations, carriers were also more likely to have a positive history of heart disease in the young.[6] Familial Ebstein anomaly was found in 1 kindred (110.647). In general, familial Ebstein anomaly is rare and only a few families with autosomal dominant inheritance have been described.[18, 19] Several genetic loci for Ebstein anomaly have been reported in humans and in animal models. Chromosomal abnormalities as well as mutations in *NKX2.5* were found in patients with Ebstein anomaly.[6, 20] Andelfinger et al[21] demonstrated linkage between tricuspid valve formation and canine chromosome 9 in a region systemic to human chromosome 1q12-q23. Of interest, penetrance of Ebstein anomaly in the dog was estimated to be 68%. This may represent the polygenic or multifactorial inheritance pattern proposed in humans with Ebstein anomaly.

A significant number of patients with Ebstein anomaly have morphofunctional abnormalities of the left ventricle, which may be explained by increased fibrosis of the left ventricular wall and septum as demonstrated by histological studies.[22] Attenhofer Jost et al[4] reviewed 106 consecutive patients with Ebstein anomaly and LVNC was identified in 18%. Also, in several other studies, Ebstein anomaly was associated with LVNC.[4, 19, 23] In 1 large family with autosomal dominant LVNC and Ebstein anomaly, an *MYH7* mutation was found.[7] Ebstein anomaly in families with autosomal inheritance of LVNC[19, 23] might represent a specific subtype with a mendelian inheritance pattern. The present study supports the association between Ebstein anomaly

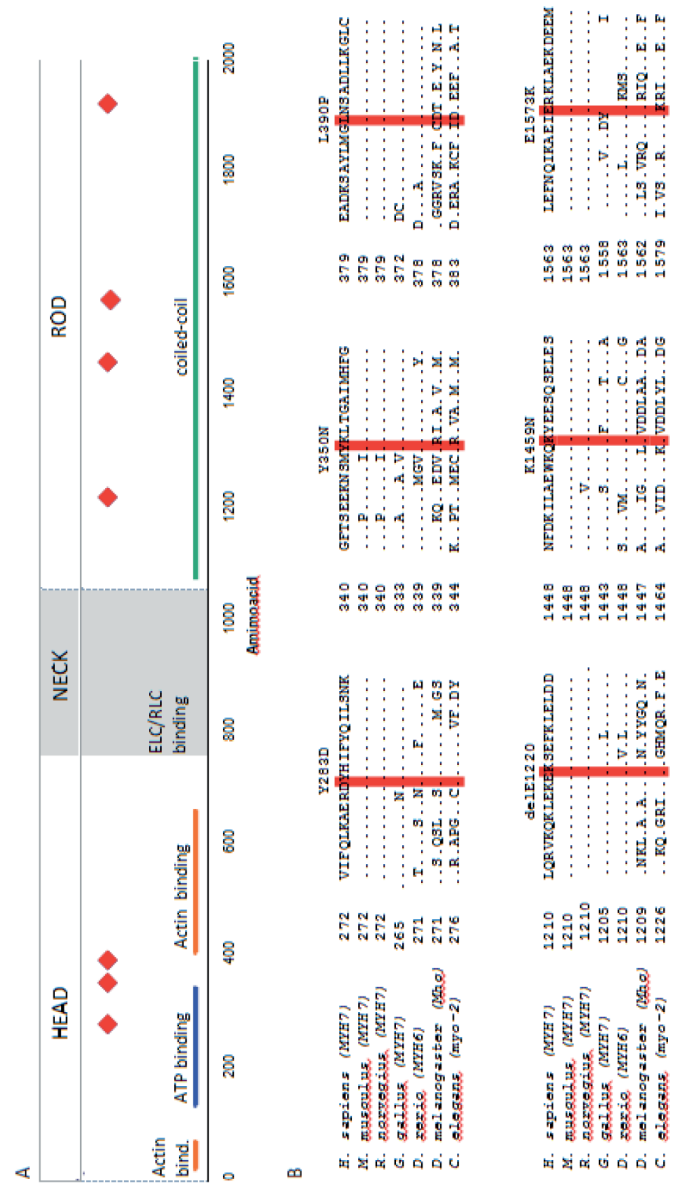

Figure 3. A, Distribution of the 7 distinct *MYH7* mutations in Ebstein anomaly. The resulting amino acid changes in the *[3*-myosin heavy chain molecule are depicted. B, Alignment of the regions flanking the mutations in *MYH7* showing evolutionary conservation of the mutated residues across species. The residues with the amino acid changes are boxed. Dots identify amino acids identical to the one in the human sequence. Accession numbers (FASTA): Human cardiac *[3*-myosin heavy chain, NP_000248; rat cardiac *a*-myosin heavy chain, NP058935; chicken fast skeletal myosin heavy chain, NP_001013414; *Danio rerio* ventricular myosin heavy chain, AAF00096; *Drosophila melanogaster* muscle myosin heavy chain, NP_723999; and *Caenorhabditis elegans* myosin heavy chain, NP_510092.

with LVNC and *MYH7* mutations. Clinical and genetic evaluation is recommended to facilitate the diagnosis of cardiomyopathy and congenital heart disease in probands and their first-degree relatives.[12, 24]

Mutations in *MYH7* are a common cause for hypertrophic cardiomyopathy and well recognized in dilated cardiomyopathy and LVNC. Mutations are distributed throughout the molecule, and the relationship between mutation location, cardiomyopathy type, and disease severity is poorly understood.[17] The first link between sarcomeric proteins and congenital heart malformations was provided by Ching et al[25] by identifying a mutation in α-myosin heavy chain (*MYH6*) through genetic linkage analysis. Later, a founder mutation in *ACTC1* was identified in 2 families with autosomal dominant ASD, in the absence of other cardiac anomalies.[15] How mutations in sacromere genes could have detrimental effects on cardiac morphogenesis and produce septal defects and valve anomalies should be subject to further investigations. Because LVNC is thought to result from altered regulation in cell proliferation, differentiation, and maturation during wall formation,[26] arrest in directional growth could account for the association of Ebstein anomaly and LVNC.[27, 28]

Conclusions

Ebstein anomaly is within the diverse spectrum of cardiac morphologies associated with mutations in the gene encoding β-myosin heavy chain. *MYH7* mutations are predominantly found in Ebstein anomaly associated with LVNC. In the subset of patients with Ebstein anomaly carrying a *MYH7* mutation, genetic and clinical evaluation of family members is recommended to identify congenital heart malformations and cardiomyopathy.

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Disclosures

None.

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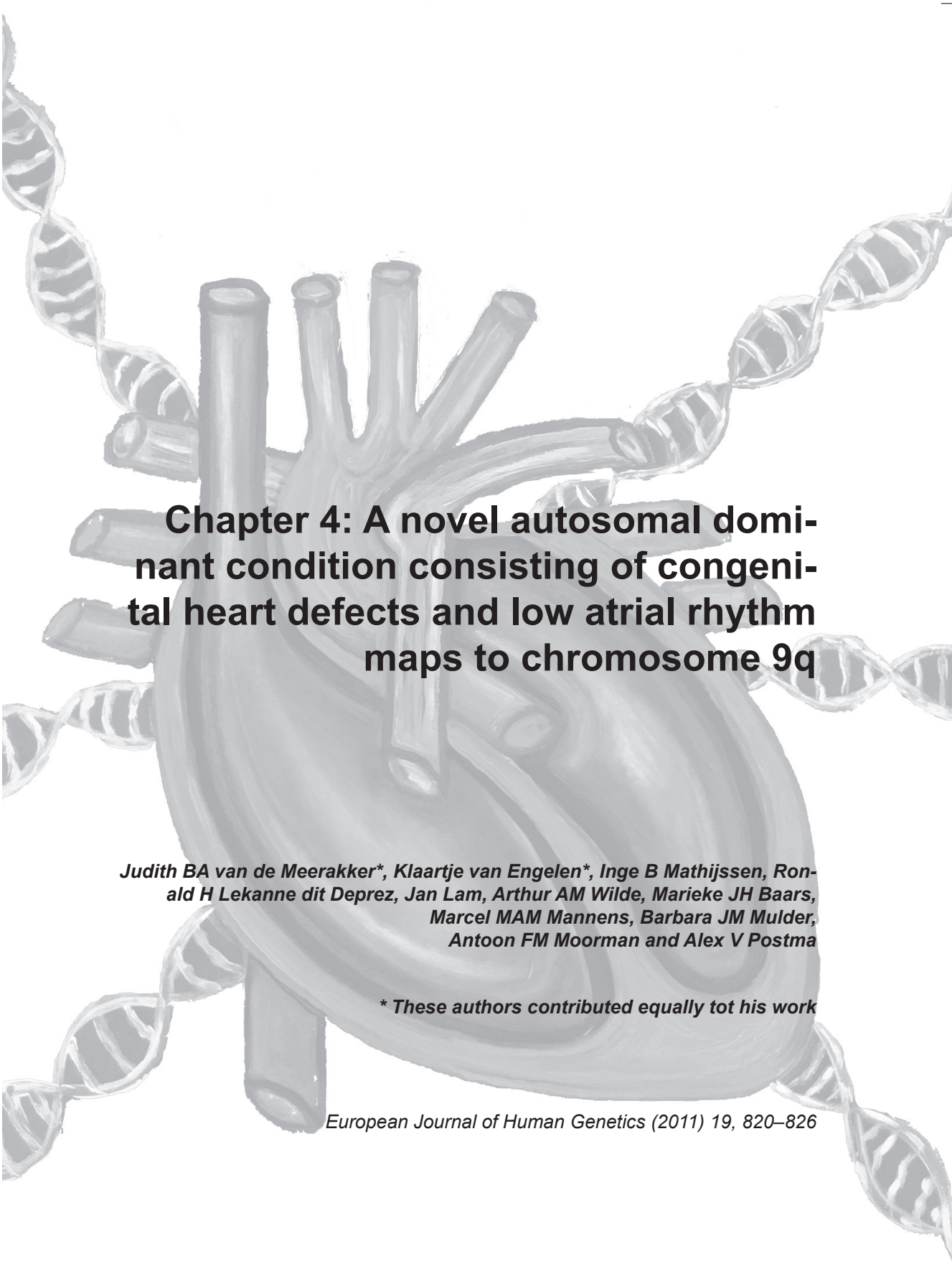
Clinical perspective

Ebstein anomaly is a rare congenital heart malformation affecting both the tricuspid valve and right ventricle, and is characterized by adherence of the septal and posterior leaflets of the tricuspid valve to the underlying myocardium. Ebstein anomaly is more common in patients with a family history of congenital heart disease but most cases are sporadic and familial Ebstein anomaly is rare. Associated abnormalities of left ventricular morphology and function have been observed. A possible association between Ebstein anomaly with left ventricular noncompaction (LVNC) and mutations in *MYH7* encoding β-myosin heavy was previously shown in one family with multiple affected members. In the present study, we describe that Ebstein anomaly is within the diverse spectrum of cardiac morphologies triggered by a sarcomere gene defect. We performed mutational analysis of *MYH7* in a cohort of 141 unrelated probands with Ebstein anomaly. Mutations were identified in 8 of 141 probands (6%), the largest resequencing study of Ebstein anomaly thus far. Mutation-positive probands and family members showed various congenital heart malformations as well as LVNC. Significant pleiotropy and reduced penetrance were characteristic of *MYH7* mutation-positive congenital heart malformations. In 6 of 8 probands with *MYH7* mutations, LVNC was identified in addition to Ebstein anomaly, whereas among 133 *MYH7* mutation-negative probands, none had LVNC. We provide further evidence for a link between structural proteins, cardiomyopathy, and congenital heart malformations. *MYH7* mutations are predominantly found in Ebstein anomaly associated with LVNC and may warrant genetic testing and family evaluation in this subset of patients.



Chapter 4: A novel autosomal dominant condition consisting of congenital heart defects and low atrial rhythm maps to chromosome 9q

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Abstract

Congenital heart defects (CHDs) occur mostly sporadic, but familial CHD cases have been reported. Mutations in several genes, including NKX2.5, GATA4 and NOTCH1, were identified in families and patients with CHD, but the mechanisms underlying CHD are largely unknown. We performed genome-wide linkage analysis in a large four-generation family with autosomal dominant CHD (including atrial septal defect type I and II, tetralogy of Fallot and persistent left superior vena cava) and low atrial rhythm, a unique phenotype that has not been described before. We obtained phenotypic information including electrocardiography, echocardiography and DNA of 23 family members. Genome-wide linkage analysis on 12 affected, 5 unaffected individuals and 1 obligate carrier demonstrated significant linkage only to chromosome 9q21–33 with a multipoint maximum LOD score of 4.1 at marker D9S1690, between markers D9S167 and D9S1682. This 48-cM critical interval corresponds to 39 Mb and contains 402 genes. Sequence analysis of nine candidate genes in this region (*INVS*, *TMOD1*, *TGFBR1*, *KLF4*, *IPPK*, *BARX1*, *PTCH1*, *MEGF9* and *S1PR3*) revealed no mutations, nor were genomic imbalances detected using array comparative genomic hybridization. In conclusion, we describe a large family with CHD and low atrial rhythm with a significant LOD score to chromosome 9q. The phenotype is representative of a mild form of left atrial isomerism or a developmental defect of the sinus node and surrounding tissue. Because the mechanisms underlying CHD are largely unknown, this study represents an important step towards the discovery of genes implied in cardiogenesis.

Introduction

Congenital heart defects (CHDs) are among the most common congenital defects, occurring with an incidence of 8/1000 live-births.[1] The aetiology of CHD is generally complex, environmental exposures, chromosomal abnormalities and gene defects can all contribute. Most CHD occur sporadic, but in recent years an increasing number of familial cases with various types of CHD have been reported.[2-4] Although mutations in several genes have been identified in a small subset of patients and families with CHD, eg, *NKX2.5*,[4] *GATA4*[3] and *NOTCH1*,[5] the mechanisms underlying human cardiogenesis and CHDs remain largely unknown. Some CHD patients and families also display cardiac arrhythmias, which can occur due to the anatomical defect itself or sometimes due to surgical interventions.[6] More-over, in some patients arrhythmias are the direct consequence of the underlying genetic defect, in absence of any structural defect. [4, 7]

Ectopic atrial rhythms originate when a focus outside the sinus node takes over the pacemaker function. Consequently, the direction of atrial activation may be altered, which can be seen as an abnormal P-wave axis on the electrocardiogram (ECG). The appearance of the P-wave depends on the site of origin of the ectopic rhythm. The P-wave can be decreased in amplitude, or it can be biphasic or negative in leads II, III and AVF. The rhythm is regular and the P–R interval is usually normal or, at times, prolonged.[8] Usually, ectopic atrial rhythms are haemodynamically insignificant. They can be isolated and idiopathic, especially in children, but can also co-occur with CHD.[9] A P-wave frontal axis oriented in a superior direction, reflecting atrial pacemaker tissue located in the lower part of the atrium, is referred to as low atrial rhythm. This can be present due to sinus node dysfunction in patients with left atrial isomerism, a laterality disorder characterized by bilateral left sidedness.[9, 10]

Here, we describe a large four-generation family with an autosomal dominant condition, comprising various types of CHD and low atrial rhythm, a unique phenotype not described before within a family. A genome-wide linkage analysis yielded linkage to a region of 39 Mb on chromosome 9q with a maximum LOD score of 4.1.

Methods

Clinical details

This study was approved by the Medical Ethical Committee at the Academic Medical Centre in Amsterdam. Written informed consent was obtained from all participants. Subjects were clinically evaluated by analysis of medical records, physical examination with attention to syndromic features, cardiologic examination, 12-lead ECG and two-dimensional echocardiography. Previously performed ECGs were obtained from the medical records if available. The number of available ECGs ranged from one to 15 in the evaluated persons. One experienced paediatric cardiologist (JL) reviewed

the ECGs and echocardiographic images. A total of 23 family members were examined. One patient (II-2) refused cardiologic examination for this study and therefore only previous medical records (including ECGs and echocardiography records) were obtained. We used magnetic resonance imaging (MRI) for evaluation of situs in the proband (IV-10). Abdominal ultrasound for evaluation of abdominal situs was performed in three individuals, and chest X-rays for evaluation of situs of the lungs were available for six individuals. Computed tomography (CT) of the thorax was performed in patient III-3.
Low atrial rhythm was defined as a P-wave frontal axis oriented in a superior direction. We defined the P-wave frontal axis to be horizontal when it was B01, which is abnormal, although not a true low atrial rhythm. A wandering atrial pacemaker was defined as a change in P-wave axis of 601 or more on subsequent ECGs.

Linkage analysis, mutation screen and array comparative genomic hybridization

Genomic DNA of family members was extracted from peripheral blood according to standard procedures. Linkage analysis was performed using the ABI linkage set v2.5 MD10 set on an ABI 3700 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).[11] Phenotype, genotype and pedigree information were combined for multipoint linkage analysis with the use of the easyLinkage software package[12] running Simwalk v2.91[13] with the assumption of an autosomal dominant pattern of inheritance, a disease-allele frequency of 0.0001 and a penetrance of 0.9. Gene frequency was assumed to be equal between males and females. Both parametric and nonparametric linkages were calculated.
PCR amplification of all the coding exons of candidate genes inversin (*INVS*), transforming growth factor-b receptor 1 (*TGFBR1)*, tropomodulin 1 (*TMOD1*), Kruppel-like factor 4 (*KLF4)*, inositol 1,3,4,5,6-pentakisphosphate 2-kinase (*IPPK*), BARX homeobox 1 (*BARX1*), patched homologue 1 (*PTCH1*) and sphingosine 1-phosphate receptor 3 (*S1PR3*), multiple epidermal growth factor-like domains 9 (*MEGF9*) (only exon 12b, as this is specifically expressed in brain and heart[14]) was performed using primers located in flanking intronic sequences (available on request). Subsequently, PCR products were analysed by direct sequencing, using the BigdyeTerminator v3.1 Kit on an ABI 3700.
MLPA probes were designed for *INVS* and *TGFBR1* using MAPD, a probe design suite for multiplex ligation-dependent probe amplification assays.[15] The MLPA procedure and analysis were carried out as described.[16]
Array comparative genomic hybridization (array CGH) was performed using 4x180K slides (average 13 kbp spacing between probes), AMADID 023363 (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol (Oligonucleotide Array-Based CGH for Genomic DNA Analysis V5.0, June 2007) with

adaptations. No amplification or restriction was used on the genomic patient and reference DNA. Fluorescent labelling of gDNA was performed using the CGH labelling kit for Oligo Arrays (Enzo Life Sciences Inc., Farmingdale, NY, USA) and purified using the MinElute PCR Purification Kit (Qiagen, Valencia, CA, USA). Hydridization was performed according to the manufacturer's protocol (Oligonucleotide Array-Based CGH for Genomic DNA Analysis V5.0, June 2007) and slides were scanned using an Agilent G2250C 2 mm scanner with Agilent Scan Control software (Version A.8.1.3.) using default settings. Spot intensities were measured with Agilent Feature Extraction software (V10.7), and further data analysis was performed using DNA Analytics software (V4.0.76) with algorithm ADM-2 using a filter of minimal three subsequent clones with a minimal absolute ratio of 0.3.

Results

Clinical details

In the presented family, several individuals had CHD with or without low atrial rhythm, whereas others had isolated low atrial rhythm. The pedigree of the family is shown in Figure 1. Table 1 summarizes the clinical features of the family members. The proband (IV-10) was diagnosed during her first year of life with an incomplete atrioventricular septal defect (AVSD), a common atrium and a small communication between the left and right ventricle. At 2.5 years of age, she underwent surgical correction including septation of the monoatrium, leaving the coronary sinus draining into the left atrium. During the surgical procedure, the presence of bilateral left atria with bilateral left atrial appendages was observed. At 33 years of age, she was asymptomatic. MRI at that age showed normal situs of the lungs and abdominal organs. ECG persistently showed left QRS axis deviation and low atrial rhythm.

The sister of the proband (IV-9) was asymptomatic. Echocardiography did not reveal any abnormalities; however, ECG at age 32 showed low atrial rhythm (Figure 2). The asymptomatic father of IV-9 and IV-10 (III-10) also had normal echocardiography, and low atrial rhythm with bradycardia (45/min) on ECG.

The paternal aunt of the proband (III-9) was diagnosed with congenital agenesis or malfunction of the sinus node at infant age because of bradycardia with AV-junctional escape rhythm and low atrial rhythm. At later age, she developed intermittent complete AV dissociation. Echocardiography repeatedly did not show any structural abnormalities. Because of chronic fatigue, a pacemaker was implanted at the age of 39 years. During implantation, the lead could not be placed in a stable position in the right atrial appendage, and therefore this appendage was assumed to be absent. Normal situs of the lungs and abdominal organs were confirmed by chest X-ray and abdominal ultrasound. The 18-year-old daughter of III-9 (IV-8) was born with a large

incomplete AVSD and ASD II, which were surgically corrected at the age of 8 months. At surgery, absence of the coronary sinus and a persistent left superior vena cava (LSVC) connecting directly with the left upper corner of the left atrium were noted. The right superior vena cava was hypoplastic and the brachiocephalic vein was absent. ECGs persistently showed low atrial rhythm. Situs of the lungs and abdomen was normal.

One of the paternal uncles of the proband (III-6) had tetralogy of Fallot and LSVC draining into the coronary sinus, which was surgically corrected at the age of 27. P-wave frontal axis was horizontal. Normal situs of the lungs and bronchi was present. In his 28-year-old daughter (IV-5), a cardiac murmur was noticed shortly after birth and she was followed at a cardiology clinic until the age of 7 years. Echocardiography performed at age 20 showed mildly elevated pulmonary artery pressure, with unknown cause. Low atrial rhythm was also present.

The paternal grandfather of the proband (II-2) had a myocardial infarction at the age 76 years. He received a pacemaker at the age of 84 years because of atrial and ventricular arrhythmias with severe bradycardia. P-wave frontal axis was horizontal. He developed atrial fibrillation at the age of 85 years. Chest X-rays showed normal situs of the lungs and bronchi.

His sister (II-1) showed low atrial rhythm on ECG at the age of 74 years. She developed atrial fibrillation when she was 77 years old. Echocardiography at this age showed dilated atria and mildly elevated pulmonary artery pressure, with normal left ventricular systolic function and diastolic dysfunction. Ultrasound showed normal abdominal situs. Two children of II-1 (III-1 and III-2) had died in the first months of life: III-1 was said to have had aortic hypoplasia, detected at autopsy, and III-2 was said to have died of pneumonia.

The daughter of II-1 (III-3) was asymptomatic at the age of 57 years. Echocardiography and CT showed an LSVC draining into the coronary sinus (Figure 2), aberrant right subclavian artery arising from the aorta distal from the left subclavian artery (arteria lusoria) and elevated pulmonary artery pressure, whereas on ECG low atrial rhythm and paroxysmal supraventricular tachycardia were present.

Pulmonary situs was normal. In the asymptomatic 28-year-old daughter of III-3 (IV-1) echocardiography showed abnormal inter- ventricular wall movements, but no ventricular septal defect or other structural abnormalities were seen. She did have low atrial rhythm. The son of III-3 (IV-2), who was also asymptomatic at the age of 25 years, showed a horizontal P-wave frontal axis on ECG with normal echocardiography.

In the youngest son of II-1 (III-5), ECG at the age of 40 years showed low atrial rhythm and first-degree atrioventricular (AV) block. On echocardiography at the same age, a mildly dilated left ventricle was present as well as moderate regurgitation of calcified aortic and tricuspid valves. The 23-year-old daughter of III-5 (IV-3) was diagnosed in her first year of life with ASD II, membrane in the left

atrium, absent brachiocephalic vein, LSVC draining into the coronary sinus and a small right superior vena cava. ECGs persistently showed low atrial rhythm. Situs of the lungs and bronchi was normal. The other daughter of III-5 (IV-4) was diagnosed with frequent paroxysmal supraventricular tachycardia and atrial extrasystoles with the origin in the lower right atrium at birth, with normal sinus rhythm in between. She was on anti-arrhythmic medication until the current age of 17 years because of persistence of the tachycardia. Echocardiography showed no structural abnormalities and chest X-ray showed normal pulmonary situs.
At the age of 78 years, the asymptomatic brother of II-1 and II-2 (II-3) was found to have bradycardia with arrests up to 2.6 s with junctional escapes, atrial extrasystoles and non-sustained ventricular
tachycardia. He also had paroxysmal atrial fibrillation and complete right bundle branch block. P-wave frontal axis was normal. Echocardiography at the age of 80 years demonstrated left ventricular hypertrophy, dilatation of the aortic root (42 mm) and mild prolapse of the mitral valve, but no congenital abnormalities.
Seven other family members were evaluated in this study (III-4, III-7, III-8, III-11, III-12, IV-6, IV-7), all with normal echocardiography and ECG. None of the individuals with an abnormal P-wave axis had a wandering pacemaker. Clear dysmorphic features were not present in any of the family members.

Linkage analysis and mutation screen

Before performing a genome-wide linkage, we excluded *GATA4*, *TBX5* and *NKX2-5* as the disease-causing gene in this family by linkage analysis. In first linkage scenario, termed nuclear family, subjects were considered affected if they had a structural heart defect and/or low atrial rhythm. Therefore, genome-wide linkage was performed on 12 affected (II-1, III-3, III-5, III-6, III-9, IIII-10, IV-1, IV-3, IV-5, IV-8, IV-9 and IV-10), 5 unaffected individuals (III-4, III-7, III-8, IV-6, IV-7) and 1 obligate carrier (II-2; Figure 1 and Table 1). The analysis demonstrated significant linkage to a single locus on chromosome 9q shared by all affected individuals. This locus was absent from unaffected individuals (Figure 1). The shared locus has a multipoint maximum LOD score of 4.1 at marker D9S1690, and is delineated by markers D9S167 and D9S1682 based on the haplotypes (Figures 1 and 3). No other loci with an LOD score higher than 1.0 was detected in this family genome wide.
To reduce the risk of phenocopies, individuals IV-2, IV-4 and II-3 (and therefore his children III-11 and III-12) were not included in the nuclear family linkage analysis. IV-2 was excluded because he had a horizontal P-wave frontal axis, and although this is abnormal, it is not a clear low atrial rhythm. II-3 an IV-4 had significant rhythm and conduction abnormalities, but normal P-wave frontal axes.

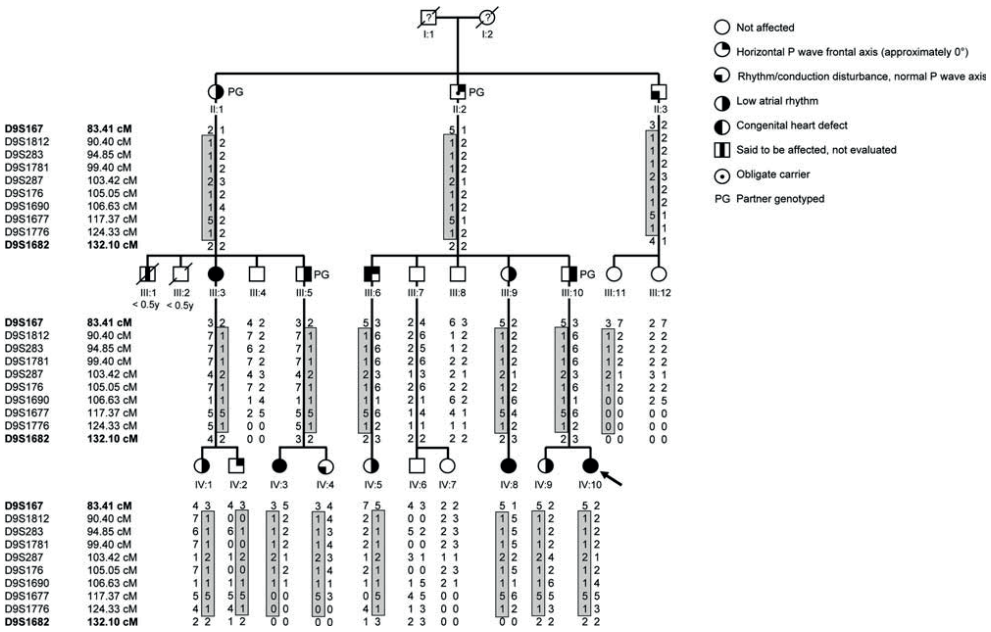

Figure 1 Pedigree of the family; circles represent females, squares males. Unaffected individuals are depicted with empty symbols and deceased individuals are indicated by slashes with age of death in years. Right-sided filled symbols denote individuals with low atrial rhythm, left-sided filled symbols represent those with congenital heart defects or persistent left superior vena cava, right upper quarter filled symbols represent individuals with horizontal P-wave frontal axis and left lower quarter filled symbols indicate those with rhythm/conduction disturbances with normal P-wave frontal axis. Dots denote obligate carriers. The proband is indicated by an arrow. Haplotypes are depicted under each individual, and the disease haplotype is demarcated by the box. For reasons of clarity not all parents are shown, markers according to the Marshfield map.

In a second linkage scenario, termed extended family, we presumed IV-2, IV-4 and II-3 to be affected and III-11 and III-12 to be unaffected. We found that all affected carried to locus, however III-11 did as well, who had normal echocardiography and ECG. The maximal LOD score for the extended family scenario is 4.45; the locus remained the same. A third linkage scenario, termed affected only, was run in which individuals II-1, III-3, III-5, III-6, III-9, III-10, IV-1, IV-3, IV-5, IV-8, IV-9 and IV-10 were included as affected. The maximal LOD score for this affected only scenario was 3.3, also delineated by markers D9S167 and D9S1682.

The shared locus comprises a 48-cM critical interval, that corresponds to 39 Mb and contains 402 genes, of which 9 (*INVS*, *TMOD1*, *TGFBR1*, *KLF4*, *IPPK*, *PTCH1*, *BARX1*, *MEGF9* and *S1PR3*) are known to be, directly or indirectly, involved in

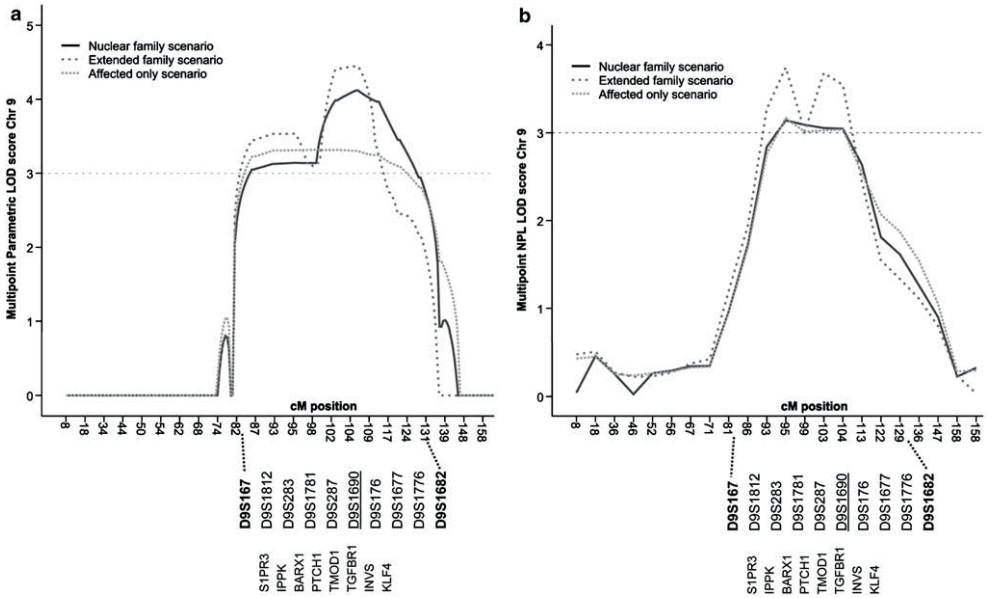

Figure 3 Graphs of the multipoint parametric (a) and nonparametric, NPL (b) LOD scores of microsatellite markers on chromosome 9 for various analysis scenarios. The area of linkage is enlarged, depicting the position of markers and candidate genes screened by sequencing.

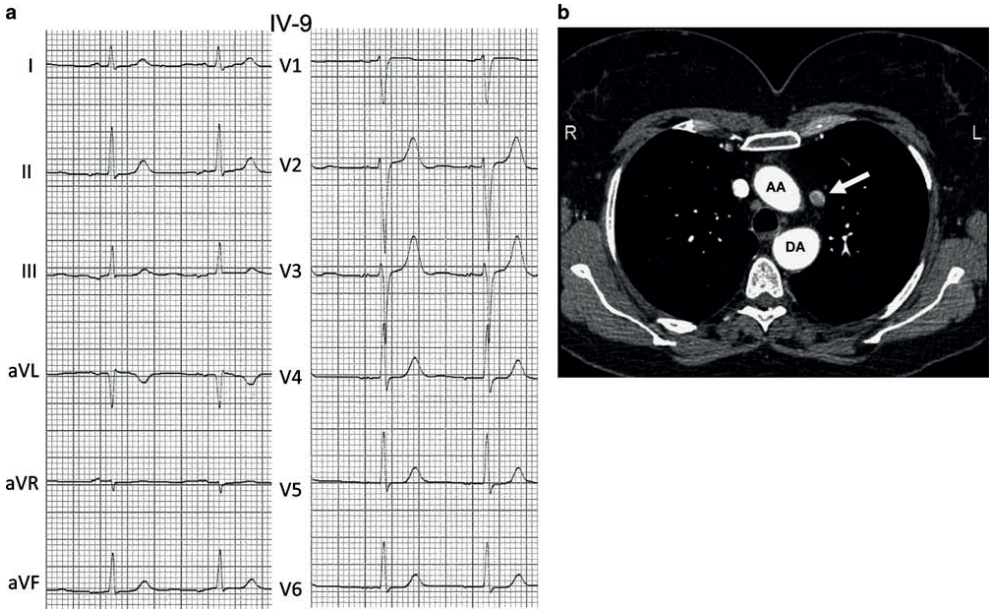

Figure 2 (a) ECG of patient IV-9 at age 32 years with isolated low atrial rhythm. The P-wave is negative in leads II, III and aVF. (b) Transverse computed tomography image of individual III-3. Persistent left superior vena cava (arrow). AA, ascending aorta; DA, descending aorta.

development and (left-right) patterning of the heart. Therefore, mutation analysis was performed in these genes in the proband (IV-10). Moreover, 10 conserved non-coding sequences within 5 kb up- or downstream of candidate genes were also screened. Nonetheless, no mutations were identified by direct sequencing. As large genomic rearrangements could also potentially underlie the phenotype, we also performed an array CGH on III-6 and IV-10, using a 180-K oligo array with genome-wide coverage. However, no genomic imbalances in or out- side the co-segregating locus were detected, besides copy number variations are also present in controls (data not shown). Moreover, MLPA analysis of the coding exons of *INVS* and *TGFBR1* did not reveal genomic imbalances for these genes.

Discussion

We describe a large family with an autosomal dominant condition with a wide spectrum of cardiovascular abnormalities. The phenotype comprises different types of CHD with an abnormal atrial rhythm. Remarkably, several family members have low atrial rhythm without detectable structural heart defects, suggesting that these individuals show a mild manifestation of the familial disorder. We identified a locus at chromosome 9q that co-segregates with this autosomal dominant disorder and excluded several candidate genes present within the shared 39-Mb disease locus.
The presence of bilateral left atrial appendages in the proband in combination with low atrial rhythm led us to hypothesize that the CHDs in this family might be the result of a defect in the establishment of left-right asymmetry, and might represent a mild or variant expression of left atrial isomerism. Left isomerism is a laterality disorder characterized by bilateral left sidedness, i.e., two morphologic left cardiac atria and atrial appendages, often in association with bilateral bilobed (left) lungs, isomerism of the bronchi, abdominal situs abnormalities, polysplenia and abnormalities of the large systemic veins.[17,18] The internal architecture of the heart is often disrupted as well, and different forms of CHD are observed in left atrial isomerism.[17–19] Besides CHD, the phenotype in this family is characterized by abnormal P-wave frontal axis on ECG, typically present in left atrial isomerism due to sinus node abnormalities. In normal hearts, the sinus node is located in the right atrium at the junction between the superior caval vein and the right atrial appendage, which results in a P-wave frontal axis orientated leftward and inferiorly.[20] Histological studies in hearts with left atrial isomerism demonstrate absence, hypoplasia and/or ectopic location of nodal tissue in a large proportion of patients.[21,22] In correspondence with these histological abnormalities, abnormal atrial rhythms are commonly found in patients with left atrial isomerism,[10,23] and a low atrial rhythm with a superior P-wave frontal axis is frequently present.[10,23] In this family, four of the five individuals with CHD or isolated LSVC had low atrial rhythm, and one individual had a horizontal P-wave

axis. In addition, seven family members without detectable structural heart defects also showed low atrial rhythm, and two had a horizontal P-wave axis. Indeed, rhythm and conduction disturbances have been described in patients with confirmed left isomerism without structural cardiac defects.[24,25] Taken together, the spectrum of CHD in this family is compatible with that seen in left isomerism. Importantly, we could not confirm signs of left isomerism outside the heart (eg, polysplenia) in the investigated family members, although imaging of the abdomen and lungs was not available in all subjects, so we cannot rule out laterality defects in every individual. In conclusion, the phenotype of this family resembles a developmental laterality defect, although affected individuals only show a mild expression, which appears to be restricted to the heart.

Alternatively, the familial phenotype might represent an underlying developmental defect of the sinus node, as most family members have an abnormal low atrial rhythm, caused by a dominance of a pace- maker located in the lower part of the right atrium over the normal sinus node. In its earliest stages, when the heart is no more than a simple tube, a dominant pacemaker activity is already found in the entire systemic inflow region of the heart (both left and right side). Subsequently, newly added myocardium at the inflow part, which differentiates from t-box transcription factor Tbx18 expressing pre- cursor cells, will eventually give rise to the right-sided sinus node, among others.[26] The developing sinus node also expresses the t-box transcription factor Tbx3, which acts to repress differentiation into working myocardium by imposing a conduction system phenotype.[27] Thus, formation of the sinus nodes requires transcription factors, such as Tbx18 and Tbx3, and knockout of these genes leads not only to under- or maldevelopment of the sinus node, but also to various forms of CHD.[26,27] Consequently, it is possible that the phenotype seen in the current family is the direct result of developmental defects in the formation of the sinus node, possibly via pathways involving t-box transcription factors.

The co-segregating locus on chromosome 9q contains 402 genes (genome build 37), and some of these were selected as candidate genes based on current knowledge, literature and data mining analysis: *INVS*, *S1PR3*, *TGFBR1*, *IPPK*, *BARX1*, *TMOD1*, *KLF4*, *MEGF9* and *PTCH1*. The coding regions and selected conserved non-coding regions of all these genes were sequenced, but no mutations were found. In addition, we looked for genome-wide genomic imbalances using array CGH; however, none were identified. No other genes known to have a role in left-right patterning or cardiac development map to the 9q region. The best option to identify the causative gene or region on the 9q locus would be to fully sequence it, and although advances in next generation sequencing have been tremendous, it is currently far from trivial to sequence our 39 Mb locus. Moreover, investigation of additional families with a similar phenotype might reduce the region of interest and could lead to the detection of the gene responsible for the phenotype in this family.

Table 1 Clinical details of family

	Patient characteristics				Clinical features		Status in linkage analysis
	Sex/age	Age at diagnosis of CHD and/or low atrial rhythm	Age (years) at last available ECG	Low atrial rhythm	Rhythm/conduction disturbances	Congenital heart defects	Nuclear family scenario
II-1	F/83	74 years	77	+	AF	—	Affected
II-2	M/89	84 years	84	±	Bradycardia, atrial/ventricular arrhythmia, AF[a]	—	Obligate carrier
II-3	M/87	78 years	80	–	Bradycardia, junctional escapes, AF, complete RBBB	—	Excluded
III-3	F/57	50 years	57	+	Paroxysmal SVT	LSVC, aberrant right subclavian artery	Affected
III-4	M/50		43	–	—	—	Not affected
III-5	M/47	40 years	40	+	First-degree AV block, incomplete RBBB	—	Affected
III-6	M/61	10 years	36	±	Complete RBBB	Tetralogy of Fallot, LSVC	Affected
III-7	M/59		58	–	Sinus bradycardia	—	Not affected
III-8	M/54		53	–	—		Not affected
III-9	F/47	4 years	38	+	Bradycardia, atrial arrests with AV junctional escapes, intermittent complete AV dissociation	—[b]	Affected
III-10	M/60	52 years	52	+	Bradycardia	—	Affected
III-11	F/52		45	–	—	—	Excluded
III-12	F/50		43	–	—	—	Excluded
IV-1	F/28	21 years	28	+	—	—	Affected
IV-2	M/25	24 years	24	±	—	—	Excluded
IV-3	F/23	2 months	16	+	Incomplete RBBB	ASD II, membrane in left atrium, LSVC, absent brachiocephalic vein	Affected
IV-4	F/17	Birth	16	–	Chronic SVT; atrial extrasystoles	—	Excluded
IV-5	F/28	4 years	28	+	Incomplete RBBB	—[c]	Affected
IV-6	M/34		33	–	—	—	Not affected
IV-7	F/31		24	–	—	—	Not affected
IV-8	F/18	<0.5 years	18	+	Incomplete RBBB	Incomplete AVSD, ASD II, LSVC draining into left atrium, absent coronary sinus, absent brachiocephalic vein	Affected
IV-9	F/36	32 years	32	+	—	—	Affected
IV-10	F/33	<0.5 years	33	+	Incomplete RBBB	Incomplete AVSD, common atrium, bilateral left atrial appendages	Affected

Low atrial rhythm + means that the P wave frontal axis was oriented superiorly; Low atrial rhythm ± means that the P wave frontal axis was horizontal (approximately 0°). AF, atrial fibrillation; RBBB, right bundle branch block; ASD II, secundum type atrial septal defect; AVSD, atrioventricular septal defect; SVT, supraventricular tachycardia; LSVC, persistent left superior vena cava; [a] After myocardial infarction, [b] Suspicion of abnormal atrial appendage, [c] Systolic murmur, for which cardiologic follow up until the age of 7 years, elevated pulmoary artery pressure at age 20, [g] Chest CT available.

A concern of our study is the possibility of phenocopies. Ectopic atrial rhythms can be seen idiopathically in healthy individuals, especially in children. We cannot rule out that individuals in our family have P-wave abnormalities due to other reasons than the familial genetic defect. To minimize the risk of phenocopies, we performed linkage analysis on the most affected patients, i.e., patients with low atrial rhythm, and excluded the patient with isolated horizontal P-wave frontal axis as well as the patients with significant rhythm disturbances and normal P-wave frontal axis. Though those patients all carried the disease locus, which implies that the familial disorder shows variable expression. Patient III-11, carrier of the risk allele, had normal echocardiography and ECG at the age of 45 years, which also indicates that the disorder is not 100% penetrant. Atrial fibrillation and other AV rhythm disturbances were mainly present in the older individuals in the family, suggesting age-dependent penetrance of these abnormalities. Although these abnormalities are common in the general population, it is unclear if they are a part of the disease phenotype.

In conclusion, we present a large four-generation family in which a condition, comprising of CHDs and low atrial rhythm, inherits as an autosomal dominant trait with variable expression, and which, to the best of our knowledge, has not been described before. A significant genome-wide linkage was demonstrated to a locus on chromosome 9q with an LOD score of 4.1. Although we did not uncover a causative mutation in this family, the mapping of this locus represents an important step towards the discovery of genes implied in cardiac development. Identification of the disease-causing gene will allow genetic screening and will ultimately provide fundamental insights in human cardiogenesis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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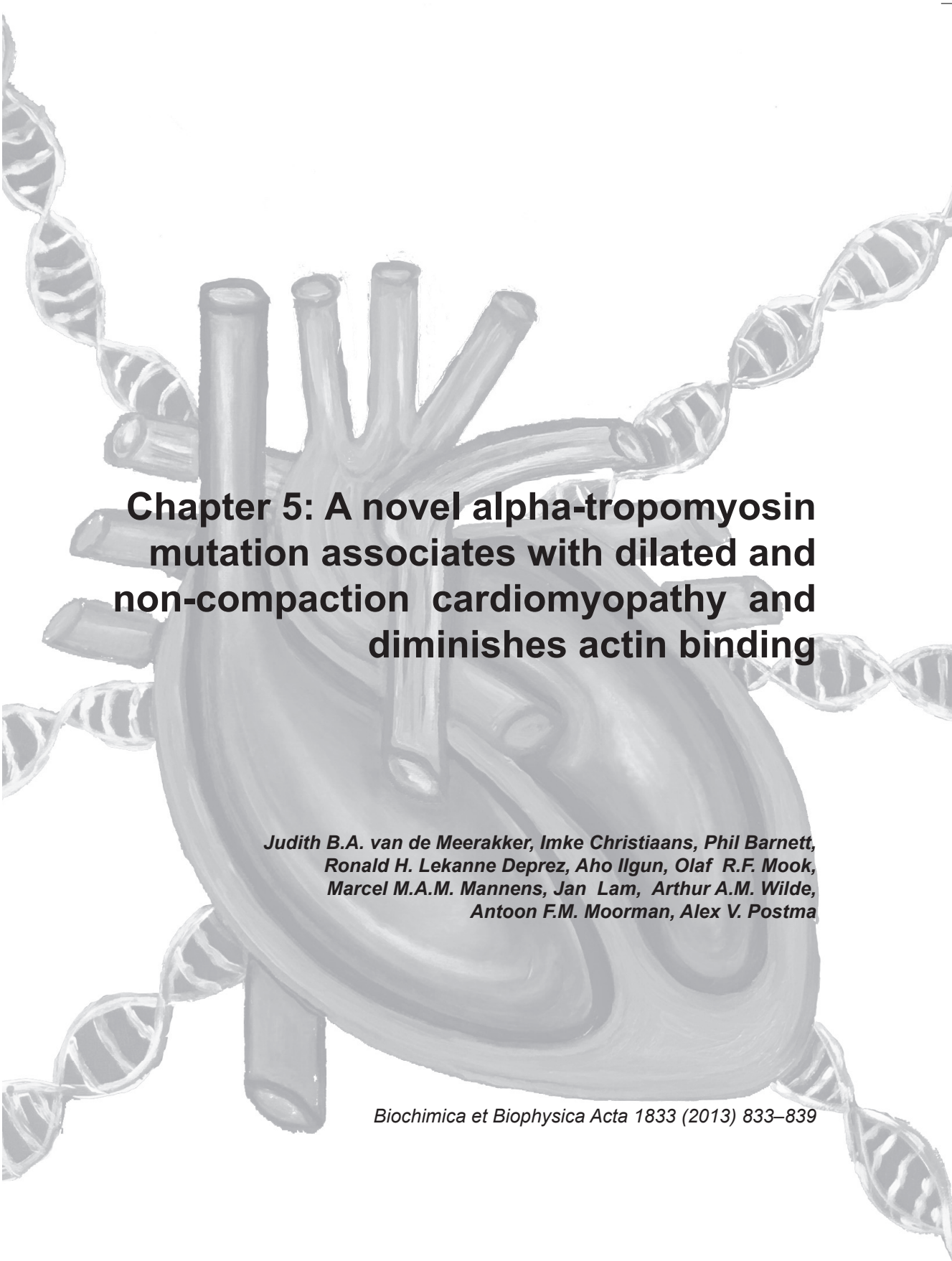

Chapter 5: A novel alpha-tropomyosin mutation associates with dilated and non-compaction cardiomyopathy and diminishes actin binding

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Abstract

Background

Dilated cardiomyopathy (DCM) is characterized by idiopathic dilatation and systolic contractile dysfunction of the ventricle(s) leading to an impaired systolic function. The origin of DCM is heterogeneous, but genetic transmission of the disease accounts for up to 50% of the cases. Mutations in alpha-tropomyosin (TPM1), a thin filament protein involved in structural and regulatory roles in muscle cells, are associated with hypertrophic cardiomyopathy (HCM) and very rarely with DCM.

Methods and results

Here we present a large four-generation family in which DCM is inherited as an autosomal dominant trait. Six family members have a cardiomyopathy with the age of diagnosis ranging from 5 months to 52 years. The youngest affected was diagnosed with dilated and non-compaction cardiomyopathy (NCCM) and died at the age of five. Three additional children died young of suspected heart problems. We mapped the phenotype to chromosome 15 and subsequently identified a missense mutation in TPM1, resulting in a p.D84N amino acid substitution. In addition we sequenced 23 HCM/DCM genes using next generation sequencing. The TPM1 p.D84N was the only mutation identified. The mutation co-segregates with all clinically affected family members and significantly weakens the binding of tropomyosin to actin by 25%.

Conclusions

We show that a mutation in TPM1 is associated with DCM and a lethal, early onset form of NCCM, probably as a result of diminished actin binding caused by weakened charge–charge interactions. Consequently, the screening of TPM1 in patients and families with DCM and/or (severe, early onset forms of) NCCM is warranted. This article is part of a Special Issue entitled: Cardiomyocyte Biology: Cardiac Pathways of Differentiation, Metabolism and Contraction.

Introduction

Cardiomyopathies are a heterogeneous group of diseases of the myocardium associated with mechanical and/or electrical dysfunction of the heart. They occur in isolated forms or as part of generalized systemic disorders and can lead to cardiovascular death or progressive heart failure-related disabilities. Primary cardiomyopathies were recently re-classified into three distinct forms: one with genetic etiology (e.g. hypertrophic cardiomyopathy (HCM)), one with a mixed genetic/non genetic etiology (e.g. dilated cardiomyopathy (DCM), restrictive cardiomyopathy), and one with an acquired etiology (e.g. myocarditis).[1] DCM and HCM are the most common forms, with DCM (including mixed/non-genetic etiology) alone accounting for 60% of the cardiomyopathies. Moreover, it is a common cause of heart failure and, importantly, represents the most frequent reason for a heart transplantation.[1-3] Idiopathic dilated cardiomyopathy is likely to be genetic and is characterized by dilatation and systolic contractile dysfunction of the left and/or both ventricles and consequently an impaired systolic function,[1] in the absence of secondary causes for the observed myocardial abnormality. Idiopathic DCM has an estimated prevalence of about 1:2500.[4, 5]

The origin of DCM is heterogeneous, but genetic transmission of the disease can account for up to 50% in diverse populations,[4-6] with multiple modes of inheritance including autosomal dominant (~ 60%), autosomal recessive (~ 16%), X-linked (~ 10%) and mitochondrial (~ 8%).[7] So far DCM has been linked to a diverse group of more than 30 loci and genes[1, 2, 8, 9] encoding cytoskeletal and costameric proteins as well as cardiac contractile proteins. Tropomyosin is one of the five proteins in the thin filaments of sarcomeres where it plays fundamental structural, developmental, and regulatory roles in muscle cells by interacting with actin.[10] Human cardiac muscle and fast twitch skeletal muscle exclusively contain alpha-tropomyosin (TPM1).[10, 11] Thus far, only two DCM associated mutations in TPM1 have been reported in nuclear families,[12] in contrast to HCM for which many TPM1 mutations have been found.[13]

Here we present a large four-generation family in which DCM is inherited as an autosomal dominant trait. Candidate gene linkage demonstrated linkage to chromosome 15 on which we subsequently identified the co-segregating p.D84N TPM1 mutation, which has not been described before and is absent from over 1100 control chromosomes. The mutation shows significantly weakened binding of tropomyosin to actin, which is corroborated by existing atomic and protein models.

Methods

Clinical details

Written informed consent was obtained from all patients or their legal representatives. Family members were evaluated using clinical history, physical examination, echocardi-

ography and ECG. DCM was diagnosed according to previously described criteria,[14] in short, the presence of a dilated and poorly functioning left ventricle (ejection fraction >40%) in the absence of abnormal loading conditions (hypertension, valve disease) or ischemic heart disease.

Linkage analysis, mutation screen and confirmation

Genomic DNA (gDNA) of family members was extracted from peripheral blood according to standard procedures. Linkage analysis was performed using the ABI linkage set v2.5 on an ABI 3700 Genetic Analyzer (Applied Biosystems).[15] Phenotype and genotype data and pedigree information were combined for multipoint linkage analysis with the use of the easyLinkage v5.02 software package[16] running the Merlin program v1.0.1[17] with the assumption of an autosomal dominant pattern of inheritance, a disease-allele frequency of 0.0001, and penetrance of 0.9 for affected individuals. Gene frequency was assumed to be equal between males and females.
Lamin A/C (LMNA) gDNA was PCR amplified using LMNA specific primers (available on request), located in flanking intronic sequences and analyzed by direct sequencing, using the BigdyeTerminator v3.1 Kit on an ABI 3700. PCR amplification of the MYH7, TPM1 and ACTC genes was performed using primers located in flanking intronic sequences (available on request). Subsequently, MYH7 and ACTC PCR products were analyzed by direct sequencing, as described above. For TPM1, PCR products were pre-screened using DHPLC according to standard protocols.[18] Certified genotypic normal controls were included in each run so that aberrant profiles could be differentiated from wildtype by visual inspection, aberrant profiles were subsequently sequenced. Exon 3 of TPM1 was additionally screened in the whole family by PCR and bi-directional sequence analysis.

Next generation sequencing

About 5 μg of DNA, isolated from peripheral blood leucocytes using an automated DNA isolator (Gentra), was fragmented according to the manufacturer's instructions (Covaris). DNA quality was assessed using a Bioanalyzer (Agilent). The sample was barcoded using standard Roche protocols. Libraries were amplified by linker-mediated PCR. This sample was part of a mixture of 10 samples that were mixed equimolarly (3 μg in total) and loaded onto the capture-array according to the manufacturer's instructions (Nimblegen). To increase target enrichment, samples were hybridized a second time on the same array. The enriched library was diluted, annealed to capture beads, and clonally amplified by emulsion PCR. After emulsion PCR, beads with clonal amplicons were enriched and deposited on a picotiter plate and sequenced on the GS FLX Titanium (Roche). The array was custom designed in such a way that all coding exons were targeted, including 100 bp flanking intronic sequences of 23 candidate genes.

In total the array contained 292 exons, targeting 117.5 kb. The following 23 genes were sequenced: 1. ACTC1 (NM_005159.4); 2. CSRP3 (NM_ 003476.3); 3. DES (NM_001927.3); 4. GLA (NM_000169.2); 5. LAMP2 (NM_002294.2, NM_013995.2, NM_001122606.1); 6. LDB3 (NM_007078.2, NM_001080116.1); 7. LMNA (NM_170707.2, ENST00000368294, ENST00000368297); 8. MYBPC3 (NM_000256.3); 9. MYH7 (NM_000257.2); 10. MYL2 (NM_000432.3); 11. MYL3 (NM_000258.2); 12. PLN (NM_002667.3); 13. PRKAG2 (NM_016203.3);14. SCN5A (NM_198056.2); 15. SGCD (NM_172244.2, NM_000337.4); 16. STA (NM_000117.2); 17. TAZ (NM_000116.3); 18. TCAP (NM_003673. 2); 19. TNNC1 (NM_003280.1); 20. TNNI3 (NM_000363.4); 21. TNNT2 (NM_000364.2; NM_001001430.1); 22. TPM1 (NM_000366.5, NM_ 001018005.1, NM_001018020.1); 23. VCL (NM_014000.2). The average coverage was 100 ± 23 reads. All variants of interest were confirmed by Sanger sequencing.

Plasmid constructs

Bacterial expression constructs were generated for human TPM1_WT and TPM1_ D84N. A GST-TPM1_WT fusion was created by ligating the *NcoI-SmaI* fragment from pOTB7 (Image ID 2962925), encompassing the entire human TPM1 ORF, into the pGEX2T- (Pharmacia) derived plasmid pRP265nb.[19] Subsequently the missense mutation D84N was introduced into this wildtype human TPM1 clone by PCR-based site-directed mutagenesis (QuickChange II Site-Directed Mutagenesis Kit, Stratagene) and verified by sequencing.

GST-TPM1 affinity purification

Escherichia *coli* BL21 cells transformed with GST-TPM1 bacterial expression constructs were grown at 37 °C to an OD600 of 0.5 in 100 ml of LB medium supplemented with 1% glucose. Cells were then induced with 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG, Gibco-BRL) and transferred to 30 °C for further incubation to minimize proteolysis and inclusion body formation. After 1–2 h growth, cells were harvested by centrifugation for 10 min at 10,000 *g* and then resuspended in 3 ml of ice-cold resuspension buffer (20 mM Tris pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mg/ ml DNAse and 1 mg/l RNAse). Cell suspensions were subsequently lysed till a clear lysate was obtained by sonication (Sonics Vibracell sonicator VC-70, 6× 20–30 s amplitude 80 at 4 °C) and then centrifuged to pellet cell debris. The cleared lysate containing the GST fusions was mixed with equal amounts of binding buffer (20 mM Tris pH 7.3, 150 mM NaCl, 0.5% NP-40, 1 mM PMFS and an appropriate amount of protease inhibitor) before passing it over a Glutathione Sepharose resin (Amersham Biosciences) column equilibrated in binding buffer. The column was then washed with 5 ml of PBS buffer supplemented with 0.05% Triton X-100 before being eluted in fractions of 100 µl of elution buffer (50 mM Tris pH 8.0, 20 mM L-Glutathione (Sigma)).

Elute fractions were collected, subjected to SDS-PAGE and PageBlue staining (Fermentas) of the gel.

Actin-TPM1 co-sedimentation assay and analysis

Rabbit muscle actin was used (Sigma, A2522, 43 kDa). For the assay, the actin (1 mg/ml) was diluted to 0.4 mg/ml in a buffer containing 5 mM Tris pH 8; 0.2 mM CaCl2; 0.2 mM ATP and 0.5 mM DTT, centrifuged at 20,000 *g* for 10 min at 4°C. The supernatant, which is monomeric actin was kept on ice for 1 h before polymerization. Actin polymerization was induced by the addition of 1/10th volume of polymerization buffer (500 mM KCl; 20 mM MgCl2 and 1 mM ATP). The polymerization occurred at room temperature for 1 h. A molar ratio of actin to purified GST-TPM1 of 4:1 was used in the binding assay, final reaction volume was 30–40 µl. The GST-TPM1 proteins (60 kDa) and F-actin were incubated in reaction buffer (10 mM Tris pH 7; 2 mM MgCl2; 1 mM ATP; 0.2 mM DTT and 1 mM EGTA) for 1h at RT. Tropomyosin from porcine muscle (Sigma, T2400, 35 kDa) served as a positive control. Following incubation, the samples were spun at 130,000– 140,000 *g* for 30 min at 4 °C using a Beckman airfuge. The rotor was carefully removed so as not to disturb the pellets. The supernatants were carefully removed and 5 × Laemmli SDS-PAGE sample buffer was added. The remaining pellets were dissolved in 1× appropriate volume and incubated overnight at −20 °C. The relative amounts of protein in the pellets and supernatants were analyzed following their separation on a SDS-PAGE gel and PageBlue (Fermentas) staining of the gel. Subsequently the staining intensity of the actin bands of both wildtype and mutant proteins in the pellet phase were measured using Aida 3.44. The experiment was repeated 5 times. Results are expressed as means±SD. To remove variations from experiments attributable to differences in protein batches and day-to-day handling, a factor correction was applied.[20] The corrected data set was used for statistical analysis. Comparisons between wildtype and mutant were performed using Student's t tests. Pb 0.05 was considered statistically significant.

Surface and ribbon model

To create the surface and ribbon model, Deepview version 3.7 was used. Positive charged side groups are colored blue, negative ones red. The structural representations are based on the coordinate files from the atomic model described by Pirani et al.[21]

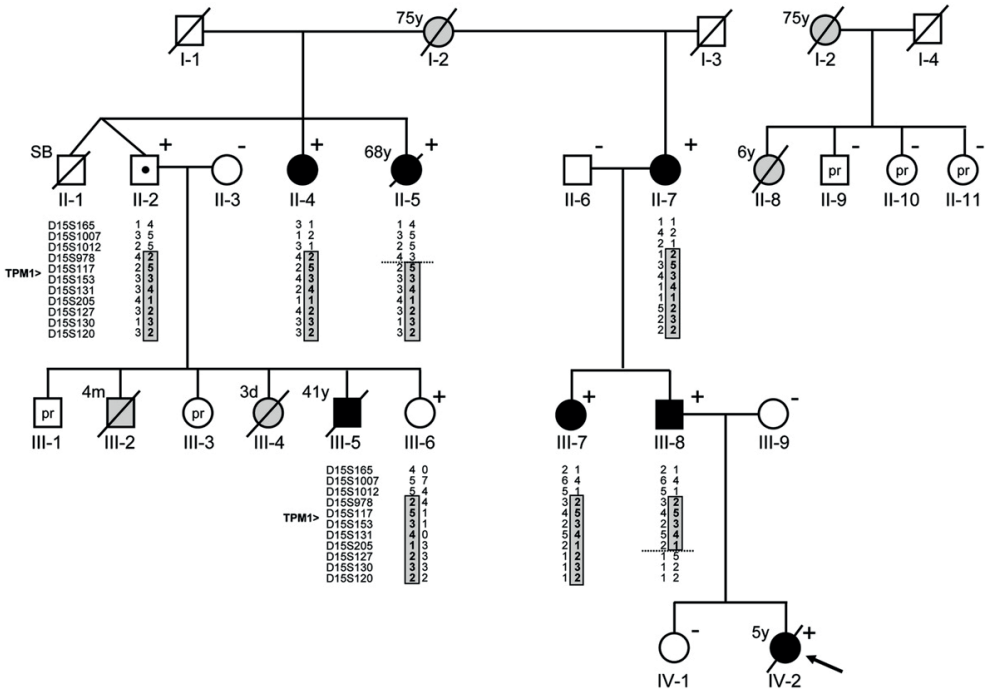

Figure 1. Pedigree of family with DCM and NCCM, including linkage with microsatellite markers surrounding *TPM1* and the resultant 5-3-4-1 haplotype co-segregating with the phenotype, dotted lines indicate the boundaries of linkage. Carriers of the p.D84N TPM1 mutation are noted with +, wildtype −. Solid symbols indicate individuals with clinically defined DCM, gray filled symbols mark individuals with suspected DCM phenotype, marks obligate carrier, and open symbols indicate individuals without DCM phenotype. pr indicates clinical status obtained from relative of patient, but not investigated by clinician, slashes denoted deceased individuals, age at death is noted at top left of individual, the proband is indicated with an arrow. For further clinical details see Table 1.

Results

Clinical phenotype

The proband, IV-2, (Figure 1) was admitted to the hospital at the age of 5 months because of progressive coughing, feeding difficulties and growth retardation. Echocardiography demonstrated an enlarged heart with severely reduced function. Subsequent investigations, by heart catheterization and a muscle biopsy, did not show other abnormalities and the girl was diagnosed as having an idiopathic dilated cardiomyopathy. At the age of 2 years the proband had often been readmitted to the hospital with pneumonia. She had developed atelectasis of part of the left lung, an insufficiency of the mitral valve and she was on anticoagulants because of a cardiac thrombus. Echocardiography later revealed

also signs of non-compaction cardiomyopathy (NCCM). At the age of 4 years and 11 months the proband was admitted to the Intensive Care Unit with heart failure after surgery of an inguinal hernia. The proband died at home during her sleep at the age of 5 years and 7 months. Examination of the family history revealed a theme of heart problems in the family of the proband's father. The proband's father, III-8, was at the time of the diagnosis of his daughter 29 years old and asymptomatic. Subsequent echocardiographic evaluation revealed mild dilatation of the left ventricle and a slightly decreased ejection fraction. His heart function has remained stable over the past decade. In addition, echocardiography of the sister of the proband's father, III-7, revealed a dilatation of the left ventricle, which has also remained stable over the last decade. The paternal grandfather, II-6, did not show any abnormalities on echocardiography, but the proband's paternal grandmother, II-7, had dyspnea on exertion, palpitations and dizziness at the time of diagnosis of the proband. She was also diagnosed with a DCM. Her mother, I-2, had nine children with three husbands. Of the four children of her first marriage, one child, II-1, one of twin siblings, was stillborn. The twin brother, II-2, had a myocardial infarction at the age of 66 years, though has not been diagnosed with cardiomyopathy. He had six children, two of whom, III-2 and III-4, died prior to the age of 6 months of a suspected cardiomyopathy. One son, III-5, died suddenly at the age of 41, pathological examination revealed multiple lung emboli and he was shown to have a factor-V Leiden mutation. His heart displayed hypertrophy and was dilated. The youngest daughter of II-2, III-6, although examined on several occasions, does not show any cardiological abnormalities. The two sisters of II-2, II-4 and II-5, have also been diagnosed with DCM. II-4 was diagnosed with DCM at the age of 51 years, she received an implantable defibrillator (ICD). Her sister, II-5, showed the presence of DCM when examined upon admission for heart failure at the age of 52 years. After a syncope and a cardiac arrest several years later, she also received an ICD. In subsequent years she had one appropriate ICD discharge and progressive heart failure developed. She died at the age of 68 years waiting for a heart transplantation. The third marriage of I-2 to I-4 bore four children. One of them, II-8, died at the age of 6 years from an undefined heart problem, the other three children have no history of heart problems. The great grandmother herself, I-2, died at the age of 75, with heart complaints. The results of phenotypic evaluation are shown in Figure 1 and Table 1.

Linkage analysis and mutation screen

Initially, the Lamin A/C gene (LMNA), associated with DCM,[22] was screened, and no mutations were identified. Subsequently, we performed linkage analysis using a list of 22 candidate genes (known to be associated with DCM, these genes were also included in the next generation sequencing performed in the proband, see below) among all affected and obligate gene carriers except for IV-2 of whom a limited amount of DNA was available. This analysis revealed 2 loci co-segregating with DCM, on chromosomes 14 and

15 (Figure 1). Maximal multi-point LOD-scores for chromosome 14 and 15 were 1.08 and 1.15 respectively, with the loci being flanked by D14S283/D14S65 and D15S978/D15S127 (Figure 1). These loci contain three genes known to be causative for DCM, namely MYH7, TPM1 and ACTC.[9, 12, 23] Therefore, mutation analysis was performed on each of these genes. No mutations in MYH7 or ACTC were detected, but in exon 3 of TPM1 (NM_001018005) a heterozygous single-nucleotide variant (c.250G→A, Figure 2A) was observed in the proband. This nucleotide change would result, in all TPM1

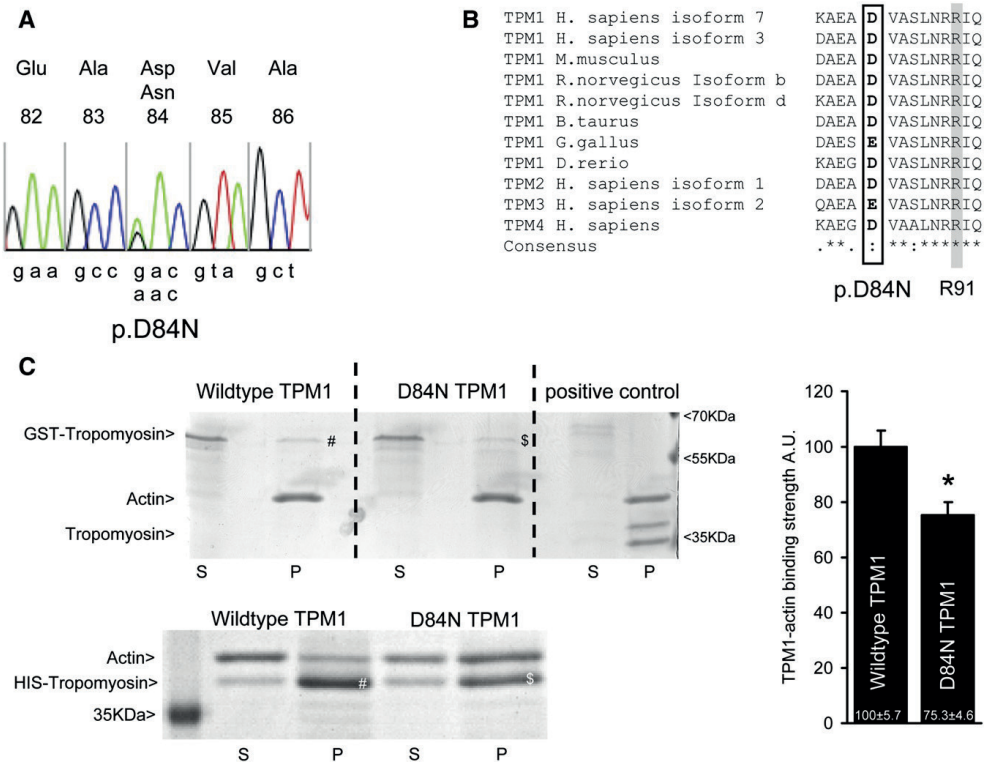

Figure 2. A. Electropherograms depicting the nucleotide changes and consequent amino acid changes of the TPM1 p.D84N mutation in the proband. B. Alignment of part of the TPM1 protein of various species, indicating that the D84N mutation occurs in an evolutionarily highly conserved area. C. Representative gel of a GST-TPM1/actin co-sedimentation assay. The left panel shows the supernatant (S) and pellet (P) phases of wildtype TPM1/actin, the middle panel D84N TPM1/actin, and the right panel the positive control of porcine muscle TPM1/actin (without GST tag, notice the lower molecular weight). A representative gel of a HIS-TPM1/actin co-sedimentation assay is also shown indicating that the mutation has a less intense binding of actin. Bar diagrams represent the averaged measured intensity of the TPM1 wildtype band (#) in comparison with that of TPM1 D84N band ($) after co-sedimentation in the pellet phase, n= 5, SD.

isoforms, in the replacement of a negatively charged aspartic acid for a polar asparagine, at the highly conserved amino acid position 84, p.D84N (Figure 2B). This variant has never been described before in cardiomyopathy patients, it was not present in 1100 control chromosomes or in over 6500 exomes.[24] In parallel, we performed mutational analysis of exon 3 of TPM1 in all family members, and found that p.D84N co-segregated with all family members diagnosed with a cardiomyopathy. Two family members carrying the mutation did not have any signs of DCM, II-2 and III-6, the first being an obligate carrier. If this nucleotide change is included in the linkage, the LOD score for the chromosome 15 locus becomes 1.73. To rule out additional mutations, we performed next generation sequencing in the proband on 23 genes known to cause either HCM or DCM. We did not find additional pathogenic mutations besides TPM1 p.D84N.

Binding of the mutant D84N TPM1 to actin is significantly diminished

To investigate the effects of the TPM1 mutation on actin binding, we produced both wildtype and D84N TPM1 protein through affinity purification (data not shown). We assayed the binding activity of wildtype and mutant TPM1 to F-actin using co-sedimentation. Any TPM1 protein bound to actin should be present in the pellet phase. After centrifugation the pellet and supernatant phase were loaded separately on a SDS-PAGE gel and stained (Figure 2C). To test the assay we initially performed co-sedimentation with tropomyosin from porcine muscle and actin (Figure 2C right panel), showing efficient, and reproducible binding of tropomyosin to actin with almost no tropomyosin in supernatant phase. Subsequently, the co-sedimentation assay was performed using the human GST-TPM1 wildtype and mutant protein. The intensity of the staining was measured for the GST-TPM1 band in the pellet (Figure 2C, symbol #) for both wildtype and D84N TPM1. The actin staining was used as an internal loading control. Although this reaction was less efficient, as the majority of GST-tropomyosin remained in the supernatant, the binding capacity of mutant D84N TPM1 for actin was modestly, but significantly reduced by $\pm 25\%$ ($p < 0.001$, $n = 5$, Fig. 2C) in comparison with that of wildtype TPM1. To rule out that the large GST-tag interfered with the assay, we repeated the assay using HIS-tagged wildtype and mutant TPM1. We found a similar reduction in binding of actin by mutant TPM1 (Figure 2C). Taken together, these results indicate that the tropomyosin mutation adversely affects actin binding.

Table 1 Clinical characteristics

Pedigree	Age at diagnosis (yrs)	Echocardiography			Electrocardiography	Cardiac phenotype	TPM1 genotype	Clinical history
		LVEDD (mm)	LVESD (mm)	FS (%)				
IV-2	0.4	57	Dilated	18	Sinustachycardia, high voltages, abnormal repolarization	DCM, with signs of NCCM	p.D84N	SD at home during sleep at age 5
III-8	29	54	42	22.22	Sinusbradycardia (58 BPM), PQ 184 ms, QRS 86 ms, normal repolarization	DCM	p.D84N	Stable disease, alive at age 39
II-6	–	Normal	Normal	Normal	Normal: sinusrhythm, normal PQ and QRS time, normal repolarization	Normal	Wildtype	Healthy at age 61
II-7	48	53	43	18.87	Normal: sinusrhythm, PQ 160 ms, QRS 76 ms, normal axis, normal repolarization	DCM	p.D84N	NYHA class II, alive at age 58
III-7	34	54	38	29.63	Normal: PQ 130 ms, QRS 84 ms, normal repolarization	DCM	p.D84N	NSVT once, alive at age 42
II-2	–	–	–	–	Sinusrhythm, normal PQ time, incomplete LBBB, QRS 100 ms, repolarization abnormalities	Normal	p.D84N	MI at age 66, alive at age 78
III-2	–	–	–	–	–	Uncertain	–	Death at age of 4 months of heart muscle disease
III-4	–	–	–	–	–	Uncertain	–	Death at age of 3 days of heart muscle disease
III-5	41	–	–	–	–	DCM	–	SD at age 41. Dilated, hypertrophied heart and lung emboli at autopsy
III-6	–	Normal	Normal	Normal	Normal: PQ 178 ms, QRS 76 ms, normal repolarization	Normal	p.D84N	Asymptomatic at age 42
II-4	51	69	62	10	–	DCM	p.D84N	ICD implanted, alive at age 77
II-5	52	Dilated	Dilated	–	Partly pacemaker rhythm, partly LBBB and ventricular extrasystoles	DCM	p.D84N	ICD implanted after cardiac arrest, assessed for transplant, SD at age 68
II-8	–	–	–	–	–	Uncertain	–	Death from heart problem at age 6
I-2	–	–	–	–	–	Uncertain	–	Heart problems, death at age 75
IV-1	–	48	–	42	Normal: PQ 136, QRS 72, normal repolarization	Normal	Wildtype	Asymptomatic at age 12
II-9	–	–	–	–	–	Uncertain	–	Asymptomatic
II-10	–	–	–	–	–	Uncertain	–	Asymptomatic
II-11	–	–	–	–	–	Uncertain	–	Asymptomatic

NCCM = non-compaction cardiomyopathy, SD = sudden death, DCM = dilated cardiomyopathy, NYHA = functional class according to the New York Heart Association, NSVT = non-sustained ventricular tachycardia, MI = myocardial infarction, ICD = implantable cardioverter defibrillator.

Putative effect of tropomyosin Asp84 to charge–charge interactions with actin

Figure 3A shows a ribbon representation of a segment of tropomyosin based on the X-ray crystal structure of tropomyosin.[25] Known mutated residues, Glu40, Glu54, Arg91(Beta-tropomyosin[26]) and the Asp84 described here, are shown in bold. In Figure 3B (stereoscopic view) and 3C (close-up) we show an atomic model of the binding of TPM1 to actin constructed by Pirani and co-workers.[21] Blue colors denote positively charged areas and residues, while red colors denote negatively charged ones. Although exact atomic distances may be under- or overestimated, it is clear that the negatively charged wildtype Asp84 (red) is in close proximity to a positively charged area (blue) on the actin molecule. As such, it is positioned in a way that allows charge–charge interactions with actin. The introduction of the mutant polar asparagine in this position would disturb such interactions, especially in the Ca^{2+}-state and would result in the weakened binding of tropomyosin to actin as experimentally observed.

Discussion

There is considerable heterogeneity in the clinical features and age at disease onset of DCM resulting from a single gene mutation. Most genetic DCM cases become apparent during the fourth or fifth decade of life, but first signs can also be noticed in young children and the severity of symptoms and survival, even in affected members of the same family, can vary considerably.[27] We have identified a novel missense mutation (p.D84N) in alpha-tropomyosin in a large four-generation family with DCM and non-compaction cardiomyopathy and we reason that it is the disease causing mutation. This is supported by several observations, firstly, linkage and haplotype analysis show that a DCM disease locus on chromosome 15 (containing TPM1) co-segregates with the disease. Secondly, we identified a mutation in TPM1 that is present in all clinically affected family members, and no other mutations in DCM or HCM genes were found. Thirdly, the mutated p.D84N residue has been highly conserved in vertebrates throughout evolution. Fourthly, the mutation is predicted to weaken the binding of tropomyosin to actin in the Ca^{2+} state and lastly the mutation significantly weakens TPM1 binding to actin.

The phenotype of the p.D84N mutation carriers ranges from dilated cardiomyopathy with signs of non-compaction, resulting in (sudden) death at the age of five, to asymptomatic at the age of 42 (or possibly even older considering I-2). Several family members have been diagnosed with DCM at an age over 50, while three young children died of suspected heart problems without a clear diagnosis. Only two TPM1 mutations associated with DCM have been described before in small nuclear families (E54K and E40K), both of which are associated with severe, early onset DCM, arrhythmias and cardiac heart transplantation, but also with asymptomatic carriers,[12]

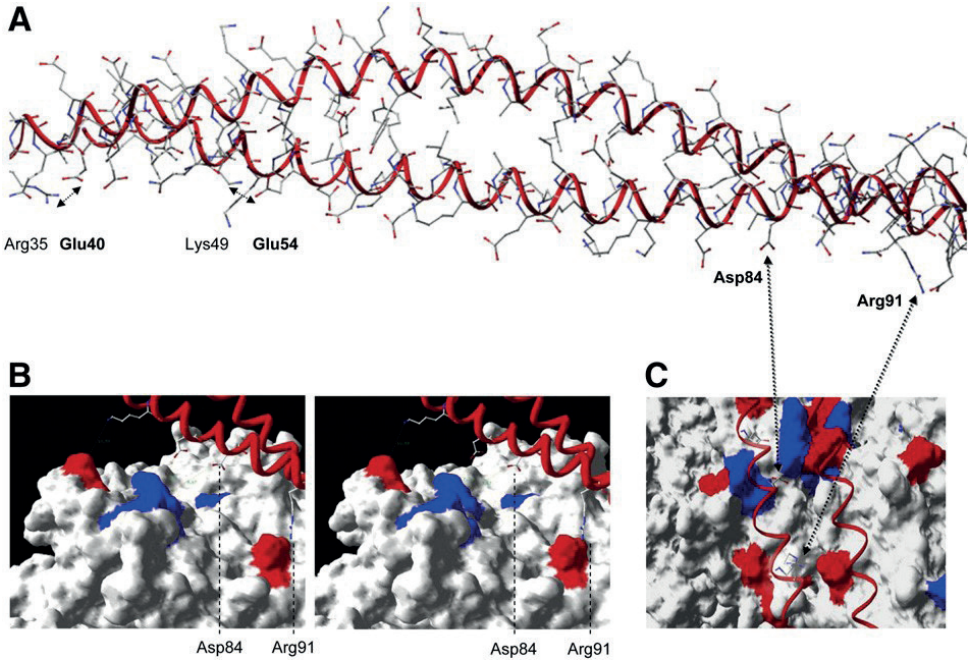

Figure 3. A. Ribbon representation of a segment of tropomyosin based on the X-ray crystal structure of tropomyosin highlighting positions of key residues involved in TPM-actin interactions. B. Stereoscopic view of an atomic model of the binding of TPM1 to actin [21], blue denotes areas of positively charged residues and red negatively charged residues. TPM1 appears as a ribbon. C. Same model as used in B, but viewed from above, showing the local charges on the actin surface in relation to those on TPM1, in particular in the area of the mutation. Note that TPM1 positively charged (red) residues (Asp84) are in close contact with negatively charged (blue) areas on actin and vice versa (Arg91).

being in line with the phenotype of the current family. This suggests that TPM1 DCM mutations not only cause a highly diverse phenotype, but importantly, can include death at young ages and severe, early onset cardiomyopathic forms. In fact, the proband, who died at the age of five, also had signs of NCCM and while this can be detected in patients with an initial diagnosis of DCM or HCM,[28] recent reports describe the co-occurrence of both NCCM and DCM co-occurring in one family, analogous to the family presented in this study.[28-30] The additional screening of 23 known DCM/HCM genes by next generation sequencing did rule out a second mutation in those genes, however we cannot rule out the possibility that the diverse phenotype in this family might be caused by additional mutations, or could be the effect of the genetic background. However, why DCM TPM1 mutations can cause such diverse and severe forms of cardiomyopathy remains to be solved. One possibility is that alterations in protein stoichiometry, caused by a point mutation, may produce a

poison polypeptide that disrupts myofibrillar organization on incorporation into the sarcomere and thereby result in the severe phenotype. The fact that mutation carriers can be asymptomatic can be explained by a regulatory mechanism that maintains the level of myofibrillar tropomyosin. Such a mechanisms was shown in TPM1 heterozygote knockout mice, who have an approximately 50% reduction in alpha-tropomyosin mRNA levels, but have no reduction in alpha- tropomyosin protein.[31] Regardless of the underlying mechanism, our data suggests that screening of TPM1 in families with both DCM and NCCM is warranted.

TPM1 mutations were initially associated with HCM, also presenting with diverse phenotypes, from a benign course to severe hypertrophy with progression to DCM and heart failure.[32-34] In Japanese families, missense mutations in the TPM1 gene have been associated with the progression of HCM to DCM.[34] However, based on all available clinical and in vitro data, our patients did not have such a HCM to DCM transition, as it would be extremely unlikely for end-stage HCM to present as DCM or NCCM at the age of 2 years or as minimally symptomatic and stable DCM diagnosed at 29 years of age. Furthermore, only one of the mutation carriers (III-5) showed hypertrophy of the heart muscle at autopsy, though in combination with a dilated heart. Moreover, the in vitro characteristics of the mutant protein point to a loss of binding, which is consistent with DCM. Our in vitro results suggest that mutant TPM1 binding to actin is significantly diminished, possibly caused by a weakened charge–charge interaction of the protein, see below. Although the wildtype tropomyosin from porcine muscle showed a more efficient binding to actin compared to the human one, we attribute that to the presence of the GST or HIS tag, necessary for the production and isolation of human wildtype and mutant protein. As mentioned earlier, only two DCM causing mutations are described for TPM1 so far, E54K and E40K.[35] Both these DCM mutations are associated with a decreased Ca^{2+} sensitivity possibly as a direct consequence of a weakened calcium-on interaction with actin. In addition, a crystallographic study of tropomyosin at 7 Å revealed that both E40 and E54 may contribute to the stability of the TPM1 filaments via interchain salt bridging to R35 and K49 respectively (Figure 3A).[25] The E40K and E54K mutations will disturb such interactions, altering local tropomyosin structure and possibly the stability of the intertwined TPM1 helices. However, even though the D84N mutant described here occurs at a similar position along the helical axis, it does not appear to form interchain salt bridges like E40 and E54. Actually, close inspection of actin–tropomyosin filament models in both the Ca^{2+} and low-Ca^{2+} state, indicates that D84 may contribute to the charge–charge interactions thought to be important for tropomyosin– actin association.[21] Negatively charged residues, such as D84 are positioned in close proximity to positively charged residues on the actin surface (and vice versa) and this may contribute to charge–charge interactions.

In fact, the positively charged residue R91 is found mutated in the highly related beta-tropomyosin (TPM2) in cases of distal arthrogryposes.[26] This R91 residue is well conserved and positioned within the charge–charge range of negatively charged areas on actin in the Ca^{2+} state. It therefore appears that reciprocally charged residues on both actin and tropomyosin play a role in the interactions between tropomyosin and actin in the Ca^{2+} state, and that disturbances herein may underlie a hypotonic response and the observed hypocontractile DCM phenotype. In the low Ca^{2+} state, the R-groups of these residues become removed from the actin charge surface by distances in excess of 20 Å, thereby probably weakening the charge-charge interaction. Unfortunately, the generated atomic model does not include E40 or E54, so no comparison can be made for these mutations regarding charge–charge interactions. Taken together, it is not entirely clear how TPM1 mutations cause DCM, but altered Ca^{2+} sensitivity, decreased tropomyosin–actin association and changed stability of the intertwined TPM1 helices, are all likely to contribute to the final phenotype. In conclusion, we present a large four-generation family with DCM, NCCM and death at young age. A co-segregating TPM1 mutation, p.D84N, was found in all clinically affected family members, and results in a diminished binding of tropomyosin to actin, possibly as a result of weakened charge–charge interactions. This suggests that screening of TPM1 in patients and families with DCM and/or severe, early onset forms of DCM or NCCM is warranted.

Acknowledgements

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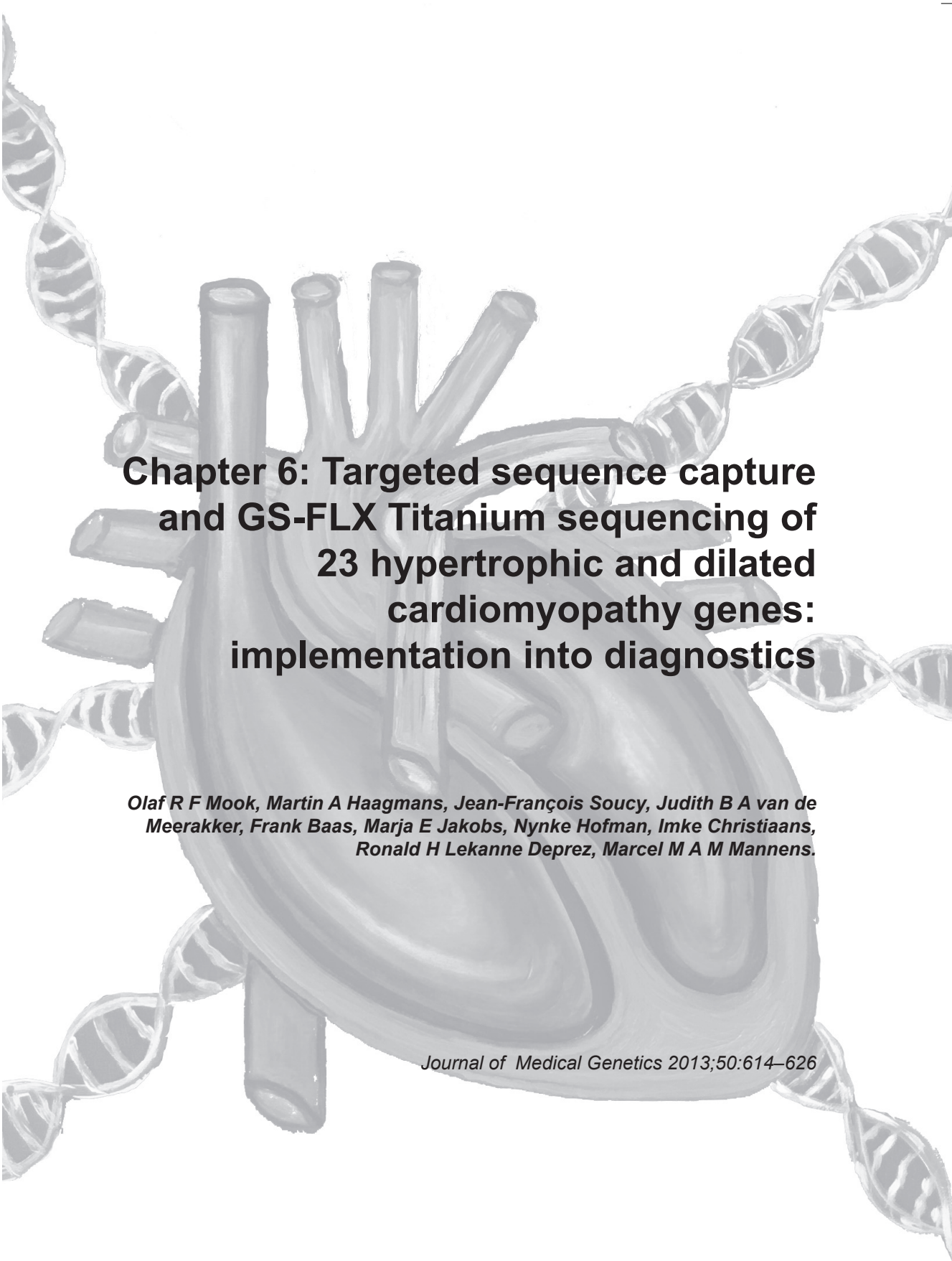

Chapter 6: Targeted sequence capture and GS-FLX Titanium sequencing of 23 hypertrophic and dilated cardiomyopathy genes: implementation into diagnostics

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Abstract

Background

Genetic evaluation of cardiomyopathies poses a challenge. Multiple genes are involved but no clear genotype–phenotype correlations have been found so far. In the past, genetic evaluation for hypertrophic (HCM) and dilated (DCM) cardiomyopathies was performed by sequential screening of a very limited number of genes.
Recent developments in sequencing have increased the throughput, enabling simultaneous screening of multiple genes for multiple patients in a single sequencing run. Objective Development and implementation of a next generation sequencing (NGS) based genetic test as replacement for Sanger sequencing.

Methods and Results

In order to increase the number of genes that can be screened in a shorter time period, we enriched all exons of 23 of the most relevant HCM and DCM related genes using on-array multiplexed sequence capture followed by massively parallel pyrosequencing on the GS-FLX Titanium. After optimisation of array based sequence capture it was feasible to reliably detect a large panel of known and unknown variants in HCM and DCM patients, whereby the unknown variants could be confirmed by Sanger sequencing.

Conclusions

The rate of detection of (pathogenic) variants in both HCM and DCM patients was increased due to a larger number of genes studied. Array based target enrichment followed by NGS showed the same accuracy as Sanger sequencing. Therefore, NGS is ready for implementation in a diagnostic setting.

Introduction

Genetic cardiomyopathies represent a group of diseases that are caused by alterations in over 50 genes lacking clear genotype–phenotype correlations.[1-3] As sequential testing of all those genes is both time consuming and expensive, a better testing strategy should therefore be cheaper and have shorter turnaround times. Over time, different approaches have been developed. In our laboratory, cardiomyopathy genes were tested using denaturing high performance liquid chromatography (dHPLC) followed by Sanger sequencing and Sanger sequencing without dHPLC as a pre-screen. Array based sequencing is a technique developed in the last few years that results in higher throughput, diminished costs, and shorter turn-around times.[4] However, this technology is less sensitive to insertion/deletion detection, is less amenable to panel modification (by adding new genes, for example), and has a very limited sequencing capacity in comparison to Illumina GAII sequencing, for example.[5] Next generation sequencing (NGS) is an attractive approach for high throughput screening of a large number of genes in a time and cost effective manner. At the moment the most popular choices are the Mi/HiSeq (Illumina), SOLiD (Life Technologies) and GS-FLX Titanium (Roche). Gowrisankar et al[5] published their results on using the Illumina GAII for the sequencing of pooled concatenated PCR products from five patients with dilated cardiomyopathy (DCM) who were previously analysed on their sequencing array. They concluded that NGS showed an analytical sensitivity that outperformed array based sequencing, mainly because of better indel detection, but it had a higher turnaround time in their hands. Meder et al[6] used the SOLiD 3 to test for mutations in 47 cardiomyopathy related and candidate genes in 10 patients diagnosed with either hypertrophic cardiomyopathy (HCM) or DCM. They concluded that an array based enrichment combined with SOLiD sequencing of 47 genes can be performed with high accuracy (but with known difficulties in detection of indels using short reads[7]) and in a cost effective manner.

For our study, we selected GS-FLX Titanium because of significantly longer reads, short run times and easy data analysis, making the transition to use in a diagnostic setting easier without the need for complex bioinformatics. Furthermore, the long reads of GS-FLX Titanium allow for more accurate mapping (pseudogene discrimination), detection of small indels, and can compensate for small untargeted regions of capture arrays.

In the present study we show that array based sequence capture can be optimised for balanced exon coverage. Optimised sequence capture in combination with GS-FLX Titanium sequencing allows for accurate detection of variants in 23 genes involved in HCM and DCM.

Methods

Subjects and clinical evaluation

This study comprises an initial pilot study followed by additional experiments in which a total of 63 patients were included. All patients have a confirmed diagnosis of HCM or DCM according to international criteria and were identified at or referred to the Department of Clinical Genetics, Academic Medical Center (AMC), Amsterdam, for screening of cardiomyopathy related genes offered at the Department of DNA diagnostics, AMC, the Netherlands.

For an initial pilot we included five HCM patients with a confirmed pathogenic mutation in either the *MYBPC3* or *MYH7* gene. In an additional experiment we included nine probands who were diagnosed with HCM but had no pathogenic mutation in eight HCM genes (*MYBPC3*, *MYH7*, *MYL2*, *MYL3*, *TNNI3*, *TNNT2*, *TPM1*, and *GLA*) screened in our laboratory by Sanger sequencing. Furthermore, we also included 19 probands diagnosed with DCM. However, DCM patients were not routinely screened for all these genes, in contrast to HCM patients. Finally, we included 30 cardiomyopathy index patients who were registered for routine DNA diagnostics for all HCM genes. These patients were used to validate the entire procedure with the intention to implement the procedure for diagnostics. Informed consent was obtained from all the patients.

Array design

We designed four custom 385K oligonucleotide microarrays according the Nimblegen Rebal algorithm to select unique probes (Roche NimbleGen). In the first design we targeted all exons, including 100 bp of the flanking upstream and down-stream intronic sequence, of 18 genes with known involvement in HCM and/or DCM. The design comprises 236 exons, targeting 92.5 kb (table 1). In the second design we rebalanced the capture probes by the addition of replicate probes at regions with low coverage in order to obtain a more evenly distributed exon coverage. For regions with a coverage between 75% and 61% of the mean coverage we added one additional probe, between 60% and 51% we added two additional probes, between 50% and 41% we added three additional probes, between 40% and 31% we added four additional probes, between 30% and 21% we added five additional probes, and for ≤20% we added six additional probes. In the third design we fine-tuned the balancing and added five more genes, making the total number of genes 23, containing 292 exons and targeting 117.5 kb. These 23 genes were selected because they were already used in diagnostics supplemented with candidate genes that were selected based on published evidence, with a focus on mutation detection rate. In the fourth design we rebalanced the last five genes that were added (table 1).

Table 1 Genes present on the capture array

Genes on V1/V2	Genes on V3	Chromosome	Exons	NM_number	Location/ function
	ACTC1	15q14	6	NM_005159.4	Sarcomere
CSRP3	*CSRP3*	11p15.1	5	NM_003476.3	Z disk
DES	*DES*	2q35	9	NM_001927.3	Intermediate filament
EMD	*EMD*	Xq28	6	NM_000117.2	Nuclear lamina
GLA	*GLA*	Xq22.1	7	NM_000169.2	Lysosome function
LAMP2	*LAMP2*	Xq24	11	NM_002294.2 NM_013995.2 NM_001122606.1	Lysosome function
	LDB3	10q23.2	13	NM_007078.2 NM_001080116.1	Z disk
LMNA	*LMNA*	1q22	12	NM_170707.2 ENST00000368294 ENST00000368297	Nuclear lamina
MYBPC3	*MYBPC3*	11p11.2	34	NM_000256.3	Sarcomere
MYH7	*MYH7*	14q11.2	37	NM_000257.2	Sarcomere
MYL2	*MYL2*	12q24.11	7	NM_000432.3	Sarcomere
MYL3	*MYL3*	3p21.31	7	NM_000258.2	Sarcomere
	PLN	6q22.31	1	NM_002667.3	Cardiac Ca++ ATPase inhibitor
PRKAG2	*PRKAG2*	7q36.1	16	NM_016203.3	AMP activated protein kinase
SCN5A	*SCN5A*	3p22.2	28	NM_198056.2 NM_001099404.1 NM_001160160.1	Sodium ion channel
	SGCD	5q33.2	8	NM_172244 NM_000337	Dystrophin associated protein complex
TAZ	*TAZ*	Xq28	11	NM_000116.3	Mitochondrial cardiolipin transacylase
TCAP	*TCAP*	17q12	2	NM_003673.2	Z disk
TNNC1	*TNNC1*	3p21.1	6	NM_003280.1	Sarcomere
TNNI3	*TNNI3*	19q13.42	8	NM_000363.4	Sarcomere
TNNT2	*TNNT2*	1q32.1	15	NM_000364.2 NM_001001430.1	Sarcomere

TPM1	*TPM1*	15q22.2	9	NM_000366.5 NM_001018005.1 NM_001018020.1	Sarcomere
	VCL	10q22.2	21	NM_014000.2	Z disk

A schematic overview of the different versions of the arrays used, the hybridisation protocol used, the array design used for a patient group and its improved performance as represented in coverage statistics is provided in figure 1.

Sample preparation

DNA was isolated from peripheral blood leucocytes using an automated DNA isolator (Gentra). Then 0.5–5 µg DNA was fragmented according to the manufacturer's instructions (Covaris). DNA quality was assessed by running the samples on a DNA7500 chip on the Bioanalyzer (Agilent). Each sample was bar coded by ligation of GSMID-adaptors or RL-multiplex identifiers (MID) adapters using standard Roche protocols. Libraries were amplified by linker mediated PCR to obtain sufficient amounts of starting material for the sequence capture; 3 µg of amplified library was

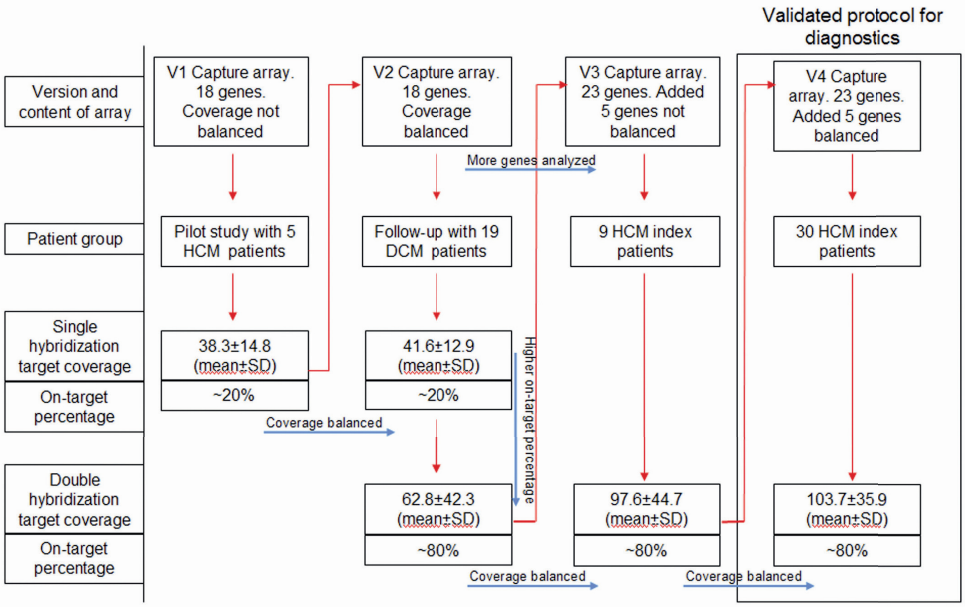

Figure 1. Flow chart of all experiments performed in this study. Red arrows show the order of the different experiments. Blue arrows indicate steps where the performance of the array was improved.

multiplexed samples, 5–10 amplified libraries were mixed equimolarly and then 3 µg of the mixture was loaded onto the array. For multiplex experiments an equimolar pool of bar code specific blocker oligo's was added in the same concentration as used for non-multiplexed hybridisations.

loaded onto the array according to manufacturer's instructions (http://www.nimblegen.com/products/lit/SeqCap_UserGuide_Titanium_Delivery_v1p1.pdf). For on-array

Target enrichment and sequencing

Hybridisation, post-hybridisation washes and elution of the enriched sample was performed according to the manufacturer's instructions (http://www.nimblegen.com/products/lit/SeqCap_UserGuide_Titanium_Delivery_v1p1.pdf). To increase target enrichment, the enriched samples were hybridised a second time on the same array used the first time. This second hybridisation reduces background sequences (off-target sequences) that are still present after the first hybridisation. It is the reduction of background that results in higher on-target percentages. To verify successful hybridisation capture, we performed quantitative PCR (qPCR) on four control loci before and after array enrichment. The relative fold enrichment was calculated using the values of delta crossing point (CP) (i.e., the difference between average CP of non-captured and average CP of captured samples) according $E^{\delta\text{-}Cp}$ where E is the efficiency of the qPCR assay for a particular amplicon.

The enriched library was diluted, annealed to capture beads, and clonally amplified by emulsion PCR. After emulsion PCR, beads with clonal amplicons were enriched and deposited on a picotitre plate and sequenced on the GS-FLX Titanium.

Mapping, variant detection and classification

The obtained sequence reads were mapped against the human reference genome (hg19) with the Roche GS Reference Mapper (V.2.6) using the default software settings. Output was restricted to the targeted regions as defined by the sequence capture design. Coverage statistics were extracted from the mapping output files using custom scripts. Variants were automatically detected during mapping and annotated with known gene (refSeq genes from http://genome.ucsc.edu/) and single nucleotide polymorphism (SNP) information (dbSNP130 from http:// genome.ucsc.edu/). They were denoted as high quality differences (HCDiffs) when the variation was present in at least three non-duplicate reads that included at least one forward and one reverse read, or when it was seen in at least five reads with quality scores over 20. Variants that did not meet the above criteria were collected in the AllDiff files. Occasionally, known variants end up in the AllDiff table but not in the HCDiff table. A custom script was written for the comparison of the HCDiff and AllDiff variants and selection of variants with a variant percentage ≥20%.

The script also identifies, at the single base resolution, regions with a coverage lower than 16×. These regions are additionally analysed by Sanger sequencing. This threshold has been reported by Hoischen et al[8] to be sufficient for diagnostic testing. We also calculated the minimal number of reads needed statistically. For the statistics we have used the following criteria:

1. for a heterozygous variant the allele frequency is 50%

2. a variant is reported when the variant percentage is ≥20%.

   All individual chances that a variant is missed at a given coverage is calculated in R using: x—seq(300) and c—pbinom((x*0.2),x,0.5).

With these criteria we calculated a 99% sensitivity (comparable to a Phred quality score of 20) at 16×. The frequency of each individual coverage given a mean coverage±SD is calculated in R using y <- pnorm(x, mean coverage, SD) with x <- seq (300). Then the chance that a variant is missed in an experiment with mean coverage±SD is calculated as: sum of (chance variant missed at given coverage)×(frequency at given coverage). Since we use a 16× threshold, the chance that a variant is missed is calculated for the 16× to 300× coverage interval. At a coverage of 100±35× this chance is 4.03E-5 (0.004%). An example of the calculations is given in figure 2.

Coverage	Chance missed	Frequency at 100±35X	Chance missed x frequency at 100±35X
1	0,5	0,000217317	0,000108659
2	0,25	0,000235415	5,88539E-05
3	0,125	0,000254813	3,18516E-05
4	0,0625	0,000275584	1,7224E-05
5	0,1875	0,000297805	5,58384E-05
6	0,109375	0,000321555	3,517E-05
7	0,0625	0,000346915	2,16822E-05
8	0,03515625	0,000373971	1,31474E-05
9	0,01953125	0,000402807	7,86733E-06
10	0,0546875	0,000433513	2,37078E-05
11	0,032714844	0,000466179	1,5251E-05
12	0,019287109	0,000500898	9,66087E-06
13	0,011230469	0,000537763	6,03933E-06
14	0,006469727	0,00057687	3,73219E-06
15	0,017578125	0,000618316	1,08688E-05
16	0,010635376	0,0006622	7,04274E-06
17	0,006362915	0,000708619	4,50888E-06
18	0,003768921	0,000757673	2,85561E-06
19	0,002212524	0,000809462	1,79095E-06
20	0,005908966	0,000864085	5,10585E-06
21	0,00359869	0,000921642	3,3167E-06
22	0,002171755	0,000982231	2,13316E-06
23	0,001299739	0,001045948	1,35946E-06
24	0,00077194	0,00111289	8,59084E-07
25	0,002038658	0,00118315	2,41204E-06
26	0,001246959	0,00125682	1,5672E-06
27	0,00075686	0,001333987	1,00964E-06
28	0,000456117	0,001414737	6,45286E-07
29	0,000273056	0,001499151	4,09353E-07
30	0,000715453	0,001587306	1,13564E-06
31	0,000438955	0,001679273	7,37125E-07
32	0,000267526	0,001775119	4,74891E-07
33	0,000162032	0,001874904	3,03794E-07
34	9,76E-05	0,001978683	1,93046E-07
35	0,00025413	0,002086502	5,30243E-07
36	0,000156276	0,002198401	3,43556E-07
37	9,55E-05	0,002314411	2,21115E-07

Coverage	Chance missed	Frequency at 100±35X	Chance missed x frequency at 100±35X
38	5,81E-05	0,002434555	1,41408E-07
39	3,51E-05	0,002558847	8,98856E-08
40	9,11E-05	0,002687289	2,44766E-07
↓↓↓↓↓↓	↓↓↓↓↓↓	↓↓↓↓↓↓	↓↓↓↓↓↓
270	2,02E-24	8,01856E-08	1,61661E-31
271	1,26E-24	6,97391E-08	8,76637E-32
272	7,83E-25	6,06041E-08	4,74558E-32
273	4,87E-25	5,26227E-08	2,56458E-32
274	3,03E-25	4,56552E-08	1,38358E-32
275	7,62E-25	3,95779E-08	3,01634E-32
276	4,75E-25	3,42815E-08	1,62907E-32
277	2,96E-25	2,96697E-08	8,7834E-33
278	1,84E-25	2,56573E-08	4,7277E-33
279	1,15E-25	2,21695E-08	2,54042E-33
280	2,88E-25	1,91402E-08	5,51523E-33
281	1,80E-25	1,65113E-08	2,96667E-33
282	1,12E-25	1,42318E-08	1,5931E-33
283	6,97E-26	1,22571E-08	8,54061E-34
284	4,33E-26	1,05477E-08	4,57098E-34
285	1,09E-25	9,06935E-09	9,88215E-34
286	6,79E-26	7,79182E-09	5,29422E-34
287	4,23E-26	6,68878E-09	2,83157E-34
288	2,64E-26	5,73721E-09	1,51192E-34
289	1,64E-26	4,91699E-09	8,05962E-35
290	4,12E-26	4,2106E-09	1,73517E-34
291	2,57E-26	3,60275E-09	9,25842E-35
292	1,60E-26	3,08014E-09	4,93188E-35
293	9,97E-27	2,63118E-09	2,62284E-35
294	6,20E-27	2,24583E-09	1,39258E-35
295	1,56E-26	1,91536E-09	2,98562E-35
296	9,72E-27	1,63218E-09	1,58662E-35
297	6,06E-27	1,38973E-09	8,41778E-36
298	3,77E-27	1,18233E-09	4,45875E-36
299	2,35E-27	1,00506E-09	2,35787E-36
300	5,90E-27	8,53676E-10	5,03413E-36
Chance missed x frequency for cov. 16-300X			4,03E-05

Figure 2. Statistical calculation of assay sensitivity. The assay sensitivity is calculated as the sum of (statistical chance that a variant is missed at a given coverage) x (frequency of that coverage). In this example the assay sensitivity was calculated for an experiment with a coverage of 100±35X. Individual chances in the coverage interval 16-300X (shown here the interval 1-40X and 270-300X) are highlighted in green. The assay sensitivity is highlighted in yellow.

The following criteria were used to classify variations/mutations. We use a list of mutation specific features based on in silico analysis using the mutation interpretation software AlaMut (V.1.5). A score is given depending on the outcome of a prediction test for each feature (i.e., Grantham distance). Then, depending on the total score and the availability of the variant in at least 300 ethnically matched control alleles (data obtained from the literature and/or the internet, e.g., 1000 Genome http://browser.1000genomes.org/index.html; Exome Variant Server: http://evs.gs. washington.edu/EVS, or from own control alleles), we classified them as: not pathogenic; as a variant of unknown clinical significance; VUS1, unlikely to be pathogenic; VUS2, uncertain; or VUS3, likely to be pathogenic. Family information (co-segregation), phenotypic features and/or functional analysis are needed to classify a variant as (putatively) pathogenic. The protocol used for the classification of variants has recently been published by van Spaendonck-Zwarts et al[9] All variants of interest identified in our HCM and DCM group were confirmed by Sanger sequencing.

Results

Target enrichment and on-target percentage

We evaluated and optimised the ability of Roche NimbleGen 385K Custom Sequence Capture Arrays to enrich patient samples for every exon in a panel of 23 genes. The degree of enrichment for the captured samples was estimated by qPCR analysis of four control loci. The average relative enrichment for the four control loci was >100-fold for all samples sequenced. The on-target percentages, calculated as number of bases present on the targeted region divided by the total number of mapped bases, varied between 12.8% and 33.1%, using the standard sequence capture protocol. When calculating the ratio of mean base coverage of the 92.5 kb targeted region divided by the mean base coverage outside the targeted region (~3 Gb), the enrichment factor is still 4720-fold and 16046-fold respectively. A mean on-target percentage using the standard protocol was on average ~20%. Although this is the equivalent of an enrichment factor of ~8000-fold, it is still not high enough to sequence an acceptable number of patients at an acceptable base coverage on the GS-FLX Titanium. Therefore we introduced a second round of hybridisation of the same sample on the previously used capture array. This procedure increased the on-target percentage to 75–92% (an 80% on-target represents an enrichment factor of 129 729-fold). Base coverage was reproducible both within and between the experiments with 99.8% of the targeted sequence covered.

On-array multiplexing

The use of capture arrays gives a limitation in the number of samples that can be processed in a given time. Moreover, the costs of one capture array per sample are

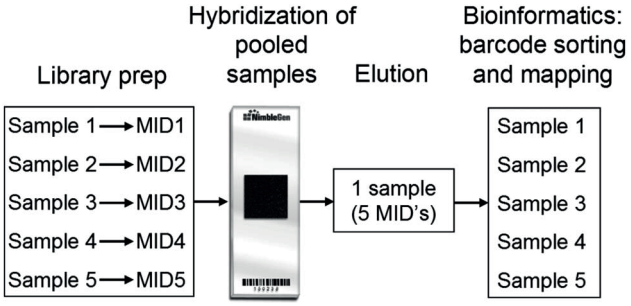

Figure 3 A schematic representation of an on-array multiplexed sequence capture experiment. After individual introduction of an unique bar code (multiplex identifiers) to each sample during the library preparation, all samples are mixed equimolarly and hybridised to one capture array. From that time on, the five multiplexed samples are treated as one sample during hybridisation, washes and elution as well as all post-capture steps including linker-mediated PCR, emulsion PCR and sequencing.

a significant part of the total costs of the entire procedure. Therefore, we used unique MIDs (bar coding) which are introduced during library preparation. After amplification and quantification of the individual libraries, equimolar mixtures of 5–10 patients were hybridised to one capture array according to the protocol. From that moment on the 5–10 patients were treated as one sample. After sequencing, the patients were separated based on their barcode and the variants were determined using the gsMAPPER (Roche) (figure 3). In general, 100K reads per patient within each multiplex experiment were sequenced. The difference in the number of reads per patient within a multiplexed sample usually was below twofold (75K to 150K reads per patient). Comparable number of reads per patient indicates that on-array multiplexing is feasible. Only occasionally a sample has less than half of the reads that was aimed for.

Proof of principle experiment

In a first proof of principle experiment we evaluated a custom design capture array (V1) to enrich patient samples for every exon of 18 genes with known involvement in cardiomyopathy (table 1). In this experiment we included five HCM patients with different known pathogenic mutations (two substitutions, a 2 bp deletion, and a 1 bp and 26 bp insertion,) in either the *MYBPC3* or *MYH7* gene. All five pathogenic variants (*MYH7* c.1436A>C, *MYH7* c.1207C>T, *MYBPC3* c.2373_2374dup, *MYBPC3* c.676_701dup26, and *MYBPC3* c.2864_2865del) were detected with sequencing on the *GS-FLX* Titanium. The whole coding region including splice junctions were Sanger sequenced for at least the gene where a pathogenic mutation was identified in the above mentioned HCM patients. Therefore, direct comparison of all the vari-

ants (pathogenic and non-pathogenic) found with Sanger sequencing and GS-FLX Titanium sequencing was possible. This showed that 57 out of 57 (100%) variants were detected with GS-FLX Titanium sequencing. Fifty-three out of 57 variants were present in the HCDiff list whereas four out of 57 were present in the AllDiff list. Evaluation of the variants present in the AllDiff list showed that three out of four variants were variants at the same position (*MYBPC3* c.1093-24C>T) and were not in the HCDiff list due to low coverage at the flank of the targeted region. The fourth variant (*MYBPC3* c.2373dup) was present in a sufficient number of reads that were of good quality, so there was no clear reason why it was not present in the HCDiff list. Therefore, in all the following analysis also the variants present in the AllDiffs with a variant percentage >20% were extracted from the list.

Optimisation of exon coverage and addition of more genes

Exon coverage was determined for all 18 genes and all exons except exon 5 of *PRKAG2* were enriched by the capture array. Coverage per exon varied significantly between genes but also within genes (figure 4A). The mean base coverage for the targeted bases was 38.3±14.8 (mean±SD) after a single hybridisation. Exons with coverage significantly below the mean coverage were rebalanced on the next design (V2) by adding more probes for poorly covered regions (both more identical and probes from the same region but at a different position were added). The number of probes added per target region was dependent on the coverage. After enrichment with this rebalanced capture array and sequencing the mean coverage per exon was calculated again. The mean base coverage for the targeted bases after a single hybridisation was 41.6±12.9 (mean±SD). Lower SD at a higher mean coverage implies a more even coverage when using the V2 array. In fact, exons that had a low coverage in the V1 array were more evenly distributed after enrichment with the V2 array (figure 4B). The missing exon from the V1 array (exon 5 of *PRKAG2*) was targeted in the V2 with seven times the standard amount of probes, but still this particular exon was not present in the reads. Evaluation of the characteristics of this region showed a high GC content of 76%. It is likely that during pre-capture and post-capture amplification there is poor amplification of this region and therefore it is not represented in the post-capture library. Since the on-target percentages were low we switched to a protocol that used a double hybridisation. After the second hybridisation the mean base coverage for the targeted bases was 62.8±42.3 (mean±SD). Introduction of a second hybridisation increased the on-target percentage drastically, however, the SD of the mean coverage also increased. In the next version of the capture array (V3), we performed an additional balancing step and added the coding exons of five more genes, resulting in targeting a total of ~300 exons of 23 genes. The mean base coverage for the targeted bases was 97.6 ±44.7 (mean±SD). The percentage of bases covered at least once was 99.8% (only missed 90 bases of exon 5 *PRKAG2*: chr7:151329155-151329224). In the final version of

the capture array (V4) we have rebalanced the final five genes. The mean base coverage for the targeted bases was 103.7±35.9 (mean±SD). With the last design there was an average of 1.64 fragment per patient that had one or more bases at <16× coverage and therefore needed to be analysed with a Sanger reaction. This represents an average of 0.36% of the analysed bases.
When comparing double hybridisation on the V2 array with the V4 array (mean±SD 62.8±42.3 vs 103.7±35.9) it clearly shows improved performance due to design optimisation.

Screening of DCM patients already seen in diagnostics but no mutation found

Seventeen patients diagnosed with DCM and two patients with a DCM family history were selected for screening of the genes present on the V2 and V3 arrays. In 10 out

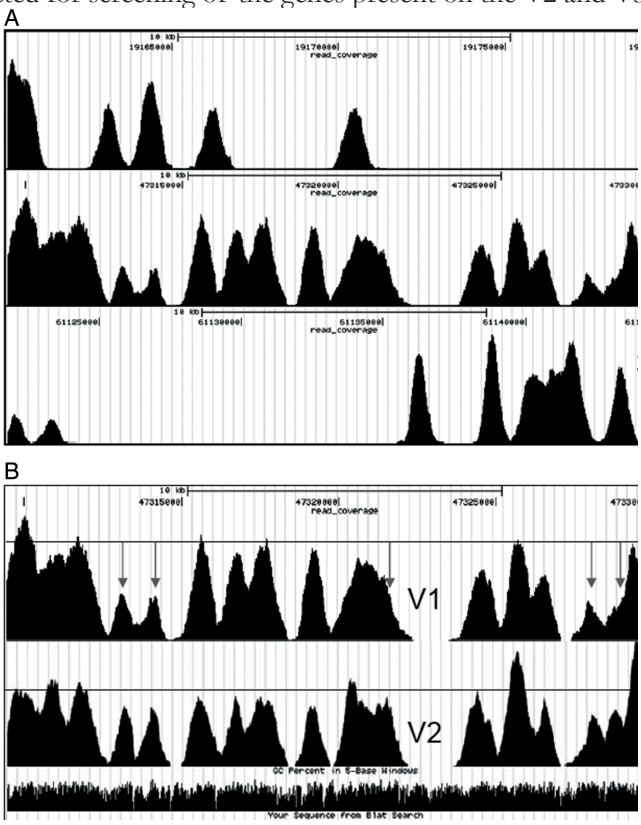

Figure 4 Representative example of a gene with even exon coverage (1), intermediate exon coverage (2), and uneven exon coverage (3) before rebalancing of the capture array (A) and exon coverage distribution before (V1) and after (V2) rebalancing of the capture array (B). Note that poorly covered exons or exon flanks (arrows) are far below the average coverage (horizontal line) before rebalancing. After rebalancing exon coverage is far more uniform.

of 19 patients we found variants with different probability of pathogenicity (for more details see table 2). Two variants, the *PLN* c.40_42del and the *MYH7* c.415G>T, were both observed twice in unrelated patients, with one patient having two variants (*MYH7* c.415G>T + *LAMP2* c.661G>A). All the other variants were present solitary. All the variants present in *TNNI3*, *EMD*, *SCN5A*, *MYH7*, and *LAMP2* were not observed in all the previously tested probands for these genes. The variant present in *PLN* has been reported as pathogenic[10, 11] and is a well-known founder mutation in the Netherlands. One of the two variants in *SCN5A* (NM_198056.2 : c.4859C>T) has been reported previously.[12-16] The variant in *LDB3* has not been found in 200 HCM/DCM/non-compaction cardiomyopathy patients screened in Erasmus Medical Center, Rotterdam (personal communication).

Comparison of variants found with Sanger sequencing and GS-FLX Titanium sequencing in HCM patients

In order to make a side by side comparison between variants that were found with Sanger sequencing and variants that were found by GS-FLX Titanium sequencing, we have selected nine patients diagnosed with HCM but with no pathogenic mutation identified. In these patients, all the coding exons of the genes offered for DNA diagnostics (*MYBPC3*, *MYH7*, *MYL2*, *MYL3*, *TNNI3*, *TNNT2*, *TPM1*,

Table 2 Clinical parameters and variants found in DCM patients

DNA nr.	Gender	Age at diagnosis (years)	EF (%)	Gene	g.Var	Reference sequence	c.Var	p.Var	PolyPhen2 HumVar	PolyPhen2 HumDiv	Grantham dist	SIFT	AGVGD	Freq. in EVS (variant alleles/wt alleles)	Classification*	Additional information
Patient 1	F	64	?	*TNNI3*	55665408 A>G	NM_000363.4	539A>G	p. Asp180Gly	Probably damaging	Possibly damaging	94	0.04	C0	Not found	VUS2	3× DCM index patients,[17 18] 1 × LVNC.[18] Mother of our index with clinical suspicion is not a carrier
Patient 2	M	51	17													
Patient 3	M	44	?	*EMD*	153609246 C>T	NM_000117.2	454C>T	p. Arg152Cys	Benign	Benign	180	0.00	C55	Not found	VUS3	No
Patient 4	F	50	13	–	–		–	–	–			–	–			
Patient 5	M	40	40	–	–		–	–	–			–	–			
Patient 6	F	29	37	*PLN*	118880124_ 118880126del	NM_002667.3	40_42del	p.Arg14del						Not looked at	Pathogenic	Founder in the Netherlands; mouse model+DCM family[19]; large DCM family.[11]
Patient 7†	F	22	51	*SCN5A*	38595955 T>C	NM_198056.2	4628T>C	p. Val1543Ala	Benign	Benign	64	0.00	C65	Not found	VUS2	No
Patient 8	F	55	20	–	–		–	–	–			–	–			
Patient 9	F	28	35	–	–		–	–	–			–	–			
Patient 10	F	27	30	*MYH7*	23901935 G>T	NM_000257.2	415G>T	p.Val139Leu	Probably damaging	Probably damaging	32	0.00	C0	Not found	VUS3	Found in 3 other index patients‡
	F	–	–	*LAMP2*	119581776 G>A	NM_002294.2	661G>A	p.Gly221Arg	Probably damaging	Probably damaging	125	0.00	C0	21/10542	VUS2	No
Patient 11	M	56	?	–	–		–	–	–			–	–			
Patient 12	F	52	38	*MYH7*	23901935 G>T	NM_000257.2	415G>T	p.Val139Leu	Probably damaging	Probably damaging	32	0.00	C0	Not found	VUS3	Found in 3 other index patients‡
Patient 13†	M	40	47	–	–		–	–	–			–	–			
Patient 14	M	35	21	*SCN5A*	38593004 C>T	NM_198056.2	4859C>T	p. Thr1620Met	Probably damaging	Probably damaging	81	0.00	C65	Not found	VUS3	Large IVF family (monoallelic with rare polymorphism).[13] Functional test not conclusive.[12-16]
Patient 15	F	41	25	–	–		–	–	–			–	–			
Patient 16	F	73	?	*LDB3*	88451727 A>G	NM_007078.2	764A>G	p.Lys255Arg	Benign	Benign	26	0.00	C25	Not found	VUS1	Not
Patient 17	V	25	41	*PLN*	118880124_ 118880126del		40_42del	p.Arg14del						Not looked at	Pathogenic	Founder in the Netherlands; mouse model+DCM family[19]; large DCM family.[11]
Patient 18	M	49	37	–	–		–	–	–			–	–			
Patient 19§	V	?	?	*MYH7*	23896054 T>C	NM_000257.2	1976T>C	p. Met659Thr	Probably damaging	Probably damaging	81	0.00	C65	Not found	Pathogenic	Large family (co-segregation in 10 affected members)‡

*For further details see Methods section.
†Patient does not have observed dilatation yet but has a strong family history with DCM.
‡Identified in cardiomyopathy patients analysed during routine diagnostics in our laboratory.
§Patient also has hypertrophy so DCM could be a result of HCM.
DCM, dilated cardiomyopathy; EF, ejection fraction; HCM, hypertrophic cardiomyopathies; IVF, idiopathic ventricular fibrillation; LVNC, left ventricular non-compaction; n.a, not available; ?, decreased EF, no exact measurement performed.

Table 3 Clinical parameters and variants found in HCM patients

	Gender	Age at diagnosis (years)	MLVWT (mm)	SCD in family	Gene	Var g.	Reference sequence	Var c.	Var p.	PolyPhen2 HumVar	PolyPhen2 HumDiv	Grantham dist	SIFT	AGVGD	Freq in EVS (variant alles/wt alleles)	Classification*	Additional information
Patient 20	M	?	?	?	*TPM1*	63356265 A>G	NM_001018020.1	775A>G	p. Lys259Glu	Not reliable	Not reliable	56	0.00	C55	4/10 754	VUS2	No (not present in transcript NM_000366.5)
Patient 21	M	65	17	No	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 22	F	58	16	Yes	*VCL*	75854219 G>A	NM_014000.2	1543G>A	p. Gly515Ser	Probably damaging	Probably damaging	56	0.00	C55	Not found	VUS2	No (splicing could be affected)
Patient 23	M	62	15	No	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 24	M	79	17	No	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 25	M	44	20	?	*LDB3*	88441437 C>T	NM_007078.2	566C>T	p. Ser189Leu	Benign	Benign	145	0.1	C0	2/10 806	VUS3	DCM/LVNC family (called S196L), co-segregation in 4 affected members.[23] Functionally tested.[24, 25]
					MYBPC3	47354463 T>C	NM_000256.3	3392T>C	p. Ile1131Thr	Benign	Possibly damaging	89	0.03	C55	7/12 331	VUS1	3 other DCM/NCCM index patients† (one of them together with the pathogenic Dutch founder mutation c.2373dup in MYBPC3[22]; in another family no co-segregation in an affected uncle)
Patient 26	M	56	23	No	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 27	M	50	19	No	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 28	M	67	28	?	*TNNT2*	201328349 C>T	NM_000364.2	877C>T	p. Arg293Cys	Probably damaging	Probably damaging	180	0.00	C0	Not found	VUS3	3 HCM index patients (called R286C)[20, 21] 3 HCM index patients†

Variants in the genes highlighted in bold are variants that were found on top of the variants that were already detected by Sanger sequencing. The variant present in VCL (patient 22) introduced a loss of a splice site on top of the amino acid change.
*For further details see Methods section.
†Identified in cardiomyopathy patients analysed during routine diagnostics in our laboratory.
DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; LVNC, left ventricular non-compaction; MLVWT, maximal left ventricular wall thickness; n.a., not available; NCCM, non-compaction cardiomyopathy; SCD, sudden cardiac death; ?, unknown or no exact measurement performed.

and *GLA* genes) were already analysed with dHPLC and/or Sanger sequencing. Within the targeted region of the V3 capture array, 258 variants were detected with dHPLC and/or Sanger sequencing.
Of these 258 variants, three were variants of unknown clinical significance (*TNNT2*, c.877C>T,[17, 18] *TPM1*, c.755A>G and *MYBPC3*, c.3392T>C[19]), while the others were non-pathogenic coding and non-coding variants.
These variants served as positive control in the direct comparison of Sanger sequencing versus GS-FLX Titanium Roche sequencing. Evaluation of the variants present in the HCDiff and AllDiff lists showed a total of 262 variants. From the 258 variants that were potentially detectable, one variant (*MYL2* c.353+46dup) was missed in two patients by the GS-FLX Titanium. This means six additional variants (one heterozygote and five homozygotes) were found with GS-FLX Titanium sequencing, but not with dHPLC. All additional variants were confirmed with Sanger sequencing.
This results in a detection rate of 101.6%, compared to the dHPLC and/or Sanger

sequencing. If one excludes the missed variants by the dHPLC, 258 variants were potentially detectable. From these, 256 were found in either HCDif or AllDiff, while one variant (*MYL2*, c.353+46dup) was missing in two patients resulting in a corrected detection rate of 99.2%. It should be noted that all the other 48 known non-coding indels of different lengths were detected correctly.
On top of the known variants and putative mutations we also found two additional amino acid changes from which one also introduced a potential splice site loss in genes that were not analysed before. These variants are *VCL*: NM_014000.2: c.1543G>A (amino acid change and loss of splice site), LDB3: NM_007078.2: c.566C>T. We classified them as VUS2 (see table 3).

Side by side comparison of GS-FLX Titanium sequencing and Sanger sequencing in 30 index patients

Finally, the fully optimised capture array (V4) was used for a second direct comparison in 30 HCM index patients by comparing the seven genes (*MYBPC3*, *MYH7*, *TNNT2*, *TNNI3*, *TPM1*, *MYL2*, and *MYL3*) sequenced with both Sanger sequencing and GS-FLX Titanium sequencing. From the 319 variants present, a total of 317 variants were detected with Sanger sequencing and 318 with GS-FLX Titanium sequencing. One variant was not detected with GS-FLX Titanium sequencing (*MYL2*: NM_000432.3: c.353+20delG) and two variants were not detected with Sanger sequencing (*MYH7*: NM_000257.2 c.1231G>A and *MYH7*: NM_000257.2: c.2967T>C).
Re-examination of these two variants by Sanger sequencing confirmed both variants. Re-evaluation of the original traces also showed the two variants but they were not picked up due to high background. Also, here it must be noted that the other 51 known indels were detected with GS-FLX Titanium sequencing. This represents a sensitivity of 317/319 (99.4%) and 318/319 (99.7%) for Sanger sequencing and GS-FLX Titanium sequencing, respectively.
In the 30 patients we found 26.7±5.8 (mean±SD) variants per patient. From these, 17 variants (0.57±0.77, mean±SD) have varying probabilities of pathogenicity. These include variants in genes routinely screened with Sanger sequencing—that is, *MYH7*: NM_000257.2: c.1178C>T,[20-22] c.1231G>A,[23-26] c.2002C>A, c.5135G>A, c.1987C>T, c.121G>A and *MYBPC3*: NM_000256.3: c.2827C>T,[27] c.3004C>T (twice), c.2864_2865del, *MYL2*: NM_000432.3: c.37G>A[28-31] and *TPM1*: NM_000366.5: c.618A>G, but also in genes that are part of the additional genes in the NG cardiomyopathy panel— that is, *SCN5A*: NM_198056.2: c.3157G>A,[32, 33] *PRKAG2*: NM_016203.3: c.253C>T, *TCAP*: NM_003673.2: c.37_39del,[34, 35] *CSRP3*: NM_003476.3: c.10T>C,[36, 37] *LMNA*: NM_170707.2: c.1804G>A,[38, 39] *DES*: NM_001927.3: c.935A>C (table 4). Ten patients had a single variant, two

patients had two variants each (*MYH7*: NM_000257.2: c.1178C>T combined with *SCN5A*: NM_198056.2: c.3157G>A, and *MYBPC3*: NM_000256.3: c.3004C>T combined with *LMNA*: NM_170707.2: c.1804G>A), and one patient had three variants (*MYH7*: NM_000257.2: c.121G>A, *MYBPC3*: NM_000256.3: c.3004C>T, and *DES*: NM_001927.3: c.935A>C). All variants were confirmed with Sanger sequencing.

Discussion

In the present study we show the feasibility of array based target enrichment of 23 HMC and DCM genes combined with NGS for diagnostic mutation screening in HCM/DCM patients. The use of DNA bar codes enabled pre-capture multiplexing, a step essential for increasing throughput and reducing the costs of analysis.[40] This approach not only enables the simultaneous and comprehensive investigation of multiple target genes but also facilitates the analysis of multiple samples in parallel. We have developed an optimised capture array that is at least as sensitive as the current standard, Sanger sequencing. For HCM, the current capture array analyses three times more genes as compared with the current Sanger sequencing panel within the same turn-around time. Screening of more genes results in a higher diagnostic yield. For DCM patients, but not for HCM patients, we have developed a similar assay for *TTN*. This enables us to screen *TTN* on top of the 23 reported genes only for DCM patients.

We and others have shown that NGS technologies are on the verge of being broadly used in clinical laboratories. It is very likely that these new technologies will replace traditional (Sanger sequencing based and array based) sequencing tests for genetically heterogeneous disorders like HCM/DCM. Targeted exome massively parallel sequencing has great potential for both research and clinical use. However, sequencing of the entire exome for diseases with 'limited' numbers of genes to be investigated is not practically feasible yet due to relatively high costs, variable depth of exon coverage, the extent of data analysis, and data storage. To overcome these problems, sequence capture based target enrichment for a limited number of genes followed by NGS can be an approach that is logistically and financially feasible.

Our first pilot study with five HCM patients with known pathogenic mutations showed that array based target enrichment and NGS could easily detect different types of known mutations (substitutions, insertions and deletions) and numerous non-pathogenic variants already detected with Sanger sequencing. All 57 variants detected with Sanger sequencing were also found with GS-FLX Titanium sequencing, including a coding 26 bp insertion in the *MYBPC3* gene (figure 5). Because we detected 100% of the variants present in five HCM patients we proceeded with testing of additional patients. Nevertheless, in this pilot study we have observed that exon coverage

varies significantly within one sample (determined by array design) resulting in lower confidentiality of particular variants (e.g., *MYBPC3*: NM_000256.3: c.1093-24C>T). A balanced representation of all targeted exons would reduce the average coverage needed to detect variants with high confidentiality, consequently lowering the false negative rate. Therefore, we have designed arrays with a more balanced coverage as has previously been proposed by others.[41, 42] The rebalanced capture array has been used to analyse nine HCM patients and 19 DCM patients. The rebalanced design showed that 99.80% of the targeted coding bases were covered at least once and 99.64% at least 16×. We and others[8] have calculated that at 15–16× coverage a 99% sensitivity is obtained. This means that for an experiment with a mean coverage of 100±35× the statistical chance that a variant is missed in a patient is 0.004% (for calculation see figure 2).

The bases with low coverage were exon 5 from *PRKAG2* (NM_016203.3) in all patients and exon 2 from *LAMP2* (NM_002294.2) in about half of the patients. This is likely due to a high GC content, a phenomenon observed before.[43] In the current test these exons are analysed by standard Sanger sequencing.
From the nine HCM patients the HCM gene panel had been analysed with Sanger sequencing. In these patients 99.2% of the present variants were detected with GS-FLX Titanium sequencing. The two undetected non-coding variants (identical in two individuals) were both times a single nucleotide insertion present in a region with

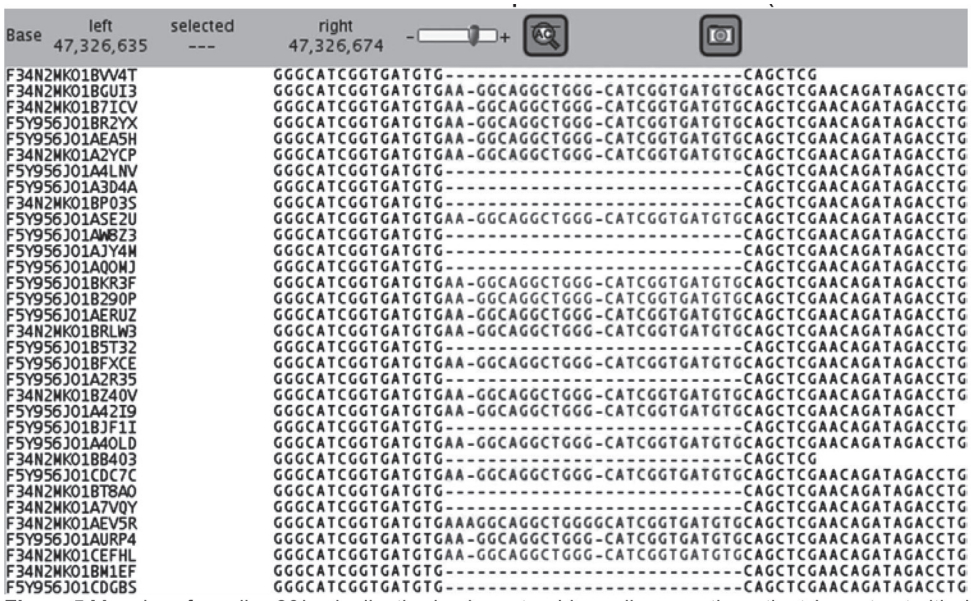

Figure 5 Mappingof acoding26bpduplicationinahypertrophic cardiomyopathypatient. In contrast withshort readplatforms, a heterozygous insertion of 26bp is easily mapped and reported.

multiple homopolymer stretches of four to six nucleotides. Variant detection and in particular indels in homopolymer stretches is a known problem with pyrosequencing due to incorrect base calling in these regions.[44] In our datasets it has become clear that homopolymer stretches up to 5-mers are generally called properly, while 6-mers and more in general result in improper base calling. Screening all coding regions for ≥6-mers showed 17 exons with ≥6-mers. For diagnostic purposes, these exons are analysed by Sanger sequencing until better base calling algorithms are available.
Apart from the variants present in the already used gene panel, numerous additional variants were found in the genes that were not Sanger sequenced previously. Two variants (indicated in bold), which both result in amino acid changes (one amino acid change also predicts a splice site loss) (table 3), are of possible clinical significance. The variant in LDB3 has been reported before[45-47] while the *VCL* variant is novel.
In the DCM panel we detected 11 potentially pathogenic mutations in 10 individuals. The genes involved are *MYH7* (three mutations), *TNNI3* (one mutation), *PLN* (two identical mutations), *LAMP2* (one mutation), *LDB3* (one mutation), *EMD* (one mutation), and *SCN5A* (two mutations) (for details see table 2). The variant in *TNNI3*, *PLN*, and *SCN5A* have already been reported in the literature,[10, 11, 48-50] and all other variants are novel. Identification of additional variants in both HCM and DCM patients shows an increase in diagnostic yield upon extension of the gene panel sequenced.
Finally, we evaluated 30 index patients in parallel with Sanger sequencing. In these experiments we especially focused on the diagnostic value of this approach. In 10 patients a single potentially pathogenic variant was found, in two patients two potentially pathogenic variants were found, and in one patient three potentially pathogenic variants were identified. From the 17 variants 11 were in the genes regularly screened with Sanger sequencing, and six were found in genes that were additionally screened due to GS-FLX Titanium sequencing (table 4). This shows that the clinical sensitivity can be increased by the addition of extra genes. In this experiment we were able to compare directly the sensitivity of Sanger sequencing and GS-FLX Titanium sequencing. Sensitivity of GS-FLX Titanium sequencing was at least as good as that of Sanger sequencing (99.7% vs 99.4%, respectively), showing that GS-FLX Titanium sequencing can replace Sanger sequencing in a diagnostic setting.

Small insertions and deletions (1–50 bp) represent the second most frequent class of variation in the human genome after SNPs.[51] Throughout all the experiments described here we have particularly focused on the detection of indels. Two non-coding variants (*MYL2* c.353+20 and c.353+46) were not reported in the GS-FLX Titanium variant list. Both variants are present in a region with multiple homopolymer stretches of four to six nucleotides, likely the underlying reason for improper variant calling. Nevertheless, the other 106 indels present in this study were called properly. In fact, pathogenic mutations caused by an insertion of a G nucleotide, deletion of CT nucleotides, deletion of AGA nucleotides, as well as an insertion of 26 bases

Table 4 Clinical parameters and variants found in index patients

	Gender	Age at diagnosis (years)	MLVWT (mm)	SCD in family	Gene	Var g.	Reference sequence	Var c.	Var p.	PolyPhen2 HumVar	PolyPhen2 HumDiv	Grantham dist	SIFT	AGVGD	Freq in EVS (variant alleles/wt alleles)	Classification*	Additional information
Patient 29	M	24	17	?	*SCN5A*	38622493 G>A	NM_198056.2	3157G>A	Glu1053Lys	Probably damaging	Possibly damaging	56	0.00	C55	Not found	Pathogenic	3 BrS index patients (one had a second SCN5A mutation: R1584C)[28]
																	Functional test: no binding to Ankrin-G[29]
					MYH7	23898517 C>T	NM_000257.2	1178C>T	Ala393Val	Possibly damaging	Possibly damaging	64	0.00	C0	Not found	VUS2	No
Patient 30	F	69	19	No	–	–		–	–	–			–	–			
Patient 31†	M	65	11	No	*MYL2*	111356964G>A	NM_000432.3	37G>A	Ala13Thr	Possibly damaging	Benign	58	0.08	C0	6/13 000	VUS1	3 families and an index sometimes together with N1327K in MYH7, not always good co-segregation.[24–26] Functional test: not conclusive[27]
Patient 32	M	?	?	?	*PRKAG2*	151478451C>T	NM_016203.3	253C>T	Pro85Ser	Possibly damaging	Benign	74	0.12	C0	Not found	VUS2	No
Patient 33	M	68	20	No	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 34	F	55	16	No	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 35	M	16	18	Yes	*MYBPC3*	47356671 C>T	NM_000256.3	2827C>T	Arg943X	–	–	–	–	–	Not found	Pathogenic	Dutch founder mutation.[23]
Patient 36‡	F	68	14	Yes	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 37	M	33	19	No	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 38	F	58	15	No	*MYH7*	23898464 G>A	NM_000257.2	1231G>A	Val411Ile	Possibly damaging	Probably damaging	29	0.00	C0	Not found	VUS3	4 HCM index patients.[29–31]
Patient 39	M	31	?	Yes	*MYH7*	23896028 C>A	NM_000257.2	2002C>A	His668Asn	Probably damaging	Probably damaging	68	0.00	C65	Not found	VUS3	Mutation was not found back in both parents (father is probably affected)
Patient 40	M	46	15	No	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 41§	M	62	?	Yes	–	–	–	–	–	–	–	–	–	–	–	–	
Patient 42	M	69	31	No	*TCAP*	37821649_ 37821651 del	NM_003673.3	37_39del	Glu13del						Not looked at	VUS1	8 index patients (LOVD); 2 HCM index patients.[31] in 0.4–0.5% of the control alleles[40]
Patient 43	M	42	22	No	–	–		–	–	–			–	–			
Patient 44	F	74	21	No	*MYH7*	23884860 G>A	NM_000257.2	5135G>A	Arg1712Gln	Possibly damaging	Probably damaging	43	0.00	C0	Not found	VUS1	9 Dutch HCM index cases (one family no co-segregation in affected daughter)

Continued

were called correctly. Furthermore, multiple known non-coding indels like insertion of a C nucleotide, deletion of a C nucleotide, insertion of AC nucleotides, deletion of CTTCT nucleotides, insertion of ATTTT nucleotides, insertion of ATTTT-GTTTT nucleotides, and insertion of ACAG nucleotides were all detected in these patients.

In conclusion, we have shown that on-array multiplexed sequence capture in combination with GS-FLX Titanium sequencing is suitable for reliable variant detection (sensitivity of 99.7%) and will increase clinical sensitivity in cardiomyopathy patients. To date, NGS is used as a research tool with high confidence and ease. In the present paper we demonstrate that due to continuing improvements in throughput, accuracy, cost and ease of data analysis, it has become feasible to apply NGS in a diagnostic setting.

Contributors

All authors included on the paper fulfil the criteria of authorship, as they contributed to the study conception and design, acquisition of data, or analysis and interpretation of data, drafting the article or revising it for intellectual content, and approving the final version submitted. In addition, ORFM designed the study and experimental set-up, performed the experiments,

analysed data, and wrote the paper. RHLD and MMAMM designed the study, analysed data, and wrote the paper. FB designed the experimental set-up. J-FS analysed data and contributed to drafting the manuscript. JvM contributed to the study design and drafting of the manuscript. NH and IC contributed to identification and selection of patients. MAH performed the experiments and developed the bioinformatical analysis. MEJ performed the experiments.

Competing interests
None

Patient consent
Obtained

Provenance and peer review
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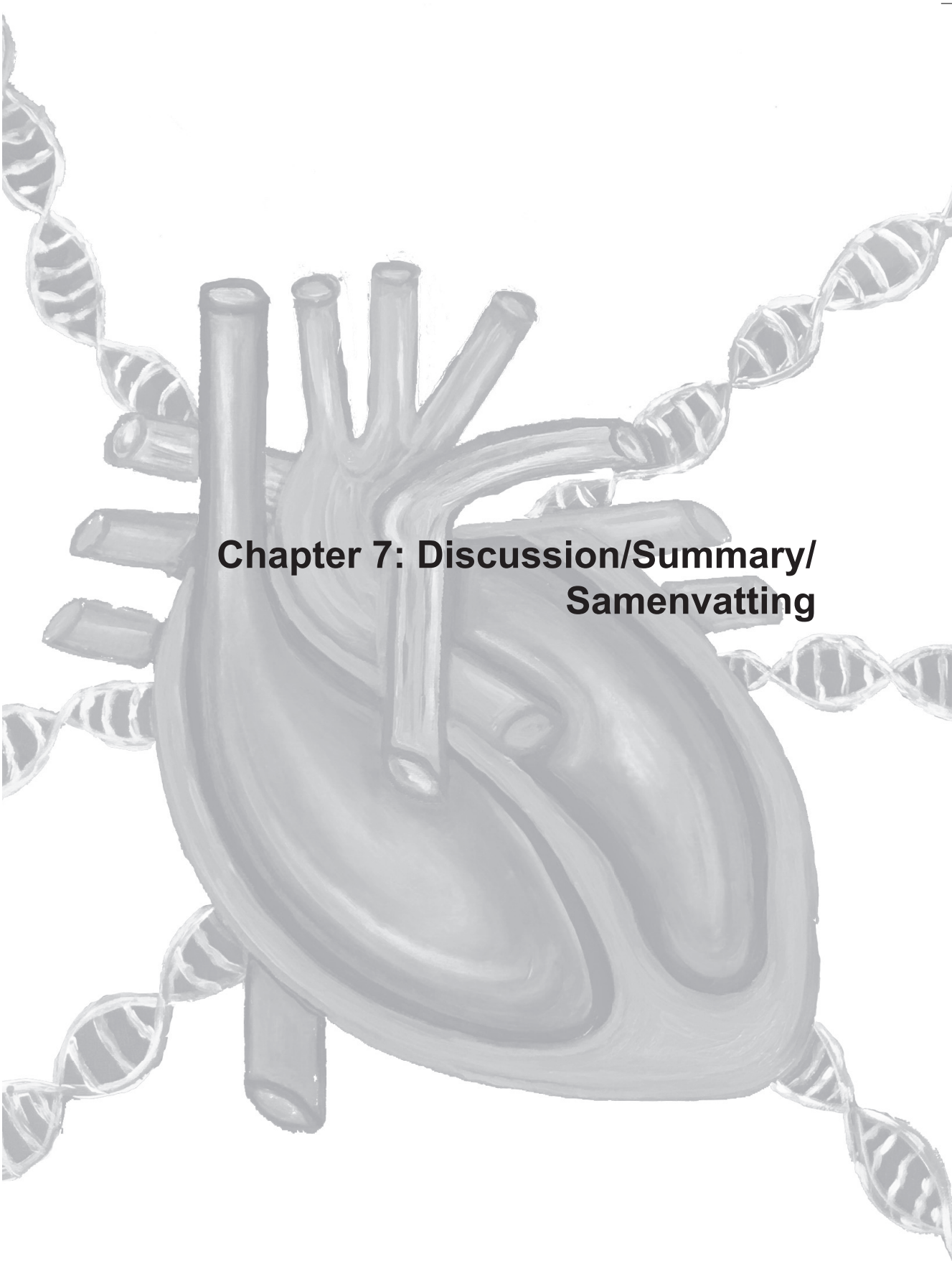

Chapter 7: Discussion/Summary/ Samenvatting

Discussion

Congenital heart disease (CHD) is the most common type of birth defect. Initially the genetic background of the different forms of CHDs was proposed to be polygenic,[1] i.e. an interaction of multiple genes and environmental factors (described in chapter one, paragraph 3.5), as it was for many genetic diseases. With the advent of new techniques, such as whole exome sequencing, array CGH (comparative genome hybridization), GWAS (genome wide association study) and others, we have now a better understanding of the genetic basis of CHD. This has shown to include monogenic inheritance, de novo inheritance, compound inheritance, polygenic inheritance and occurrence of chromosomal aberrations as causal for many different types of CHD.

Given the above, genetic testing can potentially play an important role in clinical care for CHD patients. In general, genetic analysis and diagnosis may carry the potential to improve prognosis by yielding important information with regards to personalized medical care, confidence in the clinical diagnosis and/or targeted patient follow-up and is used in patient counseling. Moreover it may also predict recurrence risk, define the pattern of inheritance in the family and the need for further family screening. While much progress has been made regarding the management of children and adults with CHD, a greater understanding of underlying etiologies could potentially lead to further advances in preventive care and therapeutic strategies.[2] This is important as patients with congenital heart disease nowadays often survive into adulthood because of better surgical interventions and care. In fact, >90% of children with CHD survive their first year of life and will live into adulthood.[3] However, survival of CHD patients into adulthood has brought new challenges in clinical management.[4] At the moment, adult CHD patients outnumber pediatric CHD patients and this population is expected to grow by 5% per year.[3] The median age of severe CHD patients has increased from 11 years in 1985 to 25 years in 2010,[5] and importantly, systemic complications arising over a lifetime of CHD differ from those in a younger population.[4] Furthermore, depending on the genetic model, they might transmit the disease to their offspring. Taken together, this establishes and important role for uncovering the (individual) genetics of congenital heart defects not only to establish the cause, but perhaps it can also guide therapy and prognosis.

One specific syndrome we investigated in this thesis is the Holt-Oram syndrome (HOS), that is normally caused by haploinsufficiency of the TBX5 gene. We have found a TBX5 gain of function mutation (G125R) in a family with atypical HOS because a lot of family members showed paroxysmal atrial fibrillation and only a minor part had congenital heart defects (chapter 2). This was recently further corroborated by a study showing that Hdac3, a histone deacetylase, regulates Tbx5 acetyla-

tion and activation of Tbx-5 dependent cardiomyocyte lineage-specific genes.[6] In fact, the TBX5 G125R mutation we identified in the HOS family disrupts the interaction between TBX5 and HDAC3.[6] This leads to a failure of repressing the transcriptional activity of the TBX5 G125R, resulting in activation of cardiomyocyte lineage-specific genes in cardiac progenitor cells.[6] This further proves that the TBX5 G125R mutation results in a gain-of-function. Another link between TBX5 and atrial fibrillation came from recent GWA studies. A common SNP in TBX5 (rs3825214) associates with prolongation of the QRS duration, the PR interval and the QT interval as well as atrial fibrillation (AF).[7] However, concerning the connection of the rs3825214 TBX5 SNP to AF, different results were reported. In earlier studies this SNP was associated with a prolonged PR interval and a higher risk of AF.[8-11] While in the study of Holm et al.[7] this SNP correlated with a prolonged PR interval and a lower risk of AF. The GWA study was repeated in the Chinese Han population and here there was a significant association between the minor allele and AF as a whole group and even bigger with lone AF.[12]. So the GWA studies give so far inconclusive results. Regardless of the effect of omen TBX5 SNPs on AF, three independent studies in the Chinese Han population also found mutations in TBX5 in patients with AF. The TBX5 p.H170D mutation was found in an individual with AF that had no apparent congenital anomalies in the heart, hands and forearms as well as a negative family history.[13] The other mutation,TBX5 p.P132S was found in a proband and the mutation co-segregated with AF in the family.[14] Four more mutations were found in a Chinese Han population by Ma et al,[15] p.R355C, p.Q376R, p.A428S and p.S372L and a novel TBX5 mutation (p.D118del) in a population of European ancestry. Taken together, we believe that these studies show that TBX5 can indeed be a factor in the development of AF.

Another type of heart defect that we studied in this thesis is Ebstein anomaly (EA) of the tricuspid valve (chapter 3). It is a rare congenital heart defect characterized by incomplete delamination of leaflets from the right ventricular endocardium, resulting in tricuspid valve regurgitation. In addition to the volume-overloaded myopathic right heart, left-sided cardiovascular lesions are common in patients with EA,[16] including left ventricular non-compaction cardiomyopathy (LVNC). Only MYH7 and TPM1 have been described so far in patients with EA as well as LVNC.[17, 18] The incidence of EA as well as LVNC is rare yet there have been several reports of EA associated with LVNC. In about 3% of LVNC patients EA can be found,[19] whereas in about 18% of EA patients LVNC can be found.[16] This suggests that EA and LVNC can have a common genetic cause. It can be hypothesized that when EA and LVNC occur concurrently, arrested development of the ventricular myocardium and atrioventricular valves may manifest differently in the right and the left part of the heart

due to the interplay of a primary mutation with other genetic signals. This may lead to an arrest in the compaction process of the LV causing LVNC in some cases.[17] This implicates an overlap in the molecular basis of structural and myopathic heart disease, so known cardiomyopathy genes may be interesting to test in patients with structural heart disease and EA in particular.[17] This is currently much easier by the use of next generation sequencing for cardiac gene panels or even sequencing of the whole exome (WES) or genome (WGS).

Indeed, next generation sequencing (NGS) can be used in the family we have described in this thesis, that suffers from congenital heart defects and low atrial rhythm, independent of the heart defects (chapter 4). At that time we could only identify a relatively large causative locus with ± 400 genes on 9q but we weren't able to identify the causative gene in this family using Sanger sequencing. However, with the use of exome or whole genome sequencing this is made much easier. In fact, continued research on this family recently identified a causative co-segregating mutation by use of WES in the TGFβR1 gene in this locus. This mutation behaves as a gain of function mutation, in contrast to loss of function mutations normally found in this gene. Currently, this is still a work in progress, but it shows that the development of new techniques such as next generation sequencing can give great stimulus to finding the underlying cause of (familial) CHDs.

As next generation sequencing (NGS) has been established as a major player in genetics, it is important to measure the diagnostic performance and yield between this and Sanger sequencing, the hitherto golden standard in clinical genetics. So we set up a NGS panel of 23 hypertrophic and dilated cardiomyopathy (HCM/DCM) genes to test in patients with HCM/DCM with and without known pathogenic mutations (chapter 6). Indeed, we proved that NGS is feasible in a diagnostic setting. This will make it easier to identify genes not known to be involved in certain disease. Case in point, our discovery of a TPM1 mutation in a family with DCM and NCCM (chapter 5). Such a mutation would nowadays be easily identified in routine diagnostic settings rather than in a research setting. This will generally result in a much faster and more accurate genetic diagnosis for the patient and hopefully will lead to better therapy focused on the genetic defect instead of only the type of disease.

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Summary

Congenital heart defects (CHDs) are among the leading birth defects in humans and occur in about 1 in 100 newborns. This incidence is even higher in spontaneous abortions. The high incidence of structural heart defects highlights the complexity of cardiac development. Congenital heart defects can occur solely or as part of a syndrome, some well-known syndromes that involve CHDs are Down Syndrome, Holt-Oram syndrome (described in chapter 2) and Noonan syndrome. Other cardiac defects that can occur after birth but also develop later in life, are the cardiomyopathies. These can be divided in several classes and can occur either as a secondary cause, or as a result of a genetic defect.

The **first chapter** is a general introduction to the embryonic development of the heart, such as the development and looping of the heart tube, the formation of the chambers, septa and cardiac valves. It also describes several congenital heart defects such as septal defects, cyanotic heart disease and cardiomyopathies as well as causes for congenital heart defects which include chromosomal anomalies, mutations in transcription factors and sarcomere genes and inherited as well as noninherited risk factors.

The Holt-Oram syndrome (HOS) is normally caused by loss of function mutations in the TBX5 gene. In **chapter 2** however a gain of function mutation in *TBX5* is discussed which causes an atypical form of HOS with paroxysmal atrial fibrillation. The most characteristic findings in HOS are upper limb anomalies and atrial septal defects (ASDs). The large family studied in this chapter was atypical for HOS as there were hardly any upper limb anomalies present, nor many congenital heart defects. Instead, 6 out of 9 affected members were suffering from atrial fibrillation (AF). The mutation identified in *TBX5* in this family caused a gain of function instead of a loss of function. The mutation results in normal *NKX2-5* interaction, but enhanced DNA binding and activation of the *NPPA (ANF)* and *GJA5 (CX40)* promoter. It is thought that the gain-of-function mechanism in this family underlies the mild skeletal phenotype and paroxysmal atrial fibrillation and suggests a possible role of *TBX5* in the development of (paroxysmal) atrial fibrillation.

Mutations in *MYH7* can cause cardiomyopathies such as left ventricular non-compaction (LVNC). In a family with LVNC a mutation in *MYH7* was found but there were also patients with LVNC as well as Ebstein's anomaly (EA). Screening of 141 individuals with Ebstein's anomaly for mutations in the *MYH7* gene, by next generation sequencing, and direct DNA sequencing is described in **chapter 3**. A total of

8 mutations were found only in individuals with EA and LVNC. This indicates that Ebstein anomaly is a congenital heart malformation that is associated with mutations in *MYH7*, particularly when associated with LVNC and may warrant genetic testing and family evaluation in this subset of patients.
In **chapter 4** we performed genome wide linkage analysis in a family with an autosomal dominant condition consisting of congenital heart defects and low atrial rhythm. The identified shared locus lies on chromosome 9q. The family has a unique phenotype as it comprises of congenital heart defects and low atrial rhythm. However several family members show low atrial rhythm without any structural heart defects. Because the proband shows low atrial rhythm and bilateral atrial appendages we hypothesized that this could be a result of a defect in the left-right asymmetry and might represent a mild or variant expression of left atrial isomerism. Besides CHD, the phenotype in this family is characterized by abnormal P-wave frontal axis on ECG, typically for left atrial isomerism. However, we could not confirm signs of left isomerism outside the heart. The shared region on chromosome 9 contains 402 genes; several of the candidate genes have been ruled out by sequencing.
Within families different kinds of cardiomyopathy can be present. In **chapter 5** we describe such a family in which both dilated cardiomyopathy (DCM) as well as non-compaction cardiomyopathy (NCCM) is present. We identified a novel alpha-tropomyosin (*TPM1*) mutation as the cause in this family and found that the mutation weakens the binding of tropomyosin to actin. So far only 2 families with DCM have been described with mutations in *TPM1*, in contrast to hypertrophic cardiomyopathy (HCM) for which many *TPM1* mutations have been found. The family in chapter 5 also demonstrates large phenotypic variability in cardiomyopathies in one family caused by the same mutation, as there is an 80-year-old carrier with no symptoms, but also a 5 year old carrier who died from the complications of NCCM.
In the past, genetic evaluation for HCM and DCM was performed by sequential screening of a very limited number of genes. To implement next generation sequencing (NGS) into the diagnostics, as a replacement of sanger sequencing of cardiomyopathies, we used GLS-FLX titanium sequencing of 23 hypertrophic and dilated cardiomyopathy genes in HCM and DCM patients. This is described in **chapter 6**. Although HCM patients were routinely screened at the DNA diagnostics department at the AMC in Amsterdam, DCM patients weren't. Also not in all the HCM patients genetic mutations were found, so NGS is used to enhance the diagnostic yield. Another advantage of NGS is that sanger sequencing is expensive and time-consuming. Initially NGS was used only in a research setting, but NGS is now routinely used in diagnostics. At the AMC in Amsterdam at the moment 50 genes are screened routinely for cardiomyopathies.

Samenvatting

Aangeboren hartafwijkingen behoren tot de meest voorkomende aangeboren afwijkingen in de mens en komen voor in ongeveer 1:100 levendgeborene. Dit aantal is nog hoger in spontane abortussen. De hoge incidentie van de structurele hart defecten toont aan dat de hartontwikkeling erg complex is. Aangeboren hartafwijkingen kunnen geïsoleerd voorkomen of als deel van een syndroom, enkele bekende syndromen met aangeboren hartafwijkingen zijn het Down syndroom, Holt-Oram syndroom (beschreven in hoofdstuk 2) en het Noonan syndroom. Andere hartafwijkingen die na de geboorte maar ook later in het leven voor kunnen komen zijn de cardiomyopathiën. Deze kunnen in verschillende klassen ingedeeld worden en kunnen ontstaan als secundaire oorzaak of als het gevolg van een genetisch defect.

Het **eerste hoofdstuk** is een algemene introductie in de embryonale hartontwikkeling, zoals de ontwikkeling en de looping van de hartbuis, vorming van de kamers, de septa en de hartkleppen. Er worden verschillende hartdefecten beschreven zoals de septum defecten, cyanotische hartdefecten en cardiomyopathiën alsook de oorzaken voor hartdefecten zoals chromosoom afwijkingen, mutaties in transcriptie factoren en sarcomeer genen, en zowel overervende als niet overervende risico factoren.

Het Holt-Oram syndroom (HOS) wordt veroorzaakt door mutaties in het *TBX5* gen die normaal een vermindering van de hoeveelheid eiwit veroorzaken waardoor het eiwit minder goed functioneert. Echter in **hoofdstuk 2** wordt een mutatie in het *TBX5* gen beschreven die een verhoging van de hoeveelheid eiwit veroorzaakt wat voor een verhoogde activiteit zorgt, die een atypische vorm van HOS met paroxysmale boezemfibrillatie tot gevolg heeft. De meest karakteristieke bevindingen in HOS zijn over het algemeen bovenste ledemaatafwijkingen en atrium septum defecten (ASD). De grote familie die in dit hoofdstuk werd bestudeerd was atypisch voor HOS omdat er amper bovenste ledemaatafwijkingen werden gevonden evenals amper aangeboren hartafwijkingen. In plaats daarvan hadden 6 van de 9 aangedane familieleden boezemfibrilleren. De gevonden *TBX5* mutatie in deze familie gaf een verhoging van de hoeveelheid eiwit wat voor een verhoogde activiteit zorgt in plaats van een vermindering van de hoeveelheid eiwit waardoor het eiwit minder goed functioneert. De mutatie resulteert in een normale NKX2-5 interactie, maar een verhoogde DNA-binding en een activatie van de *NPPA (ANF)* en de *GJA5 (CX40)* promoter. Gedacht wordt dat het mechanisme van een verhoging van de hoeveelheid eiwit die voor een verhoogde activiteit zorgt in deze familie leidt tot het milde skeletfenotype en paroxysmale boezemfibrillatie en suggereert een mogelijke rol van *TBX5* in de ontwikkeling van (paroxysmale) boezemfibrillatie.

Mutaties in *MYH7* kunnen cardiomyopathiën veroorzaken zoals links ventriculaire noncompactie (LVNC). In een familie met LVNC was een mutatie in *MYH7* gevonden maar er waren ook patiënten met LVNC die ook de ziekte van Ebstein hadden. Screening van 141 individuen met de ziekte van Ebstein voor mutaties in het *MYH7* gen, door middel van next generation sequencen, en het direct sequencen van DNA is beschreven in **hoofdstuk 3**. In totaal werden er 8 mutaties gevonden, echter alleen in individuen met de ziekte van Ebstein en LVNC tezamen. Dit duidt erop dat de ziekte van Ebstein een aangeboren hartafwijking is die wordt geassocieerd met mutaties in *MYH7*, vooral als er ook van LVNC sprake is. In deze subgroep van patiënten wordt het uitvoeren van een genetische test en familie screening aanbevolen.

In **hoofdstuk 4** hebben we een genoomwijde linkage analyse uitgevoerd in een familie met een autosomaal dominante overerving van een aangeboren hartafwijking en laag boezem ritme. Het geïdentificeerde gedeelde locus ligt op chromosoom 9q. De familie heeft een uniek fenotype dat bestaat uit aangeboren hartafwijkingen en een laag boezem ritme, hoewel verschillende familieleden een laag boezem ritme laten zien zonder structurele hartdefecten. Omdat de indexpersoon een laag boezem ritme laat zien met bilaterale hartoren veronderstelden we dat dit het resultaat kon zijn van een defect in de links-rechts asymmetrie en dit kan een milde of variabele expressie zijn van links atriaal isomerisme. Naast aangeboren hartafwijkingen wordt het fenotype in deze familie gekarakteriseerd door abnormale frontale as P-golven op het ECG, typisch voor links atriaal isomerisme. We hebben de klinische kenmerken van links isomerisme buiten het hart niet kunnen bevestigen. Het gedeelde gebied op chromosoom 9 bevat 402 genen; verschillende kandidaatgenen zijn uitgesloten door middel van sequencen.

Binnen families kunnen verschillende soorten cardiomyopathiën voorkomen. In **hoofdstuk 5** beschrijven we zo'n familie waarin zowel gedilateerde cardiomyopathie (DCM) alsook noncompactie cardiomyopathie (NCCM) aanwezig is. We vonden een nieuwe mutatie in alfa-tropomyosine (*TPM1*) als e oorzaak in deze familie en zagen dat de mutatie de binding van tropomyosine aan actine verzwakt. Tot dusver zijn er maar 2 families met DCM waarin mutaties in *TPM1* zijn beschreven, in tegenstelling tot hypertrofe cardiomypathie (HCM) waarin veel mutaties in *TPM1* zijn gevonden. De familie in hoofdstuk 5 laat ook grote fenotypische variabiliteit zien in cardiomyopathiën in één familie veroorzaakt door dezelfde mutatie, aangezien er een 80-jarige mutatiedraagster is zonder symptomen, maar ook een 5-jarige mutatiedraagster die is overleden aan de gevolgen van NCCM.

In het verleden werd genetische diagnostiek voor HCM en DCM gedaan door het screenen van een zeer beperkt aantal genen in een bepaalde volgorde. Om het next generation sequencen (NGS) te implementeren in de diagnostiek als een vervanging van het Sanger sequencen van de cardiomyopathiën gebruikten we de GLS-FLX titanium NGS sequencer om 23 hypertrofe en gedilateerde cardiomyopathie genen in HCM en DCM patiënten te sequencen. Dit is beschreven in **hoofdstuk 6**. Alhoewel HCM patiënten routinematig werden gescreend op de afdeling DNA-diagnostiek van het AMC in Amsterdam, gold dit niet voor DCM patiënten. Ook werden niet in alle HCM patiënten genetische mutaties gevonden, NGS kan hierdoor gebruikt worden om de diagnostische opbrengst te verhogen. Nog een voordeel van NGS is dat Sanger sequencen duur en tijdrovend is. In het begin werd NGS alleen gebruikt bij wetenschappelijk onderzoek maar NGS wordt nu routinematig ingezet in de diagnostiek. In het AMC in Amsterdam worden tegenwoordig 50 genen routinematig gescreend met NGS voor cardiomyopathiën.

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1. PhD training

	Year
General courses	
Introduction to Bioinformatics	2003
Genetic Epidemiology	2004
Basic Module: the AMC world of science	2004
Scientific writing in English for publication “Writing to be read”	2005
Specific courses	
CRIA basic course	2004
Seminars, workshops and master classes	
Symposium Moleculaire Cardiologie “From basics to clinics”	2003
The role of DNA polymorphisms in complex traits and diseases	2006

Presentations	
Life with a Rasopathy, Noonan syndrome (Poster presentation, Fourth International Rasopathies symposium, 2015, Seattle, USA)	2015
In silico analysis of the effect on splicing of single nucleotide changes at exon-intron junctions is predictive of pathogenicity. A study on MYBPC3 mutations (Poster presentation, Joint meeting UK/Dutch Clinical Genetics Societies & Cancer genetics Groups 2014, Leiden, The Netherlands and Poster presentation, ESHG 2014, Milan, Italy)	2014

Presentations	
A Novel Alpha-Tropomyosin Mutation in a Large Family with Dilated Cardiomyopathy (Poster presentation, Netherlands Association of Human Genetics meeting 2009, Veldhoven, The Netherlands and Poster presentation, ESHG 2009, Göteborg, Sweden)	2009
A novel TBX5 mutation leads to enhanced DNA-binding in a family with a-typical Holt-Oram syndrome and paroxysmal atrial fibrillation. (poster presentation, ESC, 2006, Barcelona, Spain and poster presentation, Heart-repair meeting, 2007, Amsterdam, The Netherlands)	2006-2007

2. Teaching

	Year
Practicum supervisor	2003-2007

3. Publications

	Year
Mook OR, Haagmans MA, Soucy JF, **van de Meerakker JB**, Baas F, Jakobs ME, Hofman N, Christiaans I, Lekanne Deprez RH, Mannens MM Targeted sequence capture and GS-FLX Titanium sequencing of 23 hypertrophic and dilated cardiomyopathy genes: implementation into diagnostics. J Med Genet. 2013 Sep;50(9):614-26	**2013**
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Curriculum Vitae

Judith Brechtje Annemarie van de Meerakker werd op 30 augustus 1976 geboren in Utrecht. Na het behalen van haar VWO diploma op het Hertog-Jan college in Valkenswaard begon zij aan haar studie Gezondheidswetenschappen met als afstudeerrichting Biologische Gezondheidkunde aan de Rijksuniversiteit Limburg te Maastricht in 1995. Na haar studie heeft ze in 2001 een onderzoek opgezet naar de genetische achtergronden van Vesico-ureterale reflux (klinische genetica WKZ). Hierna is ze in 2003 begonnen met haar promotieonderzoek naar genetische aspecten van aangeboren hartafwijkingen, op de afdeling Anatomie en Embryologie (AMC), met uiteindelijk dit proefschrift als resultaat. Haar bijzondere belangstelling voor dit onderwerp had veel te maken met het feit dat ze zelf geboren was met een hartafwijking (Noonan syndroom). Het promotieonderzoek werd begeleid door prof. dr. A.F.M. Moorman, prof. dr. M.M.A.M. Mannens en dr. A.V. Postma. Gedurende dit promotie onderzoek heeft zij in het kader van een Europees samenwerkingsverband (HeartRepair) 3 maanden in Newcastle upon Tyne gewerkt onder de supervisie van prof. dr. J. Goodship. Vanwege medische complicaties is het promotietraject meermaals vertraagd. Momenteel is zij werkzaam als kwaliteitsfunctionaris bij de afdeling DNA-Diagnostiek van het AMC in Amsterdam. Tevens is zij actief als vrijwilligster in de patiëntenwereld en dan met name op het gebied van het Noonan syndroom. Zo is zij o.a. bestuurslid van de Stichting Noonan Syndroom en lid van het medisch adviespanel van The Noonan Syndrome Association UK, en heeft zij meegeschreven aan het patiëntenmanifest 2017, de stem van patiënten en hun organisaties gehoord, hetgeen recentelijk aangeboden werd aan de 2^{e} kamer.

Curriculum Vitae

Judith Brechtje Annemarie van de Meerakker was born on the 30th of August 1976 in Utrecht. After getting her VWO degree at the Hertog-Jan college in Valkenswaard, she started her study Health sciences, with her specialty in biological health sciences at the Rijksuniversiteit Limburg at Maastricht in 1995. After she received her master degree, she performed a study on the genetic background of vesico-ureteral reflux (Clinical Genetics, WKZ, Utrecht). In 2003 she started a PhD project on the genetic aspects of congenital heart defects at the department of Anatomy and Embryology (AMC), with eventually this thesis as a final result. Her special interest for this subject had a lot to do with the fact that she herself was born with a heart defect (Noonan syndrome). The PhD research was supervised by prof. dr. A.F.M. Moorman, prof. dr. M.M.A.M. Mannens and dr. A.V. Postma. During her PhD she spent 3 months in Newcastle upon Tyne as part of the European consortium (HeartRepair) under the supervision of Dr. J. Goodship. Her PhD has been delayed several times because of medical complications. At the moment she is working as a quality officer at the department of DNA-Diagnostics at the AMC in Amsterdam. She is also volunteering in patients support and especially in the field of Noonan syndrome. She is a member of the board of the Dutch Noonan syndrome foundation, a member of the medical advisory panel of The Noonan Syndrome Association UK and a co-writer of the vision document 2017, the voices of patient and their organisations heard, which recently was presented at the Dutch Parliament.

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