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**Ventricular Remodeling in Children and Adults with Congenital Heart Disease**

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# Summary

Fundamental diagnostic and therapeutic progress in medicine have substantially increased the life expectancy of patients with congenital heart disease (CHD). Nevertheless, high morbidity and mortality persist in a subset of patients, specifically in those affected by complex CHD. Apart from intrinsic structural defects and anatomic anomalies, long-term sequelae from interventions and surgical procedures, as well as altered hemodynamics, contribute to myocardial stress and ventricular remodeling (VR). This may ultimately lead to heart failure, arrhythmias, and sudden cardiac death.

The aim of this dissertation was to investigate VR in children and adults with CHD both molecularly and clinically. To identify the molecular basis of VR, the first study involved ribonucleic acid (RNA) sequencing of right ventricular (RV) tissue samples from adults with tetralogy of Fallot (TOF) and existing right ventricular impairment (RVI). For comparison, infants diagnosed with TOF who had no evidence of RVI were selected. RV tissue samples were also sequenced from these patients. Gene expression analysis revealed that 3,010 genes were differentially regulated in the group of adult patients compared to children with TOF and no RVI. The identified genes code proteins potentially playing a critical role in RV remodeling and being involved in signaling pathways such as cell-cell communication, extracellular vesicles including exosomes, and oxidation-reduction processes, amongst others. The second study focused on the clinical impact of left ventricular (LV) remodeling in patients with pediatric onset hypertrophic cardiomyopathy (HCM). For this purpose, clinical data were comprehensively analyzed and as step one, the genotype-phenotype association and, as step two, the influence of the mutations on disease progression were investigated. Depending on the different types of mutations, no clear genotype-phenotype association could be identified in patients with single mutations. Patients with compound mutations showed instead a significantly higher risk of developing major arrhythmic events (MAEs). Consistent with the literature, this finding supports the hypothesis that compound mutations could adversely affect disease severity and pathologic remodeling. No association between different mutations and disease progression was identified.

Both studies contribute to a better understanding of pathological remodeling in the ventricles and their effects in children and adults with CHD. The identification of molecular pathways and clinical abnormalities helps to extend the diagnostic spectrum,

improves risk stratification, discovers potential biomarkers and facilitates the development of new therapeutic approaches targeting molecular and cellular factors.

# Summary in German

Durch grundlegende diagnostische und therapeutische Fortschritte in der Medizin hat sich die Lebenserwartung von Patienten mit angeborenen Herzerkrankungen (AHE) deutlich erhöht. Dennoch bleiben bei diesen Patienten eine hohe Morbidität und Mortalität bestehen, verursacht durch Herzinsuffizienz oder den plötzlichen arrhythmischen Herztod. Bedingt wird dies durch die anatomische Anomalie oder als Folge von interventionellen und operativen Eingriffen, die zu veränderter Hämodynamik, myokardialem Stress und ventrikulären Umbauprozessen führen.

Ziel dieser Dissertation war es, den pathologischen myokardialen Umbau bei Kindern und Erwachsenen mit AHE sowohl auf molekularer Ebene als auch klinisch zu untersuchen. Zur Identifizierung der molekularen Grundlagen des myokardialen Umbaus im rechten Ventrikel aufgrund erhöhter Druck- und Volumenbelastung erfolgte in der ersten Studie eine RNA Sequenzierung aus rechtsventrikulären Gewebeproben von Erwachsenen mit Fallotscher Tetralogie und entsprechendem hämodynamischen Stress. Zum Vergleich wurden Säuglinge ausgewählt, bei denen eine Fallotsche Tetralogie diagnostiziert wurde und die keine Anzeichen einer langjährigen hämodynamischen rechtsventrikulären Belastung aufwiesen. Auch von diesen Patienten wurden rechtsventrikuläre Gewebeproben sequenziert. Die Genexpressionsanalyse ergab, dass 3.010 Gene in der Gruppe der erwachsenen Patienten im Vergleich zu Kindern unterschiedlich reguliert waren. Die identifizierten Gene kodieren Proteine, die an Signalwegen wie der Zell-Zell Kommunikation, extrazellulären Vesikeln wie zum Beispiel Exosomen, sowie Oxidations-Reduktions-Prozessen beteiligt sind und potentiell eine entscheidende Rolle bei dem rechtsventrikulären pathologischen Umbau spielen. Die zweite Studie fokussierte auf die Untersuchung der Auswirkung eines genetischen Defekts auf den linksventrikulären Umbau und die klinischen Symptome bei Patienten mit hypertropher Kardiomyopathie mit Beginn in der Kindheit. Hierfür wurden die klinischen Daten umfassend analysiert und zum einen die Genotyp-Phänotyp Assoziation und zum anderen der Einfluss von Mutationen auf das Fortschreiten der Erkrankung untersucht. In Abhängigkeit der verschiedenen Mutationstypen konnte keine eindeutige Genotyp-Phänotyp Assoziation bei Patienten mit Einzelmutationen identifiziert werden. Bei Patienten mit Doppelmutationen zeigte sich jedoch ein signifikant höheres Risiko für schwerwiegende Arrhythmie-Ereignisse. Übereinstimmend mit der Literatur stützt dieses Ergebnis die Hypothese, dass Doppelmutationen den Schweregrad der

Erkrankung und den pathologischen Umbau ungünstig beeinflussen könnten. Ein Zusammenhang zwischen unterschiedlichen Mutationen und dem Krankheitsverlauf konnte nicht nachgewiesen werden.

Beide Studienergebnisse tragen zu einem besseren Verständnis der pathologischen Umbauprozesse in den Ventrikeln und deren klinischen Auswirkungen bei Kindern und Erwachsenen mit AHE bei. Die Identifikation molekularer Signalwege und klinischer Auffälligkeiten fördert die Erweiterung des diagnostischen Spektrums, die Risikostratifizierung, die Entdeckung von potentiellen Biomarkern sowie die Entwicklung neuer therapeutischer Ansätze, die auf molekulare und zelluläre Faktoren abzielen.

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# I. List of Abbreviations

| | |
|---|---|
| ACTC1 | Actin α Cardiac Muscle 1 |
| ACTN2 | Actinin α 2 |
| ADH1B | Alcohol Dehydrogenase 1B |
| ADH1C | Alcohol Dehydrogenase 1C |
| AHE | Angeborene Herzerkrankungen |
| ANKRD1 | Ankyrin Repeat Domain 1 |
| ANXA7 | Annexin A7 |
| APS | Ammoniumpersulfate |
| BTS | Blalock-Taussig-Shunt |
| CCNA2 | Cyclin A2 |
| CD63 | CD63 Antigen |
| CDK1 | Cyclin Dependent Kinase 1 |
| CDK14 | Cyclin Dependent Kinase 14 |
| CDK4 | Cyclin Dependent Kinase 4 |
| CDK6 | Cyclin Dependent Kinase 6 |
| cDNA | Complimentary Deoxyribonucleic Acid |
| CHD | Congenital Heart Disease |
| CMR | Cardiac Magnetic Resonance Imaging |
| CRYAB | Crystallin α B |
| CSRP3 | Cysteine and Glycine Rich Protein 3 |
| CTA | A-Tailing Control |
| CTE | End Repair Control |

| | |
|---|---|
| CTL | Ligation Control |
| CYP1A1 | Cytochrome P450 Family 1 Subfamily A Member 1 |
| DNA | Deoxyribonucleic Acid |
| DORV | Double Outlet Right Ventricle |
| dTTP | Deoxythymidine Triphosphate |
| dUTP | Deoxyuridine Triphosphate |
| ECG | Electrocardiogram |
| EDTA | Ethylenediamine Tetraacetic Acid |
| FPKM | Fragments Per Kilobase Million |
| GEO | Gene Expression Omnibus |
| GO | Gene Ontology |
| HCl | Hydrochloride |
| HCM | Hypertrophic Cardiomyopathy |
| HSP90AA1 | Heat Shock Protein 90 $\alpha$ Family Class A Member 1 |
| ICD | Implantable Cardioverter Defibrillator |
| IL6R | Interleukin 6 Receptor |
| JPH2 | Junctophilin 2 |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| LGE | Late Gadolinium Enhancement |
| LIG | Ligation Mix |
| LV | Left Ventricular |
| LVOT | Left Ventricular Outflow Tract |
| LVOTO | Left Ventricular Outflow Tract Obstruction |

| | |
|---|---|
| MAEs | Major Arrhythmic Events |
| MAOB | Monoamine Oxidase B |
| MDH2 | Malate Dehydrogenase 2 |
| MYBPC3 | Myosin Binding Protein C |
| MYH7 | β Myosin Heavy Chain |
| MYL2 | Myosin Light Chain 2 |
| MYL3 | Myosin Light Chain 3 |
| NYHA | New York Heart Association |
| PCR | Polymerase Chain Reaction |
| PLN | Phospholamban |
| PPAR | Peroxisome Proliferator-Activated Receptor |
| PPAR γ | Peroxisome Proliferator-Activated Receptor γ |
| PRKAG2 | Protein Kinase AMP-Activated Non-Catalytic Subunit γ 2 |
| RAB5A | Ras-Related Protein Rab-5A |
| RDH10 | Retinol Dehydrogenase 10 |
| RNA | Ribonucleic Acid |
| ROS | Reactive Oxygen Species |
| RV | Right Ventricular |
| RV-PA | Right Ventricle to Pulmonary Artery |
| RVI | Right Ventricular Impairment |
| RVOT | Right Ventricular Outflow Tract |
| RVOTO | Right Ventricular Outflow Tract Obstruction |
| RXR | Retinoid X Receptor |

| | |
|---|---|
| SCD | Sudden Cardiac Death |
| SDC2 | Syndecan 2 |
| SDS | Sodium Dodecyl Sulfate |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| SOD3 | Superoxide Dismutase 3 |
| TBS | Tris Buffered Saline |
| TCAP | Titin Cap |
| TEMED | Tetramethylethylendiamin |
| TMP1 | α Tropomyosin |
| TNNC1 | Troponin C1, Slow Skeletal and Cardiac Type |
| TNNI3 | Troponin I3 |
| TNNT2 | Troponin T2 |
| TOF | Tetralogy of Fallot |
| TPM2 | Tropomyosin 2 |
| TPM3 | Tropomyosin 3 |
| Tris | Tris(hydroxymethyl)aminomethane |
| Tris-HCl | Tris(hydroxymethyl)aminomethane-hydrochloride |
| TTE | Transthoracic Echocardiography |
| VR | Ventricular Remodeling |
| VSD | Ventricular Septal Defect |

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# 1 Introduction

## 1.1 Ventricular remodeling

Congenital heart disease (CHD) is defined as a structural malformation of the heart and/or of the contiguous vessels, existing since birth, which leads to functional cardiac impairment. CHD ranks among the most common congenital anomalies, counting for approximately one third of all congenital malformations (van der Linde et al., 2011) (Dolk, Loane, Garne, & European Surveillance of Congenital Anomalies Working, 2011). Thus, CHD represents a major global health challenge (van der Linde et al., 2011). The prevalence of CHD has increased from 0.6 per 1,000 live births to 9.1 per 1,000 live births in recent decades (van der Linde et al., 2011). In Germany, the prevalence of CHD amounts 10.8 per 1,000 live births and was determined by the PAN study (Lindinger, Schwedler, & Hense, 2010). Despite worldwide variation, a generally accepted estimation of CHD prevalence is 8 per 1,000 live births (Bernier, Stefanescu, Samoukovic, & Tchervenkov, 2010).

A few decades ago, the survival probability for children with CHD was low, only a minority with moderate to severe cardiac anomalies reached adulthood. Due to massive progress in diagnostic techniques, interventional procedures and surgical treatment, survival rates of children with CHD improved substantially since then (Warnes et al., 2001) (Mazor Dray & Marelli, 2015). Nowadays, about 85 to 95% of patients with CHD reach adulthood, creating a completely new and steadily growing population of adults with CHD (van der Linde et al., 2011).

A subset of affected patients, especially those with complex CHD, suffer from anatomical anomalies and residual surgical lesions, leading to chronic hemodynamic stressors in form of pressure and volume overload (Reddy & Bernstein, 2015a). Not only pathophysiological stimuli like excessive mechanical load, but also neurohumoral activation, ischemia and altered genetic background cause myocardial injuries and trigger myocardial stress (Schirone et al., 2017). Secondary to fundamental changes in hemodynamic conditions and increased myocardial stress, ventricular remodeling (VR) such as hypertrophy, fibrosis, arrhythmias, inflammation and cardiac dysfunction occur (Reddy & Bernstein, 2015a) (Reddy et al., 2013) (Figure 1).

In the year 2000, cardiac remodeling was commonly defined as “genome expression, molecular, cellular and interstitial changes that are manifested clinically as change in size, shape and function of the heart after cardiac injury” by the International Forum on

Cardiac Remodeling (Cohn, Ferrari, & Sharpe, 2000). Cardiac remodeling or VR are seen as progressive process, resulting in the rearrangement of normal existing myocardial structures. They are characterized as a complex sequence of structural and functional remodeling, including molecular, cellular, electrophysiological, biochemical, signaling and transcriptional alterations (Burchfield, Xie, & Hill, 2013). Remodeling processes start with changes in gene expression in response to cardiac injury, leading to molecular and cellular rearrangements and end up in a progressive loss of ventricular function.

According to recent studies, the VR process is concomitted by proliferation of fibroblasts, activation of leukocytes and upregulation of inflammatory pathways, enhancing myocardial fibrosis and inflammation (Reddy & Bernstein, 2015a) (Williams et al., 2018). Compromised myofilament movement, increased release of neurohormones, altered calcium handling as well as differences in gene regulation of cardiac metabolism and mitochondrial function further contribute to VR (Burchfield et al., 2013). A metabolic shift with energy deficiency due to fatal imbalance of oxygen supply and consumption as well as increased production of reactive oxygen species (ROS) seem to play an essential role in developing myocardial oxidative stress and cardiac remodeling (Azevedo, Polegato, Minicucci, Paiva, & Zornoff, 2016). Potential factors involved in pathophysiological mechanism of cardiac remodeling and dysfunction, include different ways of cell death in form of apoptosis, necrosis and autophagy, leading to the progressive loss of functional myocytes (Azevedo et al., 2016). Modifications in ventricular size and shape such as hypertrophy, enlarged or diminished cavity diameter are critical triggers, causing disturbed cardiac architecture and function. Cardiac remodeling may contribute to the occurrence of rhythm disturbances, including life-threatening arrhythmias. Myocardial rearrangement at a structural and molecular level such as fibrosis, altered gap junctional intercellular communication for electric coupling and increased cardiomyocyte calcium sensitivity, promotes the development of a proarrhythmic phenotype (Tsoutsman, Lam, & Semsarian, 2006) (Saeed et al., 2000) (Kawara et al., 2001). Severe arrhythmias as supraventricular and ventricular tachycardias become a major problem by being largely responsible for sudden cardiac death (SCD) (Reddy & Bernstein, 2015a). SCD is known as a decisive factor, contributing to morbidity and mortality (Keren, Syrris, & McKenna, 2008) (Ullal, Abdelfattah, Ashley, & Froelicher, 2016). Apart from severe arrhythmias and SCD, progressive heart failure equally counts for a great share of life-

threatening long-term complications and arises with a prevalence of 26% in patients with CHD (Dinardo, 2013).

According to experts, pathologic cardiac remodeling is closely associated not only with the progression of heart failure, but also with SCD, encompassing poor prognosis (Schirone et al., 2017) (Azevedo et al., 2016). Thereby, VR enables temporarily functional improvement and cardiac benefits, in the long term, presumed adaptive strategies become maladaptive, leading to increased morbidity and mortality in patients with CHD (Burchfield et al., 2013).

Widespread variability at temporal occurrence, individual clinical expression of progressive heart failure as well as therapeutic strategies for left heart failure, ineffectively used in right heart failure, support the assumption of pivotal differences in cardiac remodeling between both ventricles (Reddy & Bernstein, 2015b) (Baumgartner et al., 2010). A potential reason for this might be molecular und structural heterogeneity within the ventricles, causing diverse adaptive and maladaptive strategies to pathologic conditions (Williams et al., 2018).

Since timely diagnosis and preventive treatment are essential to avoid progressive remodeling and poor prognosis, experimental studies investigate in the identification of potential biomarkers for risk stratification. In recent years, the evaluation and detection of novel biomarkers, based on molecular and structural alterations, has become more relevant. Both, animal models and clinical studies demonstrated for example galectin-3 and matrix-metalloproteinase-9 as possible useful biomarkers, which are associated with fibrosis and inflammation, the latter known as hallmarks of cardiac remodeling (de Boer, Yu, & van Veldhuisen, 2010) (Halade, Jin, & Lindsey, 2013). However, the exact role of biomarkers in cardiac remodeling is still insufficiently studied.

Pharmacological treatment of cardiac remodeling also addresses previously described pathophysiological changes by targeting altered molecular and cellular structures. Current experimental studies in animal models provide evidence in reducing cardiac remodeling and improving ventricular function through angiotensin-converting enzyme inhibitors, β blockers and aldosterone antagonists (Azevedo et al., 2016). Despite promising therapeutic strategies, mortality rates in patients with cardiac remodeling and ventricular dysfunction remain high.

In spite of intensive research, sufficient information regarding VR in children and adults with CHD is still lacking. In particular, there is a lack of information on the genetic and molecular changes during pathologic cardiac remodeling and their impact on the clinical manifestation and clinical course of affected patients. The influence of genetic and molecular changes on possible long-term effects such as cardiac dysfunction, which can lead to malignant cardiac arrhythmias and heart failure, is also not yet well understood. Therefore, the investigation of the genetic and molecular determinants of VR, as well as the associated structural cardiac changes that influence the clinical outcome and disease progression, is of importance to gain a deeper understanding of VR in children and adults with CHD.

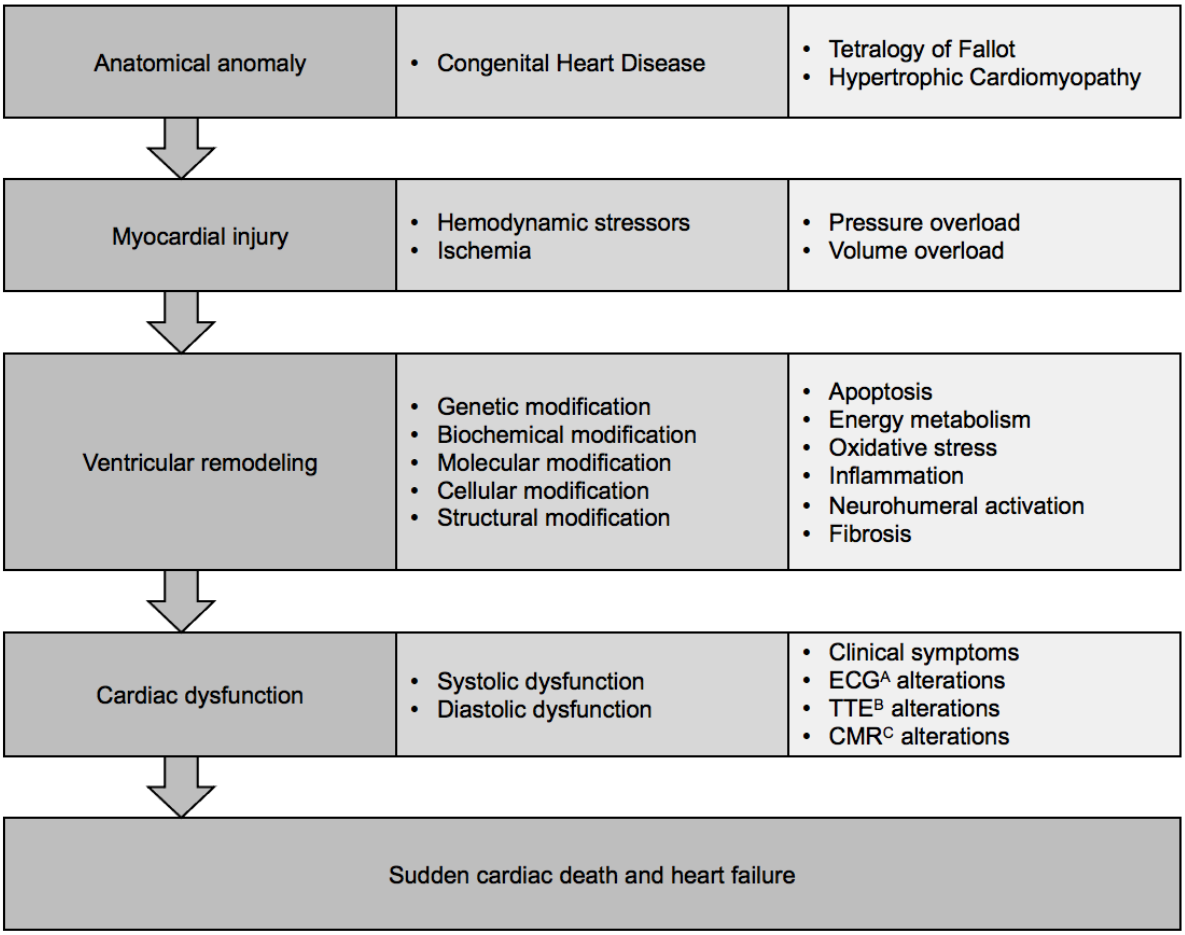


**Figure 1:** Development of cardiac dysfunction and mechanisms of pathological ventricular remodeling. [A], electrocardiographic; [B], transthoracic echocardiographic; [C], cardiac magnetic resonance imaging.

## 1.2 Medical Background

### 1.2.1 Tetralogy of Fallot

The anatomical features of Tetralogy of Fallot (TOF) were first described by thae Danish anatomist and scientist Niels Stensen in 1671 (Warnes et al., 2001) (Van Praagh, 2009). Years later in 1888, the French physician Étienne-Louis Arthur Fallot published similar findings, former defining it as “la maladie bleu” or “cyanosis cardiaque” (Allwork, 1988). Yet, the specification of TOF was coined and popularized by Maude Abbott, a Canadian pediatric cardiologist, in 1924 (Geva, Ayres, Pac, & Pignatelli, 1995) (Van Praagh, 1989). Precise descriptions of Stensen, Fallot and Abbott, combined with further fundamental publications (Van Praagh, 1989) (Peacock, 1847), contributed to a refined definition of the main characteristics of TOF. Namely right ventricular outflow tract obstruction (RVOTO) with pulmonary stenosis, ventricular septal defect (VSD), overriding aorta and concentric right ventricular (RV) hypertrophy (Figure 2).

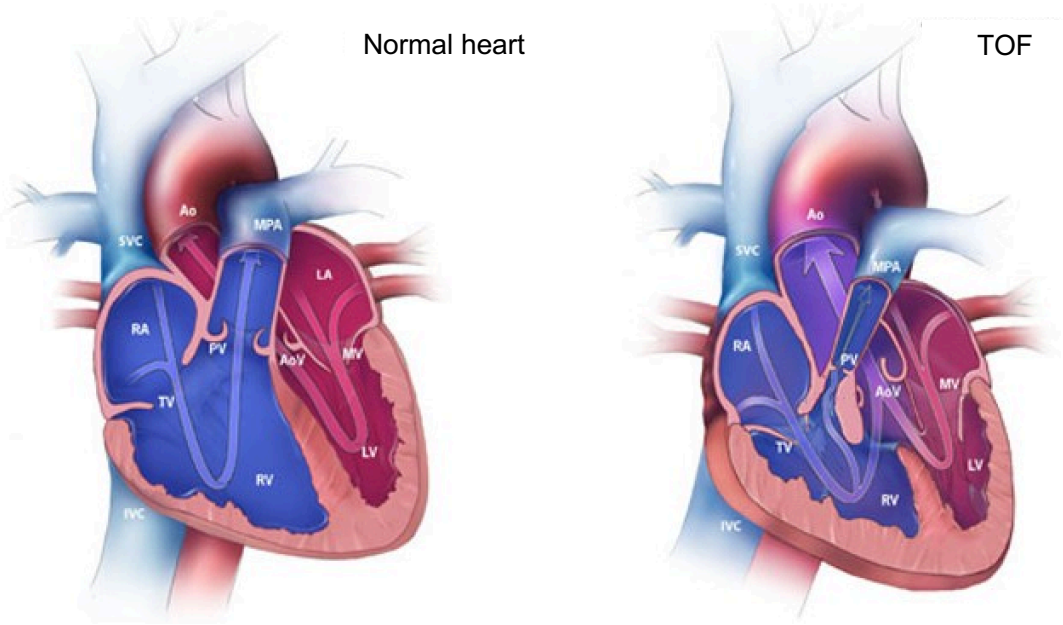


**Figure 2:** Anatomical features of TOF compared to a normal heart. Modified from (National Center on Birth Defects and Developmental Disabilities, 2020). Source: Material developed by Centers for Disease Control and Prevention, National Center on Birth Defects and Developmental Disabilities. Disclaimer: Reference to specific commercial products, manufacturers, companies, or trademarks does not constitute its endorsement or recommendation by the U.S. Government, Department of Health and Human Services, or Centers for Disease Control and Prevention. The illustration is available on the agency website for no charge. SVC, superior vena cava; IVC, inferior vena cava;

RA, right atrium; TV, tricuspid valve; RV, right ventricle; PV, pulmonary valve; MPA, main pulmonary artery; LA, left atrium; MV, mitral valve; LV, left ventricle; AoV, aortic valve; Ao, aorta.

With males being slightly more often affected than females, TOF represents the most common form of cyanotic heart disease of all complex congenital heart defects (Bailliard & Anderson, 2009) (Starr, 2010). According to the 2010 PAN study, TOF occurs with a prevalence of 2.7 out of 1,000 live births in Germany (Lindinger et al., 2010).

Exact causes leading to the development of congenital cardiac abnormalities are still largely unknown (Apitz, Webb, & Redington, 2009). A multifactorial genesis, including genetic and environmental factors, is assumed.

Clinical manifestation and the extent of hemodynamic effects depend mainly on anatomical conditions such as VSD size and RVOTO severity (Apitz et al., 2009). The majority of patients with mild RVOTO present with asymptomatic heart murmur discovered on routine auscultation, decreased exercise capacity and visible cyanosis between 2 to 6 months of age (Syamasundar Rao, 2009). In most cases there is a large, non-restrictive VSD, ensuring equal pressure ratios between both ventricles. As a consequence, the indicative loud, rough and spindle-shaped systolic murmur, which typically can be auscultated in most infants, has its origins in the narrowing right ventricular outflow tract (RVOT). Patients with severe RVOTO become conspicuous rapidly after birth by the occurrence of severe cyanosis, since an insufficient blood amount passes through the pulmonary bloodstream for oxygenation. Newborns indicating severe cyanosis due to right to left shunts at ventricle level or insufficient pulmonary blood flow require early intervention to avoid life-threatening hypoxia in the perinatal period. In contrast to so called “blue Fallot’s” with severe cyanosis, “pink Fallot’s” are non-cyanotic due to mild RVOTO, ensuring adequate pulmonary blood flow. Indeed, severe cyanosis, recurrent hypercyanotic spells and squatting are nowadays rare, since patients with TOF undergo early corrective repair within the first year of life.

Once the lesion is clinically suspected, an electrocardiogram (ECG), chest radiograph and transthoracic echocardiogram (TTE) are performed. Persistent right axis deviation and signs of RV hypertrophy, such as large R waves in the anterior precordial recordings and large S waves in the lateral precordial recordings, are typically seen in the ECG of patients with TOF (Bailliard & Anderson, 2009). As a consequence of RV

hypertrophy, the RV apex is displaced upwards which can be detected as a boot shaped cardiac silhouette in chest radiograph (Bailliard & Anderson, 2009). TTE is the gold standard to confirm the diagnosis and precisely assesses the intracardiac anatomy. Cardiac catheterization and cardiac magnetic resonance imaging (CMR) are not routinely required for confirming the definitive diagnosis, but can provide useful information for decision making and planning of operative treatment (Syamasundar Rao, 2009). Fetal echocardiography is successful in identifying structural cardiac anomalies antenatally, whereby TOF is frequently diagnosed during fetal life (Bailliard & Anderson, 2009). Hence, optimal planning of management and operative care helps to minimize perinatal morbidity and mortality.

TOF repair accounts to one of the successful stories of modern medicine since palliative and corrective surgery provide the opportunity for those born with TOF, to expect survival into adulthood. The first complete surgical correction of TOF was performed and documented by Lillehei et al in 1954 (Lillehei et al., 1955). In 1959, Kirklin was the first surgeon reporting the use of a transannular patch to expand the RVOT (Kirklin, Ellis, Mc, Dushane, & Swan, 1959). Safely performed early corrective repair within the first six month of life, which is still favored today, was made possible by the knowledge and surgical techniques of Castaneda and Barrat Boyes (Castaneda, Freed, Williams, & Norwood, 1977) (Barratt-Boyes & Neutze, 1973) (Caspi et al., 1999). Until today, several studies illustrate advantages of early corrective repair, outlining a low perioperative mortality rate of approximately 3% for affected patients (Hirsch, Mosca, & Bove, 2000). Corrective repair includes complete patch-closure of VSD to separate right and left ventricle (Figure 3, Panel A). In order to achieve dilation and relief of RVOT, the infundibular muscular stenosis is resected. In case of an additionally existing valvular pulmonary stenosis, surgical expansion of the narrowed valve will be undertaken by valvulotomy or commissurotomy. Occasionally, a pulmonary valve ring incision or extension with a transannular patch of autologous pericardium from the RVOT to the pulmonary artery is necessary.

Patients with particularly narrow pulmonary arteries or pulmonary valve atresia require removal and replacement of the pulmonary valve by creating a new connection between the right ventricle and pulmonary artery. Thereby, a valve-bearing conduit, made out of human (Homograft) or animal (Xenograft) material, is often be used (Figure 3, Panel B).

Nowadays palliative surgery with aortopulmonary shunt application is less common, due to evident success of corrective repair (Starr, 2010). In this respect, there are a variety of options, all of which serve to improve lung perfusion and reduce severe cyanosis prior to elective corrective repair. Especially infants with grave pulmonary artery hypoplasia, severe pulmonary valve stenosis, low birth weight or frequent cyanotic spells benefit from a two-stage strategy. First, the infants receive a Blalock-Taussig-Shunt (BTS) for rapid symptom improvement followed by corrective repair. Application of a modified BTS, comprising anastomosis between the subclavian artery and the ipsilateral pulmonary artery, is a commonly used method for this purpose (Figure 3, Panel C). Since serious complications such as distortion of the pulmonary artery, persistent hypoxia or shunt thrombosis can occur, subsequent corrective repair is necessary.

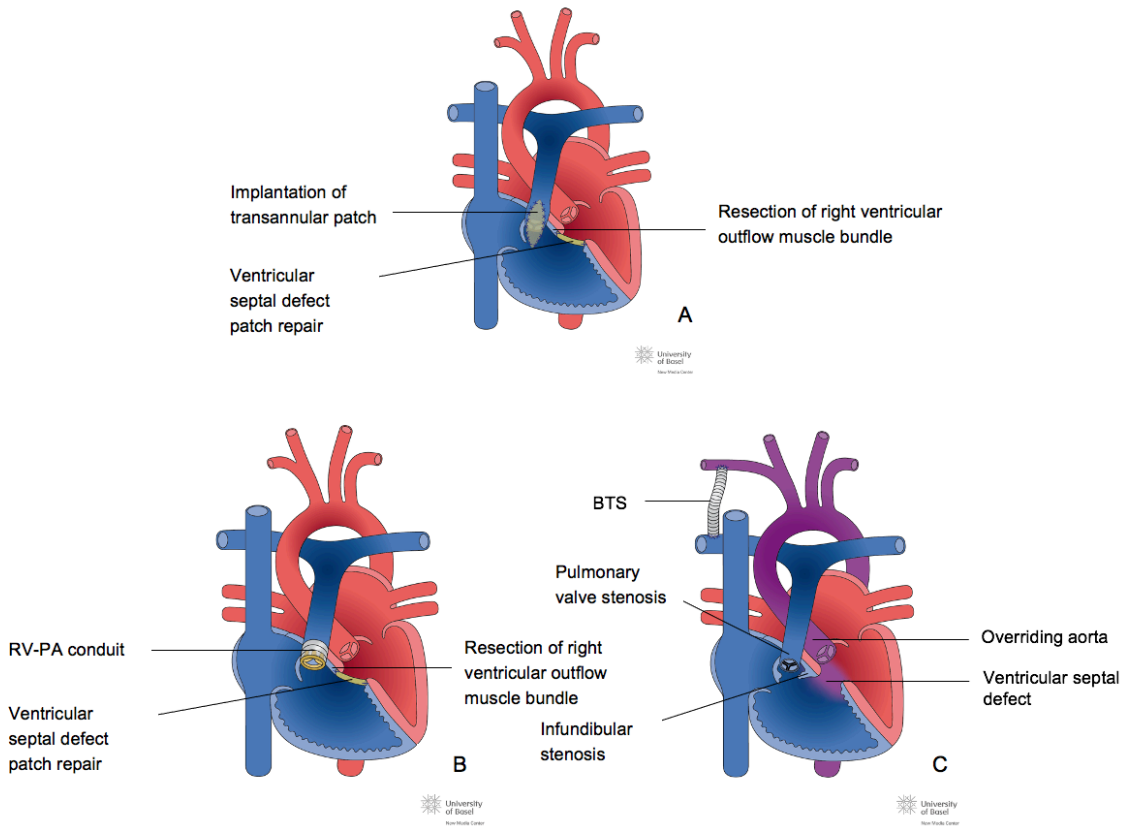


**Figure 3:** Surgical treatment in infants with TOF. Panel A, TOF repair with transannular patch; Panel B, TOF repair with right ventricle to pulmonary artery (RV-PA) conduit; Panel C, modified right Blalock-Taussig-Shunt (BTS) for palliative TOF repair. Modified illustration taken from http://www.chd-diagrams.com and licensed under Creative Commons Attribution-Non-

Usually postoperative recovery and course is uncomplicated in most of the patients (Apitz et al., 2009). However, only a minority present with early complications such as low cardiac output despite adequate corrective repair (Apitz et al., 2009). The most common described reason for this phenomenon is the manifestation of RV restrictive physiology due to myocardial damage during corrective repair (Apitz et al., 2009) (Cullen, Shore, & Redington, 1995).

Late complications include harmful VR secondary to chronic hemodynamic stressors. Residual pulmonary insufficiency occurs as sequelae of corrective repair, specifically using valve-less RV-PA conduits and after transvalvular patch (Apitz et al., 2009). Given the lack of alternative correction in infants secondary to the small size of vessels, resulting pulmonary regurgitation needs to be accepted in those cases in order to relief RVOTO. Long-term consequences include progressive exercise intolerance (Carvalho, Shinebourne, Busst, Rigby, & Redington, 1992) (Ammash, Dearani, Burkhart, & Connolly, 2007), right heart failure (Gregg & Foster, 2007) (Chaturvedi & Redington, 2007), ventricular arrhythmias and SCD (Zahka et al., 1988) (Gatzoulis et al., 2000) (Silka, Hardy, Menashe, & Morris, 1998). These results indicate that such hemodynamic determinants are indeed important and have to be taken into account, in order to prevent problems occurring decades after surgery (Redington, Oldershaw, Shinebourne, & Rigby, 1988). In response to pulmonary regurgitation the vulnerable and poorly resistant right ventricle reacts to volume overload with RV remodeling (Apitz et al., 2009) (Redington et al., 1988). Consequently, RV diastolic and systolic dysfunction (Davlouros et al., 2002) occurs, leading to right ventricular impairment (RVI) and failure. Major alterations at the molecular level involve cell signaling, activation of inflammatory pathways and increased myocardial fibrosis (Reddy & Bernstein, 2015a) (Reddy et al., 2013). Also, mitochondrial bioenergetic dysfunction plays an important role in the development of RVI. A reduction of mitochondrial enzymes leads to inefficient energy production and increased mitochondrial ROS production with simultaneous failing antioxidant defense, making the right ventricle more prone to oxidative stress, and therefore being uniquely at risk for earlier damage than the left ventricle (Reddy & Bernstein, 2015a) (Reddy & Bernstein, 2015b). Alterations in metabolic profile, including a myocardial metabolic shift towards a greater

dependence on glycolysis for energy production is only beneficial in acute ventricular impairment. In contrast, glycolysis provides insufficient quantities of adenosine triphosphate, leading to inadequate energy management which aggravates ventricular failure (Reddy & Bernstein, 2015a). Reduced angiogenesis in hypertrophic cardiomyocytes increases the susceptibility to ischemia, myocardial scarring or apoptosis. Modified release of neurohumoral factors results into impaired ventricular function. Furthermore, volume overload due to pulmonary regurgitation plays a major role in causing symptomatic supraventricular and ventricular arrhythmias (Zahka et al., 1988). Increased myofilament calcium sensitivity and diminished potassium channel expression are responsible for prolonged action potentials and QT intervals, enhancing electric remodeling (Reddy & Bernstein, 2015a). Metabolic derangements are additionally suspected to be involved in ventricular electric remodeling (Piao, Marsboom, & Archer, 2010).

Chronic pressure overload due to remaining pulmonary stenosis stimulates ventricular hypertrophy of cardiomyocytes. This results not only in increased contractility, but also in reduced filling capacity of the right ventricle during diastole. Limited active ventricular relaxation and increased passive stiffness are the result, leading to early diastolic and late systolic dysfunction (Nollert et al., 1997). Currently less is known in terms of molecular adaption processes in chronic pressure overload in adult patients with repaired TOF. To date, upregulation of proapoptotic pathways, altered regulation of calcium handling in cardiomyocytes and decreased survival rates have been described in few studies conducted (Urashima et al., 2008).

Pathological RV remodeling due to long-term hemodynamic injuries leads to RVI and right heart failure (Kaufman et al., 2008) (Buermans et al., 2005). Limitations in diagnostic techniques complicate precise detection of right heart failure onset in early stage. The transition from compensated to non-compensated right heart failure can only be inaccurately predicted by clinical examination and imaging techniques. Consequently, the identification of biomarkers to optimize patient care and determine the extent of myocardial remodeling is of utmost importance. Additional to insufficient knowledge, limited diagnostic techniques and contemporary ineffective therapeutic strategies, promising causal therapies are not available to protect patients with complex congenital heart defects from life-threatening heart failure. Of particular relevance are the delay of right heart failure onset as well as the reduction of right heart

failure severity. The identification of cardiac changes at the molecular level contributes to the discovery of new therapeutic approaches and the development of new therapeutic strategies.

### 1.2.2 Hypertrophic Cardiomyopathy

Hypertrophic Cardiomyopathy (HCM) was first described as “idiopathic hypertrophic subaortic stenosis” by Vulpian and dates back to 1868 (Liew, Vassiliou, Cooper, & Raphael, 2017). Further descriptions concerning the striking anatomical anomaly which were similar to those of Vulpian, followed shortly by Liouville and Hallopeau in 1869 (Liew et al., 2017). Years later, the British pathologist Teare published his autopsy findings in 1957, reporting of eight patients being affected by asymmetric septal hypertrophy, leading to left ventricular outflow tract obstruction (LVOTO). Seven of whom died of SCD (Teare, 1958). In 1963, first publications appeared, describing another variant of the heart disease (Liew et al., 2017). The presence of left ventricular (LV) hypertrophy without obstruction of the left ventricular outflow tract (LVOT) in fourteen patients was reported by Braunwald et al. These first historical descriptions in recent decades have contributed significantly to the current definition and classification of HCM. HCM is defined as a primary cardiomyopathy by the presence of abnormal and isolated hypertrophy of the left ventricle and septum, in the absence of altered loading conditions, simultaneously causing hypertrophy (Keren et al., 2008) (Semsarian & Group, 2011).

Following the guidelines of the European Society of Cardiology, HCM in adults is characterized by LV wall thickness of > 15 mm, which cannot be explained solely by altered loading conditions (Authors/Task Force et al., 2014). Children diagnosed with HCM require LV wall thickness with more than two standard deviations greater than the predicted mean, consequently exhibiting z-score > 2 (Authors/Task Force et al., 2014).

HCM can be classified into hypertrophic non obstructive cardiomyopathy and hypertrophic obstructive cardiomyopathy, whereby the hypertrophy of the left ventricle is additionally accompanied by LVOTO (Authors/Task Force et al., 2014) (B. J. Maron et al., 2006). Approximately 70% of HCM patients present with obstruction and 30% with no obstruction (B. J. Maron, Gottdiener, & Epstein, 1981).

HCM is the most common genetically inherited cardiovascular disorder, occurring with a prevalence of 1:500 to 1:200 in the general population worldwide (Semsarian, Ingles, Maron, & Maron, 2015) (B. J. Maron et al., 1995). The prevalence of childhood onset HCM is still unknown, but according to population-based estimations, it occurs to a lesser extent with an annual incidence of 0.3-0.5 per 100,000 live births (Lipshultz et al., 2003) (Nugent et al., 2005). Both male and female patients are equally affected by HCM (Olivotto et al., 2005).

During the last few decades, it has become apparent that about 60% of patients with HCM present with mutations responsible for the disease (Keren et al., 2008). Since then, more than 1.400 mutations in 11 or more genes have been identified to be causative for HCM (B. J. Maron & Maron, 2013). Most of the mutations are inherited in an autosomal dominant manner and are predominantly in genes, encoding for essential cardiac sarcomere proteins (B. J. Maron, 2002). Proteins of the contractile apparatus of cardiomyocytes, frequently affected by mutations are ß-myosin heavy chain (*MYH7*) and myosin binding protein C (*MYBPC3*) (Seidman & Seidman, 2011) (Richard et al., 2003). In addition to the two main causative mutations, multiple other mutations in genes, coding for various important components of the cardiac sarcomere, such as cardiac troponin T2 (*TNNT2*) (Thierfelder et al., 1994), cardiac troponin C (*TNNC1*) (Landstrom et al., 2008), cardiac troponin I3 (*TNNI3*) (Kimura et al., 1997), myosin light chain 2 (*MYL2*) (Seidman & Seidman, 2011) and $\alpha$-tropomyosin (*TMP1*) (Seidman & Seidman, 2011), were identified (Figure 5). Most of the patients carry single mutations in one of the mentioned genes, however nearly 5% are so called multiple-mutation-carrier, showing more than one mutation (Authors/Task Force et al., 2014).

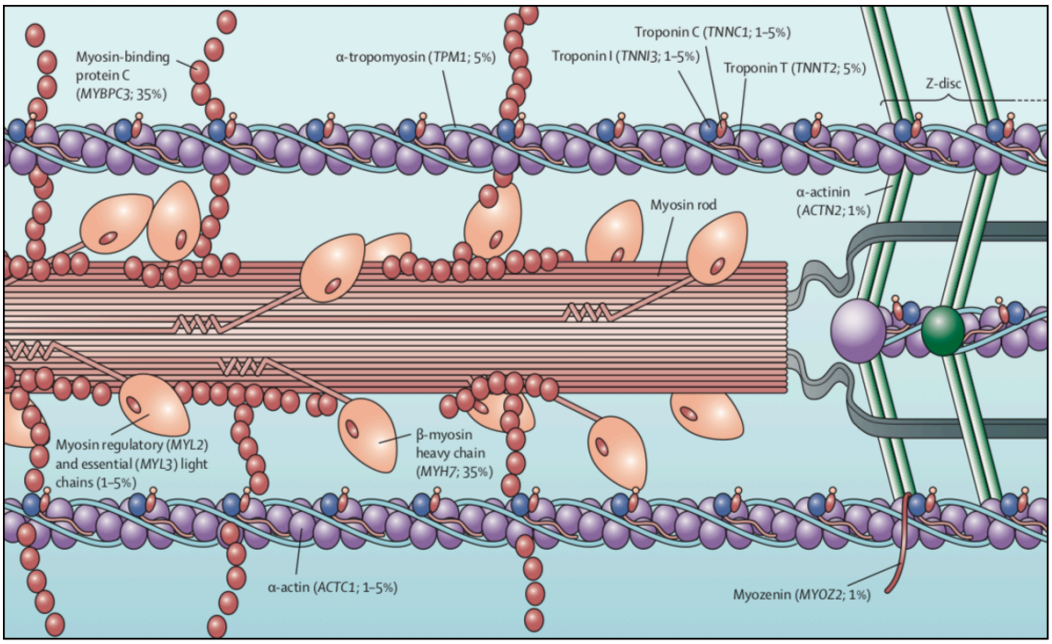


**Figure 4:** Location of sarcomere proteins affected by HCM causative mutations. Illustration and modified legend with permission from Elsevier taken from (B. J. Maron & Maron, 2013).

Apart from causative gene mutations in sarcomere proteins, metabolic, endocrine disorders, neuromuscular and mitochondrial diseases as well as malformation syndromes such as Noonan Syndrome have been identified as other genetic and non-genetic causes of HCM (Elliott et al., 2011) (Charron et al., 2004) (Limongelli, Masarone, D'Alessandro, & Elliott, 2012) (Wilkinson et al., 2012) (Huddle, Kalliatakis, & Skoularigis, 1996).

The wide range of genes that can be affected by mutations makes HCM a genetically heterogeneous disease (Wolf, 2019). Genetic variability might be a reason for the different expression of pathological characteristics, leading to a large spectrum of diverse phenotypes with manifold clinical course, including great variance in expressivity, penetrance and clinical outcome (Arad, Seidman, & Seidman, 2002).

Recent studies have shown that gene mutations entail primary and secondary effects, influencing patients phenotypes (Wolf, 2019). Initially, mutations directly modify protein structure and function, resulting in altered conditions for cardiomyocytes and their functionality (Tardiff, 2005) (Sequeira et al., 2013). For instance, modifications in intracellular calcium handling and compromised energy balance were described (Watkins, Ashrafian, & Redwood, 2011) (Olivotto, Cecchi, Poggesi, & Yacoub, 2012) (Ashrafian, McKenna, & Watkins, 2011). Altered myocardial expression of genes

encoding sarcomere and non-sarcomere proteins, metabolic enzymes and extracellular matrix proteins have also been demonstrated (Olivotto et al., 2012). Secondary to modified protein structure and function, other signaling pathways can be activated as well as molecular and cellular changes initiated. Both ultimately lead to individual variability in phenotypes and pathological VR (Watkins et al., 2011) (Ferrantini et al., 2009) (M. S. Maron, Olivotto, et al., 2009). LV remodeling occurs over lifetime with mostly severe asymmetric septal hypertrophy, contributing to serious LVOTO. Abnormal proliferation of fibroblasts is an adverse sequel of massive myocardial hypertrophy, provoking microvascular ischemia, cardiomyocyte loss due to energy depletion and apoptosis (Olivotto et al., 2012). Emerging myocardial fibrosis and myocyte disarray result from the substitution of cardiomyocytes by fibroblasts. Consequently, pathological VR contributes to systolic dysfunction with altered LV ejection fraction (Harris et al., 2006) as well as diastolic dysfunction (Melacini et al., 2010), including reduced filling capacity and atrial dilatation (Nistri et al., 2006). Furthermore LV apical aneurysms and life-threatening arrhythmias occur (Wolf, 2019) (M. S. Maron et al., 2008). Items for the development of supraventricular and ventricular arrhythmias include cardiomyocyte automaticity, increased calcium sensitivity and myocardial fibrosis, which promotes reentry tachycardia (Tsoutsman et al., 2006) (Saeed et al., 2000) (Baudenbacher et al., 2008) (Wolf & Berul, 2008). Myocardial ischemia and hemodynamic injuries concomitantly trigger rhythm disturbances in HCM patients (Elliott & Spirito, 2008) (Wood & Ellenbogen, 2000).

HCM is known as a disease with variable progression, which can be classified into different stages (Wolf, 2019). At stage one, patients carry mutations in HCM specific genes, but remain clinically unremarkable. LV hypertrophy cannot be detected in the clinical assessment with ECG, TTE or CMR. Due to sensitive imaging techniques and advanced molecular genetic testing, the prevalence of so-called genotype positive and phenotype negative individuals increased. Approximately 50% of HCM patients develop LV hypertrophy with or without LVOTO and become symptomatic between the age of 20 and 30 (Olivotto et al., 2012) (Charron et al., 1997). Usually, patients present with excessive LV function and mild symptoms, such as palpitations, decreased exercise tolerance, dyspnea, syncope and intermitting chest pain (Authors/Task Force et al., 2014).

In the consequence of lifelong VR, LV hypertrophy is progressive, leading to increased fibrosis, myocyte disarray and atrial dilatation (Olivotto et al., 2012). Typical for this stage is adverse LV diastolic dysfunction with initially preserved systolic function. Potentially malignant arrhythmias can occur. Increased morbidity and mortality in the terminal stage of HCM is not uncommon, due to life-threatening arrhythmias, heart failure and an increased risk of SCD, especially in young people and in competitive athletes (Keren et al., 2008) (Ullal et al., 2016). The terminal stage of HCM is characterized by irreversible remodeling processes, severe systolic dysfunction and hemodynamic decompensation, including worse LV wall stiffness (Wolf, 2019).

However, timely diagnosis and clinical management still remain challenging because of unpredictable clinical onset and course (Semsarian & Group, 2011). Some patients swiftly develop moderate to severe symptoms, while others maintain asymptomatic into adulthood (Semsarian & Group, 2011). Hence, ongoing detailed clinical work-up is necessary.

Family history and physical examination is important in order to identify patients at risk for disease development. Suspected patients with positive family history for HCM in other family members, SCD under the age of 40, unexplained heart failure, heart transplantation or implantable cardioverter defibrillator (ICD) implantations, carry higher risk for a potentially positive genetic test (Authors/Task Force et al., 2014). Due to the autosomal dominant inheritance, the probability that affected family members will pass on the mutation to the next generation is 50% (Richard et al., 2003). Molecular genetic testing is a powerful diagnostic tool to identify patients prior to the onset of HCM (Bagnall, Ingles, & Semsarian, 2011). Once HCM has been confirmed in genetic testing, timely monitoring, detection of HCM onset and adequate therapeutic management can optimize patient care.

Cautious physical examination can provide initial indicators of HCM. Patients present with angina, dyspnea, palpitations, episodes of syncope and typical systolic heart murmur with increased extensity while physical strain or Valsalva maneuver (Authors/Task Force et al., 2014). ECG may show ST and T wave abnormalities and left bundle branch block, as well as pathologically elevated R waves and deep Q waves, suggestive of LV and septal hypertrophy (Authors/Task Force et al., 2014). Rhythm disturbances as atrial fibrillation and ventricular tachycardia are frequently associated with HCM and can be detected in a 24-hour ECG. Imaging modalities as

TTE are essential for confirming diagnosis and disease monitoring. By means of echocardiographic examination, the severity of hypertrophy can be determined and the presence of LVOTO or systolic anterior motion of the anterior mitral valve can be detected (Authors/Task Force et al., 2014). Measurement of intraventricular pressure gradient in patients with LVOTO contribute to the decision of indication for surgical therapy, reducing LVOTO. Ventricular morphology and adverse remodeling can be assessed by CMR (M. S. Maron, Maron, et al., 2009) (Rickers et al., 2005). In particular positive late gadolinium enhancement (LGE) uncovers focal myocardial fibrosis as well as necrosis (Briasoulis, Mallikethi-Reddy, Palla, Alesh, & Afonso, 2015). Interstitial fibrosis can also be detected by increased extracellular volume fraction on CMR T1 mapping (Ho et al., 2013). Even before LV hypertrophy develops, myocardial fibrosis can be detected in genotype positive patients, supporting the assumption that fibrotic remodeling already begins in early disease state (Ho et al., 2013). Further diagnostic procedures such as cardiac catheterization for LV pressure measurement and endomyocardial biopsy, identifying hypertrophied cardiomyocytes and fibrosis, are not part of routine clinical work-up. Though, both techniques might be also helpful to provide transparency and support diagnosis in patients presenting with unclear phenotypes (Wolf, 2019).

Disease variability requires early diagnosis, continuous disease monitoring, timely interventions, optimal risk stratification and individualized therapies. To date, there is no causal therapy available for HCM. Therefore, patient's management focus on preventive therapeutic strategies to avoid disease progression with irreversible myocardial remodeling (Authors/Task Force et al., 2014). Patients with mild to moderate symptoms can benefit from the avoidance of severe physical strain and competitive sports. In cases with ventricular dysfunction and heart failure related complications, patients require pharmacotherapy, including β blocker, diuretics or calcium antagonists. Invasive therapies, including surgical septal myectomy and alcohol septal ablation are necessary in patients, presenting with drug-refractory symptoms of severe LVOTO (Figure 5).

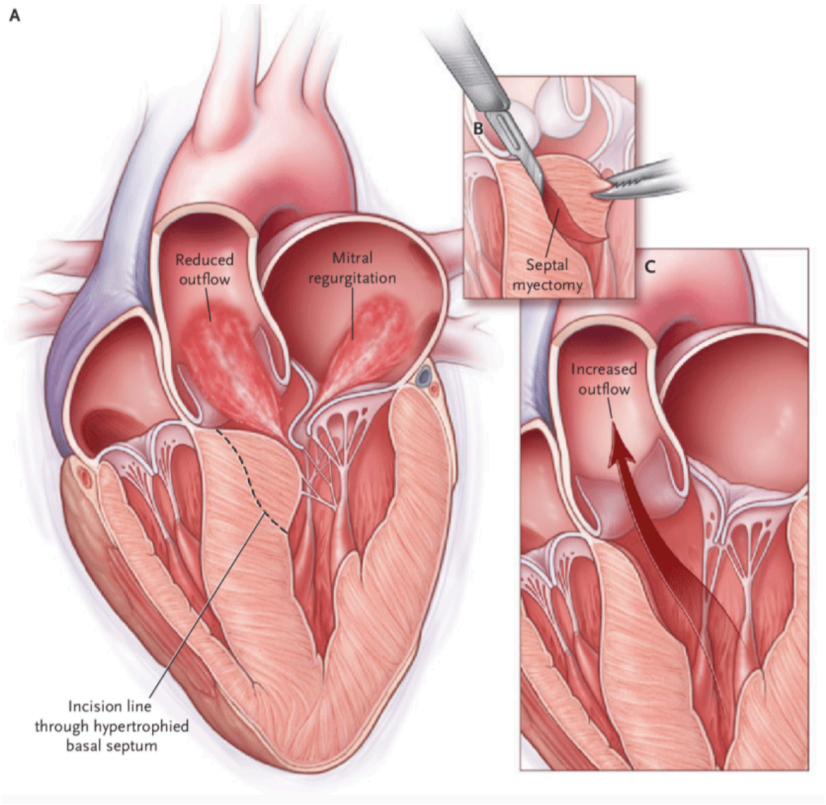


**Figure 5:** Illustration of surgical septal myectomy. Panel A, hypertrophied LV myocardium causing LVOTO; Panel B, surgical procedure; Panel C, extended LVOT after surgery. Illustration and modified legend taken from (Nishimura & Holmes, 2004). Reproduced with permission from (Nishimura & Holmes, 2004), Copyright Massachusetts Medical Society.

In the presence of rhythm disturbances, long-term anticoagulation for thromboembolism prevention as well as rhythm and ventricular rate control are used. Equally important is the protection of patients at risk from potential life-threatening arrhythmias, leading to SCD, which can be the first symptom in undetected HCM. ICD implantation as secondary prevention is consistently recommended in patients who have survived cardiac arrest due to life-threatening arrythmias (Authors/Task Force et al., 2014). Estimation of SCD risk for primary prevention depends on multiple clinical characteristics identified as risk factors for malignant arrythmias. These include massive LV hypertrophy, syncope of unknown etiology, family history of SCD under the age of 40, detected non sustained ventricular tachycardia and abnormal increased blood pressure due to stress test (B. J. Maron et al., 2003) (Gersh et al., 2011a). Five years risk stratification for SCD in HCM patients can be calculated online, taking measurement of LVOT gradient, left atrial diameter and age at evaluation as risk factors into account (O'Mahony et al., 2014). Based on defined criteria, ICD implantation is recommended. However, online risk calculation cannot be used for

children under the age of 16, consequently leaving a gap of adequate risk ratification. Still, current therapeutic strategies have not been shown to guarantee consistent efficacy with reducing morbidity and mortality, attenuating disease progression or averting adverse remodeling in young HCM patients. The development of causal therapeutic approaches, targeting molecular mechanisms involved in VR are promising to improve morbidity and mortality.

# 2 Study purpose

There is consensus that anatomical cardiac anomalies such as those present in patients with CHD lead to adverse VR, predisposing morbidity and mortality in this population. Research into the fundamentals and mechanisms acting in VR helps developing strategies to counteract, mitigate, or prevent progression of this process. Adequate information about molecular and cellular factors as well as the effect of clinical manifestation is still lacking, particularly in patients with CHD. Identification of molecular signaling pathways and determination of their putative clinical impact in patients with CHD affected by VR lead to a more precise understanding of fundamentals of remodeling processes in the human heart. Therefore, the purpose of this dissertation was to further investigate molecular mechanisms contributing to right and left ventricular remodeling, respectively, in two distinct subtypes of CHD.

The focus of the first study was the identification of altered gene expression and molecular signaling pathways, playing a role in RV remodeling in patients with TOF and long-term hemodynamic stressors.

The second study aimed on determining the influence of genetic alterations on LV remodeling and clinical outcome of patients with pediatric onset HCM.

# 3 Methodology

## 3.1 Study design

Two different approaches were used to evaluate VR in children and adults with CHD (Figure 6). In study I molecular fundamentals of VR in impaired right ventricles of adult patients with TOF were investigated. The second study evaluated the influence of underlying genotype on VR of the left ventricle in children with pediatric onset HCM and affected left ventricles. Both studies are monocentric investigations at the German Heart Centre Munich, a tertiary care university hospital.

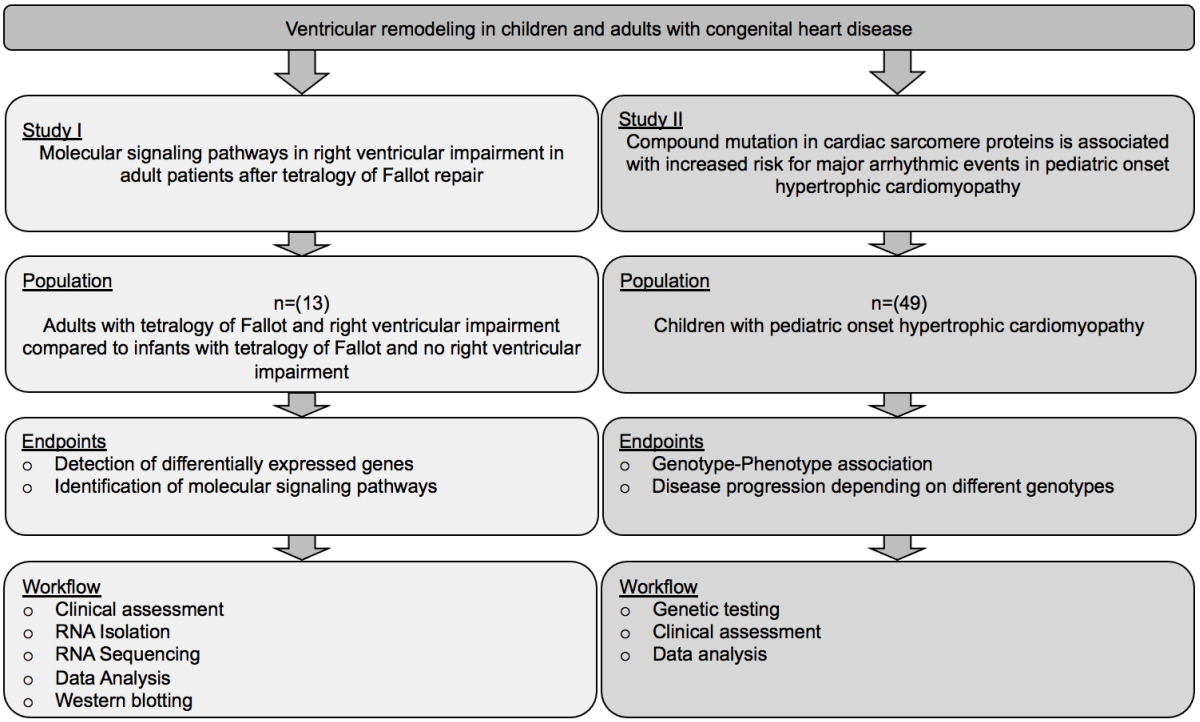


**Figure 6:** Study design.

Prior to the beginning of study I, the application was submitted and approved by the ethical committee at the Technical University of Munich (approval numbers 242/17S, 592/16S). Before study inclusion, all patients or parents gave written informed consent. Due to the complete retrospective design of study II, the need for an ethical board evaluation was waived. For a subset of patients who were also included in other studies, informed patient consent and approval of the respective institution's ethical committee at the Technical University of Munich was available (approval numbers 243/17S, 14/15S, 332/15). Both studies conform to the ethical standards of the Declaration of Helsinki as revised in 2013.

## 3.2 Study population

Study I

Between April 2009 and May 2016, 122 patients with a primary diagnosis of TOF underwent surgical treatments including RV tissue collection at the German Heart Centre Munich. RV-PA conduit surgery was performed for adult patients due to long-term hemodynamic consequences of anatomical anomalies and residual surgical lesions from former procedures. For infants, surgical treatment was conducted in the form of corrective repair. Solely RV tissue samples necessarily removed for RVOTO relief were stored at the institutional biomaterial bank. In total, 31 patients with sufficient quantity of stored RV myocardium had signed the declaration of consent for tissue sampling and usage for research purposes. To assess the clinical status of all patients, demographic and clinical information as well as data of ECG, TTE, chest x-ray, CMR, computed tomography scans and cardiac catheterization were collected by retrospective medical chart review and were analyzed. Based on recommendations of the International Right Heart Foundation Working Group (Mehra, Park, Landzberg, Lala, & Waxman, 2013) and the scientific statement of the American Heart Association (Konstam et al., 2018) criteria for RVI were selected (Table 1). Due to the lack of clear clinical characterization, genetic disorders and insufficient signs of RVI, 18 patients were not selected for the study purpose.

Study I included 6 adult patients diagnosed with TOF or Double Outlet Right Ventricle (DORV) of the type of Fallot suffering from long-term pressure and/or volume overload due to persistent hemodynamic stressors. To be eligible for inclusion as “cases”, patients had to be at least 18 years of age and already clinically and/or diagnostically confirmed RVI. For comparison, 7 infant patients with a maximum age of 5 months and no detectable signs of RVI due to short-term altered hemodynamics were enrolled as so-called “controls”. RV tissue from infants with TOF obtained at the time of corrective surgery during infancy was selected for comparison due to the similar underlying structural heart defect and due to the absence of long-standing RV hemodynamic stressors. Exclusion criteria encompass all patients with confirmed genetic disorders, additional syndromes, the presence of CHD with affected left ventricles and patients with indications of another organ failure.

**Table 1:** Criteria for RVI in adult patients with TOF and Fallot repair.

| Criteria for right ventricular impairment | |
|---|---|
| Clinical parameter | • Functional capacity<br>• Peripheral edema<br>• Engorgement of jugular veins<br>• Enlargement of liver<br>• Dyspnea<br>• Cyanosis |
| Laboratory marker | • Glomerular filtration rate<br>• Creatinine<br>• Blood urea nitrogen<br>• Blood bilirubin<br>• γ-glutamyl transpeptidase<br>• Alkaline phosphatase |
| Electrocardiogram | • Right-axis deviation<br>• Incomplete/complete right bundle branch block<br>• Length of QRS interval<br>• Abnormal repolarization<br>• Atrial/ventricular tachyarrhythmias<br>• Implantable cardioverter defibrillator implantation |
| Transthoracic echocardiography | • Right ventricular ejection fraction<br>• Pulmonary regurgitation<br>• Maximum velocity over pulmonary valve<br>• Mean pressure gradient over pulmonic valve<br>• Maximum pressure gradient over pulmonic valve<br>• Right ventricular hypertrophy<br>• Right ventricular pressure |
| Cardiac magnetic resonance imaging | • Right ventricular ejection fraction<br>• Left ventricular ejection fraction<br>• Pulmonary regurgitation fraction<br>• Right ventricular stroke volume<br>• Right ventricular end diastolic volume index<br>• Right ventricular end diastolic volume<br>• Right ventricular end systolic volume index<br>• Right ventricular end systolic volume |
| Preoperative medication | • β-blocker<br>• Diuretic<br>• Angiotensin converting enzyme inhibitor |

Study II

The second study included patients with HCM who presented to the pediatric cardiology outpatient clinic of the German Heart Center Munich between November 1981 and November 2019. Primary diagnosis of pediatric onset HCM was set in all 49 patients until the end of the 18$^{th}$ year of age. The definition of pediatric onset HCM with LV remodeling comprised a positive molecular genetic testing or echocardiographic evidence of HCM.

All genetic variants detected in molecular genetic testing were categorized according to the classification system and guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards et al., 2015) (Amendola et al., 2016). Patients identified with pathogenic or likely pathogenic mutations were grouped as genotype positive. Patients with negative molecular testing due to absent mutations, likely benign or benign mutations and presence of variants of unknown significance were classified as genotype negative. Based on the guidelines of the European Society of Cardiology (Authors/Task Force et al., 2014) and guidelines for the diagnosis and treatment of patients with HCM of the American Heart Association and the American College of Cardiology (Ommen et al., 2020), patients were defined as phenotype positive by the presence of isolated hypertrophied left ventricle with a z-score of ≥2 on TTE evaluation or LV wall thickness of > 15 mm, not explained merely by altered loading conditions. Children with no echocardiographic detection of HCM were classified as phenotype negative. Based on that, the study population was classified into 35 genotype positive/phenotype positive patients, 10 genotype negative/phenotype positive patients as well as 4 genotype positive/phenotype negative patients. Genotype positive/phenotype negative patients were also included due to existing genetic predisposition to develop HCM phenotype during childhood. Furthermore patients with proven mutation and still absence of detectable LV hypertrophy were also considered to be affected by VR due to genetic predisposition (Ho et al., 2013) (Ho, 2009). The defined groups were compared, and subgroup analysis was performed depending on the present mutations (Figure 7).

Patients with secondary factors as severe aortic stenosis, leading to equivalent LV hypertrophy as well as the presence of other CHD were not selected. Also, patients with confirmed genetic diseases, metabolic, endocrine, mitochondrial or neuromuscular disorders associated with HCM were excluded from the study.

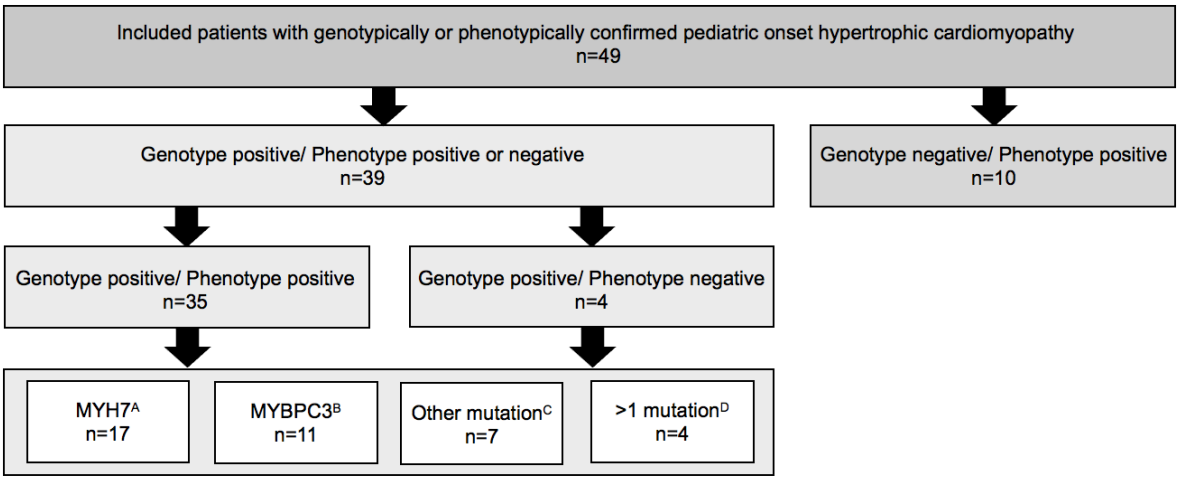


**Figure 7:** Classification and subgrouping of patients diagnosed with pediatric onset HCM. [A], β myosin heavy chain (*MYH7*); [B], myosin binding protein C (*MYBPC3*); [C], other mutation: cardiac troponin T2 (*TNNT2*, n=4), cardiac troponin I3 (*TNNI3*, n=1), α tropomyosin (*TPM1*, n=1), myosin light chain 2 (*MYL2*, n=1); [D]; compound mutation: *MYH7* with *TNNT2* (n=3) and *MYH7* with *TNNI3* (n=1).

## 3.3 Research methods study I

### 3.3.1 RV tissue sample collection and storage

RV tissue was collected during RV-PA conduit surgery in adult patients and during corrective repair in infant patients. Immediately after tissue removal, all samples were frozen in liquid nitrogen and stored at -160°C at the institutional biomaterial bank. Prior to RNA isolation, all samples were weighed, cut to size and instantaneously stored at -80 °C at the pediatric cardiology laboratory at the German Heart Centre Munich. On the day of RNA Isolation, RV tissue samples were transferred to the Helmholtz Centre Munich under permanent cooling on dry ice.

### 3.3.2 RNA isolation

Following manufacturer's instructions, total RNA was isolated at the institute of human genetics at Helmholtz Centre Munich, using miRNeasy Mini Kit and QIAcube robotic workstation (Qiagen Hilden, Germany) (Qiagen, 2014). The procedure of total RNA isolation is depicted in Figure 8.

For RV tissue protection and prevention of RNA degradation, the workflow was performed expeditiously and carefully under continuous cooling of RV tissue samples. Prior to the beginning of RNA isolation, required amounts of ethanol (96-100%) were added to the concentrated RWT and RPE buffers, which were provided by miRNeasy Mini Kit. RWT and RPE buffers serve as washing buffers to remove excessive and unbound contaminants from the spin column during the washing procedure. This promotes elution of the purified RNA, which adheres to the silica-membrane of the spin column.

700 µl QIAzol Lysis Reagent, containing phenol and guanidine thiocyanate, was added to RV tissue samples. QIAzol Lysis Reagent facilitates tissue lysis, inhibits RNase and removes cellular DNA and proteins. In order to preserve high quantity and quality of total RNA and optimal RNA binding at the silica-membrane, complete tissue destruction and homogenization were performed with Tissue Lyser II by a shaking procedure at 30 Hz for a total of 4 minutes.

After placing lysed and homogenized samples at room temperature (15-25°C) for 5 minutes, 140 µl chloroform was added and samples were shaken for 15 seconds. Chloroform leads to an improved phase separation and serves the inhibition of a stable and highly active RNase, minimizing RNA degradation through enzymes. Again,

samples were placed at room temperature for 3 minutes. Separation of total RNA from DNA particles and proteins was performed by centrifugation at 12,000 x g at 4 °C for 15 minutes. The upper aqueous phase containing total RNA was extracted and the lower organic phase containing DNA and proteins was removed.

After 1.5 volumes of ethanol (100%) were added, resulting in improved binding conditions of RNA and facilitated adhesion to the silica-membrane, 700 µl of RV tissue sample was pipetted into a RNeasy Mini spin column for RNA binding.

Thereafter, samples were centrifuged at 8,000 x g at room temperature for 15 seconds, the flow-through was removed and centrifugation was repeated.

Washing procedure was performed by adding 700 µl of RWT Buffer and 500 µl of RPE buffer, centrifugation of the samples at 8,000 x g at room temperature for 15 seconds and removal of the flow-through each time. In order to dry the spin column membrane, the tubes were again centrifuged at 8,000 x g at room temperature for 2 minutes, after adding 500 µl RPE buffer. Centrifugation at maximum speed for 1 minute was performed to ensure RNeasy Mini columns without residual RPE Buffer and excessive flow-through.

RNA elution was conducted by transferring the spin columns in a sterile tube, pipetting 30-50 µl of RNase free water directly onto the silica-membrane and centrifugation at 8,000 x g at room temperature for 1 minute.

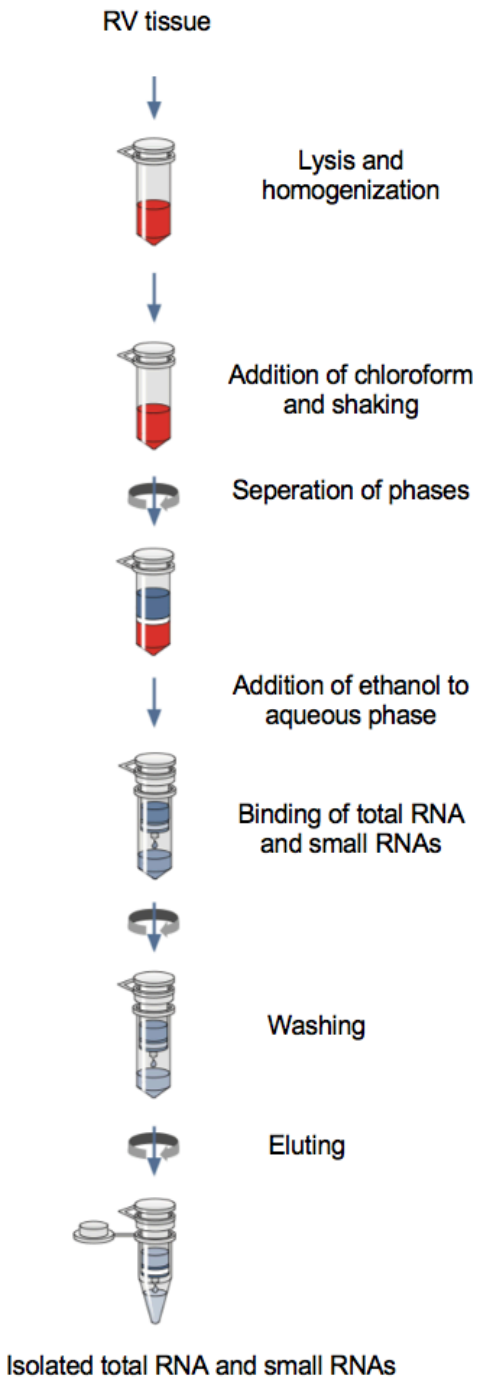


**Figure 8:** Procedure of total RNA isolation of RV tissue samples. Modified from (Qiagen, 2014). RV, right ventricular; RNA, ribonucleic acid.

### 3.3.3 RNA quantity and quality assessment

RNA quantity was assessed by measuring the concentration of isolated total RNA, using NanoDrop 2000 spectrophometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

RNA purity was screened by examining the 260/280 absorbance ratio, according to the manufacturer's recommendation (Thermo Fisher, Scientific, Waltham Massachusetts, Unites States). This ratio is an essential indicator for RNA purity, whereby eligible values should be between 1.8 and 2.1. Only samples with respective values were accepted for further analysis. Performing automated capillary electrophoresis with Agilent 2100 Bioanalyzer (Agilent, Santa Clara, United States) and RNA 6000 Nano Kit, is the recommended standard procedure for RNA quality assessment by estimating RNA integrity number. RNA integrity number values represent a measurement of available RNA quality and range from 1 to 10, symbolizing almost completely RNA degradation (value 1) or virtually completely intact RNA (value 10). According to experts, results of gene expression analysis are unreliable for samples with an RNA integrity number of less than 5. Therefore, only samples with higher RNA integrity numbers should be used for further experimental processing. For this reason, samples reaching an RNA integrity number value above 6.3 were selected for gene expression analysis. 10 out of 13 samples even reached an RNA integrity number above 8.

### 3.3.4 RNA sequencing

Total RNA sequencing is a common method to investigate complex transcriptome profiles in human beings. The determination of the nucleotide sequence of the RNA facilitates a snapshot of total RNA at a certain cellular timepoint, uncovering gene expression modification customized to various cell conditions. Total RNA sequencing of RV tissue samples was performed as previously described, encompassing Illumina's sequencing by synthesis, a worldwide adopted next generation sequencing technology (Haack et al., 2013) (Quail et al., 2012). Figure 9 summarizes the performed workflow of total RNA sequencing.

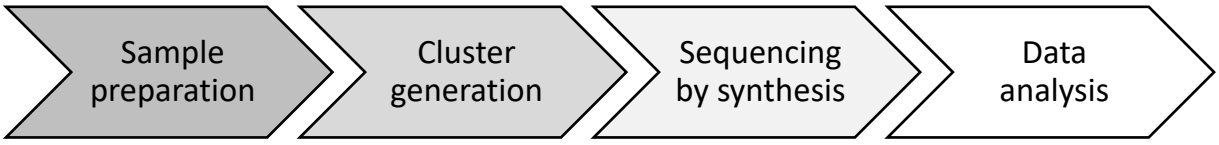


**Figure 9:** Workflow of total RNA Sequencing.

*3.3.4.1 Sample preparation*

Prior to actual sequencing, tissue samples have to be prepared by converting the purified RNA into complimentary deoxyribonucleic acid (cDNA), followed by cDNA amplification to enrich deoxyribonucleic acid (DNA) fragments for DNA library creation. Thus, the cDNA is used for cluster generation and sequencing.

Preparation of DNA library of RV tissue samples was performed by using 1 µg of RNA adhered to Illumina's information (Illumina, San Diego, California United Stated). Sample processing was performed as previously described, complying the manufacturer's recommendation (illumina, 2017).

In brief, ribosomal RNA was depleted and removed from total RNA, using 5 µl of ribosomal RNA binding buffer, 5 µl of ribosomal RNA Removal Mix and 35 µl of ribosomal RNA Removal Beads. The remaining RNA was purified with 99 µl of RNAClean XP Beads and washed with 200 µl of ethanol (70%). 11 µl of Elution Buffer and 8.5 µl of Elute, Prime and Fragment High Mix were added, and Elution 2-Frag-Prime program on the thermal cycler was started to fragment and prime RNA for cDNA synthesis.

In order to synthesize the first strand cDNA with reverse transcriptase, First Strand Synthesis Act D Mix was mixed with 50 µl of SuperScript II. 8 µl of First Strand Synthesis Act D Mix and SuperScript II mixture were added to each sample and Synthesize First Strand program on the thermal cycler was commenced. The improvement of strand specificity was warranted by actinomycin D, which avoids spurious DNA-dependent synthesis and provokes RNA-dependent synthesis.

5 µl of diluted CTE (End Repair Control), 5 µl of Resuspension Buffer and 20 µl of Second Strand Marking Master Mix were added to each well, centrifugated at 280 x g for 1 minute and incubated at 16°C on the thermal cycler for 1 hour. Thereby, RNA templates were removed, and the second cDNA strand was produced. To ensure strand specificity, dUTP (deoxyuridine triphosphate) nucleotides were used to generate the second cDNA strand, instead of dTTP (deoxythymidine triphosphate) nucleotides, which were used to generate the first cDNA strand. The incorporation of dUTP nucleotides enables second cDNA strand elimination during amplification. For the separation of double-stranded cDNA from the reaction mix and cDNA purification,

90 µl of AMPure XP Beads were added and washing procedure with 200 µl of fresh ethanol (80%) was performed. Finally, 17.5 µl of Resuspension Buffer was added.

2.5 ml diluted CTA (A-Tailing Control) and 12.5 µl A-Tailing Mix were added to each sample in order to attach the nucleotide adenine to the 3´end of the blunt cDNA fragments, preventing ligation to each other. Adenylation of the 3´end of the blunt cDNA fragments is important for proper adapter ligation. Consequently, the adapter ligates solely to the 5´end of the blunt cDNA fragments by providing a complementary overhang of a single thymine nucleotide on the 3´end of the adapter.

All samples were placed on the thermal cycler and the ATAIL70 program was run. Adapter ligation was executed by adding 2.5 µl of diluted CTL (Ligation Control), 2.5 µl Ligation Mix and 2.5 µl RNA adapters, and running of the ligation program on the thermal cycler. The ligation of the adapters is a fundamental preparation step for the hybridization of the cDNA fragments onto a flow cell for sequencing. To terminate adapter ligation, 5 µl of Stop Ligation Buffer was added to the samples. Two washing processes were carried out with in total 92 µl of AMPure XP beads, 400 µl ethanol (80%) and 75 µl Resuspension Buffer. The bundling of purified cDNA fragments with the attached adapters is called a DNA library.

Enrichment of cDNA fragments was performed with Polymerase Chain Reaction (PCR), enriching the fragment with adapters on both ends and amplifying the amount of cDNA in the library. The enzyme polymerase used in the procedure can only synthesize the complementary strand of the first cDNA strand because of the inability to read dUTP which is contained in the second strand. As a consequence, the second cDNA strand is effectively quenched during the amplification. Using 5 µl PCR Primer Cocktail and 25 µl Master Mix for each sample, the PCR program, including 15 cycles of 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds, was applied. Subsequently, the amplified cDNA was cleaned with 50 µl of AMPure XP beads on magnetic stand and two times washing process using 200 µl ethanol (80%) and 32.5 µl Resuspension Buffer. This procedure results in enriched cDNA fragments of forward and reverse strands of the first cDNA strand with adapters on both ends.

Quality and quantity of the final cDNA library were identified, using quantitative PCR and Agilent 2100 Bioanalyzer as well as Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The identification

of library quality provides information about the purity of the sample. Quantification of cDNA library is important to optimize the cluster density on the flow cell for RNA sequencing.

Normalization and pooling of cDNA libraries are necessary to prepare the cDNA fragments for cluster generation an RNA sequencing. When normalizing the measured concentrations of each cDNA library, the libraries with different concentrations are diluted to the same concentration of 10 nM. For this purpose, 10 µl of each cDNA library was added to the required amount of Tris-HCl 10 mM pH 8.5 and 0.1% Tween 20. Afterwards 10 µl of each normalized cDNA library was pooled in a single well of the provided plate. Pooling is the process of combining cDNA libraries of multiple samples for simultaneous sequencing in a single run. In order to assign the individual sequenced cDNA fragments to the corresponding sample, the cDNA fragments of a library were marked with specific indices. The unique indices are short sequences contained in the ligated adapters.

#### *3.3.4.2 Cluster generation and sequencing by synthesis*

Cluster generation and RNA Sequencing of 100 base pair paired-end runs were performed by the Illumina HiSeq 4000 platform (Illumina, San Diego, California, United States). First, the normalized and pooled libraries were loaded onto a flow cell for cluster generation. During this procedure, the clusters of cDNA fragments were amplified by bridge amplification. For this purpose, two different types of oligonucleotides are located on the surface of the flow cell, with one type of them being complementary to the adapter region of one of the cDNA fragments. This leads to hybridization of the adapter region of the cDNA fragments with the oligonucleotides on the flow cell. The complementary strand of the hybridized cDNA fragment is created by a polymerase. After cleavage of the double-stranded fragments, the first template is removed. With the binding of the appropriate adapter region to the second oligonucleotide, the second template forms a bridge with the second oligonucleotide on the flow cell, facilitating bridge amplification. After again creating a complementary strand, the double stranded bridge is denatured, establishing two single stranded fragments sticking on the flow cell. The process of bridge amplification is multiply repeated, simultaneously for all different clusters, leading to clonal amplification of all cDNA fragments. After removal of reverse strands, sequencing of the remaining forward strands was initiated.

Sequencing by synthesis was done by incorporating a single fluorescently labeled nucleotide to the cDNA fragment through natural complementarity during each sequencing cycle. Subsequently, the single incorporated nucleotide was activated by a laser and the emitted fluorescence from each cluster was recorded. The intensity of the emitted fluorescence and the specific wavelength were used to identify the incorporated nucleotide. The sequencing cycle was repeated until the last complimentary nucleotide of the cDNA fragment was incorporated and detected. Consequently, the first reads, derived from the forward strands, were produced. For sequencing the reverse cDNA fragments, the first reads were removed, and the remaining cDNA fragments again bound to the second oligonucleotides on the flow cell for bridging. After the reverse strand of the cDNA fragments was produced by a polymerase, both strands were cleaved, and the original forward strand was washed off. Now, the second reads, derived from the reverse strands, were generated through sequencing by synthesis. This procedure is called paired-end sequencing, since both, the forward DNA strand and the reverse DNA strand were synthesized.

#### *3.3.4.3 Data analysis*

To identify sequencing differences, data were aligned and compared to the reference genome human genome assembly hg19 (GRCh37). Additionally University of California Santa Cruz (UCSC) known gene annotation was done by using STAR aligner (v2.4.2a) (Dobin et al., 2013). Quantification of the number of reads mapping to annotated genes was accomplished by using HT-seq count (v0.6.0), in which Fragments Per Kilobase of transcript per Million fragments mapped (FPKM), was selected as the unity of measurement (Anders, Pyl, & Huber, 2015). By applying R Bioconductor package DESeq2, RNA sequencing data were analyzed and tested for differentially expressed genes (Love, Huber, & Anders, 2014). Pathway and Gene Set Enrichment Analysis with R Bioconductor package gage (Luo, Friedman, Shedden, Hankenson, & Woolf, 2009), pathview (Luo & Brouwer, 2013) and goseq (Young, Wakefield, Smyth, & Oshlack, 2010) were used to further interpret RNA sequencing data. Significant genes and pathways were validated by applying tools like Gene Ontology (GO) (Department of Genetics, Stanford University School of Medicine, Stanford, California, United States) (Ashburner et al., 2000) (The Gene Ontology, 2019), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Institute for Chemical Research, Kyoto University, Uji, Kyoto, Japan) (Kanehisa & Goto, 2000), ToppFun (Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center,

Cincinnati, Ohio, United States) (Chen, Bardes, Aronow, & Jegga, 2009) and PANTHER Classification System (Mi, Muruganujan, Ebert, Huang, & Thomas, 2019).

With the help of the National Center for Biotechnology Information Gene Expression Omnibus (GEO) DataSets (GSE50704), genes switching in their regulation during early childhood development, were identified and excluded in the evaluation of results (Omnibus, 2013). For this purpose, GEO2R was applied in order to compare individually selected transcriptome profiles from two fetal ventricular cardiomyocytes with two adult ventricular cardiomyocytes. The results of differentially expressed genes were compared with our findings, excluding all genes switching during early childhood development.

### 3.3.5 Western blotting

Western blotting, as a common technique to identify proteins of differentially expressed genes in certain tissue samples, was used to validate RNA sequencing results. Western blots were performed as previously described (Kracun, Riess, Kanchev, Gawaz, & Gorlach, 2014) (Chalupsky, Kracun, Kanchev, Bertram, & Gorlach, 2015).

First, cell lysis buffer (Cell Signaling) was substituted with Complete Mini$^{TM}$ protease inhibitor (Roche) and $Na_3VO_4$ and added to RV tissue samples for tissue lysis and protein isolation. For tissue homogenization, RV tissue samples were sonified. To prevent protein degradation, all samples were stored on ice for 20 minutes. By centrifugation at 4°C and 13,000 rpm for 5 minutes, cell debris was removed. Laemmli buffer (187 mM Tris(hydroxymethyl)aminomethane (Tris) pH 6.8, 6% sodium dodecyl sulfate (SDS), 30% glycerol, 0.06% bromphenol blue, 15 mM dithiothreitol, 60 mM ethylenediamine tetraacetic acid (EDTA) and 4.325 ml $H_2O$), which promotes protein isolation, was added and samples were again incubated at 95°C and 500 rpm for 5 minutes. Samples were then centrifuged at 10,000 rpm for 1 minute. Protein concentration was determined by the photometric method according to Bradford (Bradford, 1976). In this method, isolated proteins bind to Commassie Brilliant Blue G-250 dye in an acidic environment, resulting in a spectral shift. More precisely, the unbounded brown form of the dye can be measured at the absorbance maximum of 465 nm and the bounded blue form of the dye at the absorbance maximum of 610 nm. Consequently, the formation of protein-dye-complexes lead to a spectral shift with a novel absorption maximum. The optimal wavelength for detecting protein-dye-complexes is at 595 nm. Thus, quantification of proteins can be determined (scientific, 2017).

Protein separation was performed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), facilitating protein fractionation, depending on protein size and molecular weight. Mini-Protean 3 system (Biorad) was loaded with running gel ($H_2O$, 30% acrylamide, 1 M Tris-HCl, 10% SDS, 80 µl ammoniumpersulfate (APS), 10 µl tetramethylethylendiamine (TEMED)), stacking gel ($H_2O$, 30% acrylamide, 1 M Tris-HCl, 10% SDS, 15 µl APS, 3 µl TEMED), running buffer (25 mM Tris, 200 mM glycine, 0.5% SDS), 5 µl of protein marker and 7 µl of each sample for SDS-PAGE. Thereby, SDS causes protein denaturation and a balanced distribution of negative charges due to the attachment of SDS sulfate groups to the proteins. As a result, protein separation solely depends on protein size and molecular weight, unaffected by

different protein charges. Voltages of 100 V for 30 minutes and then 150 V for 30 minutes were applied for protein migration, generating an electrical gradient on the electric field. Small and light molecules migrate faster, while large and heavy molecules are rather held back by the gel, resulting in a characteristic pattern of different protein bands.

For protein visualization, separated proteins were transferred to a labeled, protein binding nitrocellulose membrane by using Whatman filter paper inserted into transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol). Wet Whatman filter paper framed the nitrocellulose membrane and the gel panel, forming a sandwich layer structure. Voltage of 100 V was applied for 1 hour, leading to electrochemical protein transfer, preserving the characteristic pattern of separated proteins. Methanol contained in the transfer buffer washes out the SDS of the SDS-protein-complexes, allowing the proteins to renature and partially regain their original structure. To visualize the protein bands, Ponceau S was added for 2 minutes to the nitrocellulose membranes. After a washing procedure with running buffer, all membranes were scanned.

Before separated proteins were identified by primary and secondary antibodies, free binding sites of the nitrocellulose membrane were saturated with milk. Milk acts as a blocking buffer, blocking the non-specific interaction between the membrane surface and the antibodies by binding to the remaining sites on the nitrocellulose membrane, since milk has the property of having no binding affinity for the antibodies (scientific, 2014). Hence, unspecific binding of primary antibodies to the nitrocellulose membrane was reduced and antigen-specific detection of separated proteins was possible. Protein blocking was performed with 5% milk diluted with Tris Buffered Saline (TBS) (50 mM Tris, 150 mM sodium chloride, 0.3% HCl) and 0.3% Tween.

Probing of the nitrocellulose membranes with the primary antigen-specific antibody was done by diluting the antibody in 5% milk, TBS and Tween and incubating it with the blot on the shaker at 4°C overnight for about 16 hours. Primary antibody against alcohol dehydrogenase 1B (ADH1B, Abcam), monoamine oxidase B (MAOB, Merck), peroxisome proliferator-activated receptor γ (PPAR γ, Thermo Fischer), superoxide dismutase 3 (SOD3, Thermo Fischer), interleukin 6 receptor (IL6R, R&D Systems) were used. For verification purposes, β actin controls (Santa Cruz) were performed. β actin is a so-called "housekeeping" protein, as it is continuously and stably expressed in high amounts in the tissue under investigation. Therefore, it is frequently used as a

positive control for equal protein loading in the lanes. In the absence of loading variance, the different expression levels of the proteins of interest in the different samples can be compared and interpreted (scientific, 2014). Three times washing procedures with TBS and Tween were carried out, removing weak and unspecific bounded antibodies.

Goat anti-rabbit, goat anti-mouse or rabbit anti-goat secondary enzyme-conjugated antibodies were added, incubated at room temperature with the blot for 2 hours, followed by washing procedure repetition. In this process, the secondary antibodies bind to specific areas of the primary antibody, facilitating the following protein detection.

Protein detection and protein band visualization is based on an enzymatic reaction leading to the emergence of a light emission at the location of the protein of interest (scientific, 2021). Hereby, the enzyme horseradish peroxidase, which is conjugated to the secondary antibody, catalyzes the conversion of the chemiluminescent substrate luminol to an oxidized luminol molecule. The emitted light signal was detected on a photographic film in the darkroom. To generate the enzymatic reaction the enhanced chemiluminescence (ECL) solution which contains the appropriate substrate was added to the nitrocellulose membrane. For this purpose, the ECL 1 (100 mM Tris pH 8.8, 2.5 mM luminol, 0.4 mM coumaric acid, destilled $H_2O$) and ECL 2 (100 mM Tris pH 8.8, 0.15% hydrogen peroxide, destilled $H_2O$) solutions were first prepared and mixed in a 1:1 ratio. The ECL mixture was then evenly distributed on the membrane surface and incubated for 1 to 2 minutes. Before the membrane was placed next to the photographic film in the translucent cassette for exposure, it was inserted between two pieces of transparent paper and small air bubbles were removed with a roller. Exposure of the membranes in the cassette results in autoradiography and thus visualization of protein bands. The exposure time was individually determined for each antibody. The membrane was swirled in the developer solution until the first bands became visible and was then placed in the fixing solution to fix the protein bands. The developed films were scanned, and quantity assessment was performed, using the image-editing software program Image J (National Institute of Health, Rockville Pike, Maryland, United States). The different optical densities of the individual protein bands were determined, which thus serves as a measure of the strength of the protein expression. Using the software package Prism 5.01 (GraphPad Software, San Diego, California,

United States), statistical evaluation was implemented by using two-tailored student's test.

### 3.3.6 Statistics

For statistics, R Bioconductor package DEseq2 was applied to transcriptome profiles and was tested for differentially expressed genes in adults compared to infants. The Benjamini and Hochberg procedure was used to correct p-values for the purpose of multiple testing. Based on a significance level of p-value < 0.01 and fold change value > 2 or < -2, differentially expressed genes were selected for further analysis. An adjusted p-value < 0.01 for results of GO analysis and a p-value < 0.05 for pathway analysis were assigned as significant.

## 3.4 Research methods study II

### 3.4.1 Molecular genetic testing

Molecular genetic testing has become a beneficial method in HCM diagnostics, enabling the identification of causative genes in approximately 60% of patients fulfilling HCM diagnostic criteria (Richard et al., 2003). Not only for pre-symptomatic diagnosis in relatives of patients with familial HCM molecular genetic testing is indicated, it is also a powerful tool to confirm suspected diagnosis in individuals with typical phenotype (Monserrat, 2018).

Following the recommendations of European and North American guidelines, 1 ml of EDTA blood samples were obtained from all patients (Authors/Task Force et al., 2014) (Ackerman et al., 2011) (Charron et al., 2010) (Marian & Braunwald, 2017). For implementation of molecular genetic testing, blood samples were sent to certified laboratories. Either amplification with PCR followed by Sanger sequencing or next generation sequencing as sequencing by synthesis or whole exome analysis was performed to identify genetic variants. In case of single gene analysis, the *MYH7* or *TNNT2* genes were sequenced. Panel diagnostic included gene sequencing of *MYH7* and *MYBPC3* or *MYH7* and *TNNT2*. Extended panel diagnostic comprised sequencing of actin $\alpha$ cardiac muscle 1 *(ACTC1),* actinin $\alpha$ 2 *(ACTN2),* ankyrin repeat domain 1 *(ANKRD1),* cysteine and glycine rich protein 3 *(CSRP3),* junctophilin 2 *(JPH2), MYBPC3, MYH7, MYL2,* myosin light chain 3 *(MYL3),* phospholamban *(PLN*), protein kinase AMP-activated non-catalytic subunit $\gamma$ 2 *(PRKAG2),* titin cap *(TCAP),* troponin C1, slow skeletal and cardiac type *(TNNC1), TNNI3, TNNT2* and *TPM1* gene.

Results of molecular genetic testing were interpreted pursuant to the classification system and guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards et al., 2015) (Amendola et al., 2016). Based on literature research information, frequency of variants in large populations, computational (in silico) analysis as well as disease and sequence databases, variants were classified for pathogenicity. Accordingly, genetic variants were ranked in the categories pathogenic, likely pathogenic, uncertain significance, likely benign and benign. In this study, only patients carrying pathogenic and / or likely pathogenic variants were defined as genotype positive. Additionally, a re-analysis with ClinVar, hosted by the National Center for Biotechnology Information and founded by the National Institutes of Health, was performed (Landrum et al., 2018).

### 3.4.2 Data acquisition and analysis

To assess the presence of pathological LV remodeling, acquisition and analysis of demographic, clinical, electrocardiographic, long-term ECG, echocardiographic, cardiorespiratory exercise testing, CMR, surgical and medication data were performed retrospectively. Data were collected at the time of first presentation at the outpatient clinic of the German Heart Centre Munich and at the time of latest follow-up.

Besides the assessment of clinical functional status by the New York Heart Association (NYHA) class or modified age-adjusted Ross classification (Ross, 2012), criteria for LV remodeling and disease phenotype, to evaluate genotype-phenotype association and disease progression, were determined (Table 2) (Wolf, 2019). Parameters of the latest follow-up were utilized to assess differences in disease phenotype. For the analysis of disease progression, the delta of respective parameters was calculated. CMR data were excluded for evaluation of disease progression since CMR examination was performed only once.

Medical records which were stored in the archive of the German Heart Centre Munich, electronically saved patient files from “Stationsarbeitsplatz” (clinic information system) and information documented in the institutional File maker (software for data acquisition) were used to obtain patient related data.

For offline analysis of lacking echocardiographic data, the Echopac Software (General Electric, Vingmed, Horten, Norway) was applied.

**Table 2:** Criteria for LV remodeling and disease phenotype in patients with pediatric onset HCM.

| Criteria for left ventricular remodeling and disease phenotype | |
|---|---|
| Morphology | • End diastolic volume index (CMR[A])<br>• End systolic volume index (CMR)<br>• Left ventricular outflow tract obstruction (TTE[B]) |
| Hypertrophy | • Myocardial mass (CMR)<br>• End-diastolic inter-ventricular septal z-score (TTE)<br>• Left ventricular posterior wall diameter z-score (TTE) |
| Fibrosis | • Late gadolinium enhancement (CMR)<br>• Localization of late gadolinium enhancement (CMR)<br>• Late gadolinium enhancement, mean (CMR)<br>• Extracellular volume fraction, total mean (CMR)<br>• Extracellular volume fraction, septal mean (CMR) |
| Systolic function | • Ejection fraction (TTE and CMR)<br>• Stroke volume index (CMR) |

| | |
|---|---|
| | • Global longitudinal strain, average (TTE)<br>• Global longitudinal strain, minimum-maximum (TTE)<br>• Global longitudinal strain, minimum (TTE)<br>• Global longitudinal strain, maximum (TTE)<br>• Global longitudinal strain, septal basal (TTE)<br>• Global longitudinal strain, septal middle (TTE)<br>• Global longitudinal strain, septal apex (TTE)<br>• Global longitudinal strain, lateral basal (TTE)<br>• Global longitudinal strain, lateral middle (TTE)<br>• Global longitudinal strain, lateral apex (TTE) |
| Diastolic function | • Mitral valve E/A Ratio (TTE)<br>• Mitral valve E Deceleration (TTE)<br>• Mitral valve E maximum (TTE)<br>• Mitral valve E´ septal (TTE)<br>• Mitral valve E´ lateral<br>• E/E´ septal (TTE)<br>• E/E´ lateral (TTE)<br>• Left atrium diameter (TTE) |
| Arrhythmia[C] | • None<br>• Mild: premature ventricular or supraventricular beats<br>• Severe: non-sustained or sustained ventricular or supraventricular tachycardia or salves<br>• Sudden cardiac death equivalent: reanimation and/or ICD discharge and/or sudden cardiac death |
| Morbidity | • Number of cardiac medications<br>• β-blocker dosage<br>• Need to hospitalization<br>• Need to surgery<br>• ICD[D] implantation for primary or secondary prevention<br>• Appropriate ICD discharge for ventricular fibrillation or ventricular tachycardia |
| Mortality | • Death |

[A], cardiac magnetic resonance imaging; [B], transthoracic echocardiography; [C], arrhythmias on electrocardiogram, long-term electrocardiogram or cardiopulmonary exercise test; [D], implantable cardiac defibrillator.

### 3.4.3 Statistics

For statistical analysis the SPSS software program version 25.0.0 (SPSS Inc., IBM Company, Chicago, IL, USA) was used. Kruskal-Wallis test was performed to calculate differences in disease phenotype and disease progression in multiple defined groups. Differences between subgroups were analyzed by Mann-Whitney U test. Pearson Chi Square test was applied for categorical variables. The estimation of survival probability was performed with Kaplan-Meier calculation. Kaplan-Meier curves were compared by log-rank test. Continuous variables were expressed as median with minimum and maximum. Two sided statistical tests with a p-value of $< 0.05$ were considered significant.

# 4 Publications

## 4.1 Molecular signaling pathways in right ventricular impairment in adult patients after tetralogy of Fallot repair (Pollmann, Raj Murthi, et al., 2021)

Authors: Kathrin Pollmann[a], Sarala Raj Murthi[a], Damir Kračun[a,b], Thomas Schwarzmayr[c], Andreas Petry[a,b], Julie Cleuziou[d,e], Jürgen Hörer[d], Mathieu Klop[a,b], Peter Ewert[a,f], Agnes Görlach[a,b,f], Cordula Maria Wolf[a,f]

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Journal: Cardiovascular Diagnosis and Therapy

Series: Current Management Aspects in Adult Congenital Heart Disease (ACHD): Part IV

Doi 10.21037/cdt-20-894

Summary:

The right ventricle of adults with CHD is subject to increased stress due to anatomical anomalies, surgical lesions and chronic altered hemodynamics, leading to RV remodeling and RVI. Both contribute significantly to morbidity and mortality in this population. To date, knowledge about molecular and cellular fundamentals in RV remodeling in patients with CHD is still limited. Therefore, the purpose of this study was to identify molecular signaling pathways acting part in RV remodeling in adult patients with CHD and RVI.

During surgical procedures RV tissue samples were collected from 6 adults and 7 infants. The underlying CHD was TOF in all patients. Subsequently, RNA isolation and RNA Sequencing were performed. Differentially expressed genes of adults with chronic hemodynamic stressors and relevant signs of RVI were compared with those of infants without sustained RV hemodynamic stressors and no RVI. In contrast to infant patients, medical charts review revealed signs of RVI in all adult patients.

Whole transcriptome sequencing of RV specimen identified altered gene expression, including a total of 3,010 differentially expressed genes in adult patients after TOF repair. Using Gene Ontology and Kyoto Encyclopedia of Genes databases, pathways involved in cellular metabolism, cell-cell-communication, cell cycling, and cellular contractility were highlighted to be dysregulated. Particularly, genes involved in retinol metabolism, PPAR $\gamma$ signaling, extracellular vesicle and exosomes were identified, contributing to RV remodeling.

RNA Sequencing enables identification of altered gene expression profiles and dysregulated molecular signaling pathways in adult patients, facing RVI after TOF repair. Identifying molecular and cellular modifications of RV remodeling is essential for the development of novel therapeutic strategies and the discovery of potential biomarkers. Notably, molecular characterization contributes to the generation of screening assays for risk stratification and optimization of current disease monitoring, improving morbidity and mortality in patients with complex CHD, surviving into adulthood.

Individual Contribution:

The analysis of patient's characteristics by medical chart review was performed by Kathrin Pollmann. Based on the collected patient data she was responsible for selecting the study population and verifying the availability of sufficient RV tissue in the biomaterial bank of the German Heart Center Munich. Kathrin Pollmann organized the RV tissue sample procurement. Experimental sample processing was performed at the Institute of Human Genetics at Helmholtz Center Munich, where she had the chance to observe and partly participate in the sample preparation. After analyzing data of RNA sequencing, Kathrin Pollmann presented the study results at the annual meeting of the Association for European Pediatric and Congenital Cardiology (AEPC) in May 2019 in Sevilla, Spain. She wrote the manuscript and after the co-author's review, she was responsible for submitting the manuscript. Further revisions she composed together with PD Dr. med. Cordula Wolf.

All actions were taken in consultation with the co-authors. Prof. Dr. med. Agnes Görlach and PD Dr. med. Cordula Wolf were responsible for study conception and design. Administrative support was provided by Prof. Dr. med. Peter Ewert. RV tissue samples and medical charts were provisioned by Prof. Dr. med. Jürgen Hörer, PD Dr. med. Julie Cleuziou and Prof. Dr. med. Peter Ewert. Collection and assembly of data were kindly supported by PhD Sarala Raj Murthi, PhD Damir Kračun, PhD Andreas Petry, Mathieu Klop and PhD Thomas Schwarzmayr. Analysis and interpretation of research results were assisted by Sarala Raj Murthi, PhD Damir Kračun, PhD Andreas Petry and PhD Thomas Schwarzmayr. All co-authors approved the manuscript and contributed to the improvement of it. Prof. Dr. med. Agnes Görlach and PD Dr. med. Cordula Wolf supported the doctoral candidate in the preparation of the lecture.

Submission and publication:

The original article was submitted on October 29th, 2020. The revised manuscript was submitted on November 27th, 2020 and accepted for publication on February 26th, 2021.

Original Article on Current Management Aspects in Adult Congenital Heart Disease (ACHD): Part IV

# Molecular signaling pathways in right ventricular impairment of adult patients after tetralogy of Fallot repair

**Kathrin Pollmann[1], Sarala Raj Murthi[1], Damir Kračun[1,2], Thomas Schwarzmayr[3], Andreas Petry[1,2], Julie Cleuziou[4,5], Jürgen Hörer[4], Mathieu Klop[1,2], Peter Ewert[1,6], Agnes Görlach[1,2,6], Cordula Maria Wolf[1,6]**

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**Background:** Right ventricular impairment (RVI) secondary to altered hemodynamics contributes to morbidity and mortality in adult patients after tetralogy of Fallot (TOF) repair. The goal of this study was to describe signaling pathways contributing to right ventricular (RV) remodeling by analyzing over lifetime alterations of RV gene expression in affected patients.

**Methods:** RV tissue was collected at the time of cardiac surgery in 13 patients with a diagnosis of TOF. RNA was isolated and whole transcriptome sequencing was performed. Gene profiles were compared between a group of 6 adults with signs of RVI undergoing right ventricle to pulmonary artery conduit surgery and a group of 7 infants, undergoing TOF correction. Definition of RVI in adult patients was based on clinical symptoms, evidence of RV hypertrophy, dilation, dysfunction or elevated pressure on echocardiographic, cardiovascular magnetic resonance, or catheterization evaluation.

**Results:** Median age was 34 years in RVI patients and 5 months in infants. Based on P adjusted value <0.01, RNA sequencing of RV specimens identified a total of 3,010 differentially expressed genes in adult patients with TOF and RVI as compared to infant patients with TOF. Gene Ontology and Kyoto Encyclopedia of Genes databases highlighted pathways involved in cellular metabolism, cell-cell communication, cell cycling and cellular contractility to be dysregulated in adults with corrected TOF and chronic RVI.

**Conclusions:** RV transcriptome profiling in adult patients with RVI after TOF repair allows identification of signaling pathways, contributing to pathologic RV remodeling and helps in the discovery of biomarkers for disease progression and of new therapeutic targets.

**Keywords:** Right ventricular impairment (RVI); congenital heart disease; transcriptome profiling; molecular signaling pathways

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## Introduction

Due to improvements in diagnostic techniques, interventional procedures and surgical treatment more children with complex congenital heart disease (CCHD) survive into adulthood (1,2). The right ventricle is often stressed by the underlying anatomical abnormalities, surgical lesions, or pulmonary valve defects leading to pressure and/or volume overload (3). Right ventricular impairment (RVI) subsequently occurs in about 26% of CCHD patients (4) and might lead to sudden cardiac death and life-threatening long-term complications. Secondary to altered hemodynamics and myocardial stress, right ventricular (RV) remodeling, such as hypertrophy, fibrosis, arrhythmias, inflammation and dysfunction, occurs (3,5). Activation of fibroblasts, upregulation of inflammatory pathways, increased release of neurohormones and differences in gene regulation of cardiac metabolism, energy production and mitochondrial function occur in the stressed right ventricle (3,6). Additionally, the right ventricle has been suggested to suffer earlier than the left ventricle from oxidative stress in response to hemodynamic stress, which might contribute to the development of pathologic conditions (3). Preventing the transition from compensated- to non-compensated right heart failure (HF) in affected patients by timely interventions, operations, or targeted medical therapy would decrease morbidity and mortality in affected CCHD patients. However, at this point, the severity of pathologic RV remodeling can only inaccurately be predicted by clinical examination and imaging techniques (7). Consequently, the identification of biomarkers to optimize patient treatment and determine the extent of RV myocardial remodeling is of utmost importance. Furthermore, patients currently receive standard left ventricular (LV) failure therapies which are often ineffective in the right ventricle (7,8). A potential reason for this failure is the differences in molecular signaling pathways between the right and the left ventricle which cause diverse adaption strategies to pathologic conditions (6,7). To date, data referring to molecular pathways contributing to RV remodeling and RVI in patients with CCHD are scarce. The aim of this study was to identify differential gene expression over time in affected patients and to define molecular signaling pathways which might play a pivotal role in the development of RVI in patients with CCHD.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/cdt-20-894).

## Methods

The study protocol is depicted in *Figure 1*. Briefly, RV tissue was obtained from patients with a primary diagnosis of tetralogy of Fallot (TOF) at the time of surgical treatment after informed consent. Surgeries included corrective repair for infants or elective replacement of right ventricle to pulmonary artery (RV-PA) conduit for adult patients. Clinical, laboratory, electrocardiogram and imaging data were analyzed by medical chart review. The criteria for RVI were selected based on the International Right Heart Foundation Working Group recommendations (9) and the scientific statement of the American Heart Association (10). Patients with syndromes and any other organ failure were excluded. Total RNA was isolated, RNA quantity was measured, and RNA quality was assessed. Whole transcriptome analysis was performed by total RNA sequencing of cardiac tissue samples as previously described (11). RNA library was prepared, quality and quantity of the RNA library were estimated, and RNA sequencing of 100 bp paired-end runs was performed with Illumina HiSeq 4000 platform (Illumina, San Diego, California, United States). The number of reads mapping to annotated genes was quantified after alignment and differential gene expression between groups was performed. Pathway and Gene Set Enrichment Analysis, which are common approaches to interpret gene expression data based on functional annotation, were performed. Bioinformatical tools used to validate pathway analysis, biological activity and allocation of individual genes to Gene Ontology categories are depicted in *Figure 2* and selected suitable genes are shown in Figure S2.

The total of 3,010 differentially expressed genes were compared to 50 most significant genes, regulating cardiac development and heart maturation, which were identified by RNA sequencing of murine cells during embryonic and postnatal period. Overlapping genes were not included in further interpretation of results (12) (Figure S1 and Table S9). Western blot was performed as previously described (11) for selected proteins isolated from RV myocardial tissue of affected patients.

Statistic evaluation was implemented by applying R Bioconductor package DEseq2 to transcriptome profiles and tested for differential gene expression between adult and infant patients. The P values were corrected for the purpose of multiple testing by Benjamini and Hochberg procedure. The level of significance was set at a P value of less than 0.01 and a fold-change value of greater than 2 or less than –2. Based on that, all significant differentially

 

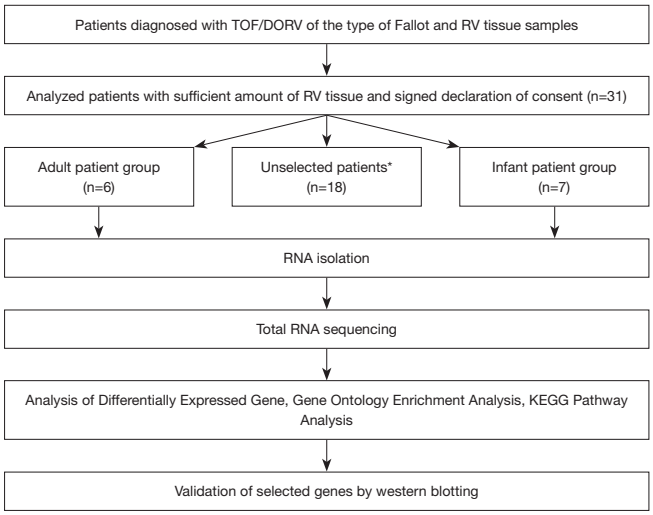


**Figure 1** Flow chart of the performed process of the study design. Patient enrollment with underlying anatomical diagnosis, group classification and process of sample preparation with analysis of results. *, unselected patients due to genetic disorders, lacking clear clinical characterization and insufficient signs of RV impairment. DORV, double outlet right ventricle; KEGG, Kyoto Encyclopedia of Genes and Genomes; RNA, ribonucleic acid; RV, right ventricular; TOF, tetralogy of Fallot.

expressed genes were selected for further analysis. The results of Gene Ontology (GO) and Pathway analysis with an adjusted P value less than 0.01 (GO) and a P value less than 0.05 (KEGG) were assigned as significant.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics committee (approval 10/16/2017, number 242/17S, and approval 01/11/2017, number 592,16S) and individual consent for this retrospective analysis was taken from all patients.

Please see Supplementary file (Appendix 1) for detailed description of Material and Methods.

## Results

Myocardial tissue samples were collected from a group of 6 adult patients with signs of chronic RVI undergoing RV-PA conduit surgery ("cases") and from a group of 7 infant patients undergoing elective TOF correction ("controls"). Patients in the "case" and the "control" group were in median 34 and 0.5 years, respectively, old at the time of sample collection (range, 29 to 62 years and 4.8 to 5.8 months, respectively). Follow-up time since corrective surgery was 34 years in median (range, 27 to 50 years) in adult patients with CCHD and RVI. Underlying structural heart disease was TOF in 12 patients and double outlet right ventricle (DORV) of the type Fallot in 1 patient (*Tables 1,2*). Clinical, laboratory, cardiopulmonary exercise testing and imaging parameters of adult patients are depicted in *Table 3* (13,14). All patients of the case group had progressive exercise intolerance and were in New York Heart Association functional class II and higher. Most patients showed engorgement of their jugular veins or enlarged liver on physical examination. Three of 6 patients showed severe stenosis of the pulmonary valve and 3 of 6 patients showed severe pulmonary regurgitation. There was RV hypertrophy and enlargement in all patients on transthoracic echocardiography and/or cardiovascular magnetic resonance tomography (CMR). Exemplary imaging is depicted in *Figure 3*.

The statistical evaluation with Principal Component Analysis visualized well separated samples, demonstrating difference in the compared groups and homogeneity (*Figure 4*). Transcriptome profiling of RV specimens identified in total

 

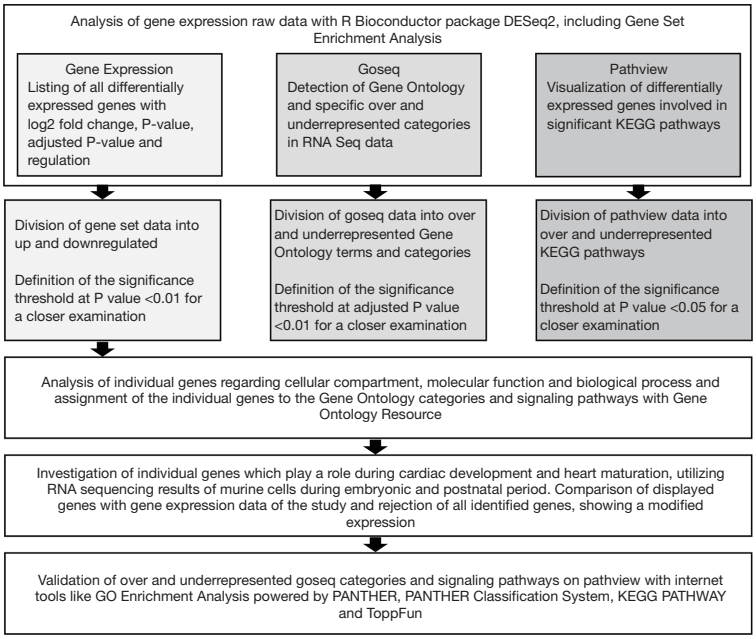


**Figure 2** Bioinformatical analysis of whole transcriptome differential expression data. Bioinformatical analysis performed to evaluate significant biological pathways derived from whole transcriptome differential gene expression data. GO, Gene Ontology; goseq, gene ontology for RNA Sequencing; KEGG, Kyoto Encyclopedia of Genes and Genomes; PANTHER, Protein Analysis Through Evolutionary Relationships; RNA, ribonucleic acid; RNA Seq, RNA Sequencing.

**Table 1** Patients characteristics of the adult patient group

| Adult patients | Identification number | | | | | |
|---|---|---|---|---|---|---|
| | 111087 | 111088 | 111089 | 111091 | 111092 | 111094 |
| Age (years) | 31 | 43 | 29 | 37 | 61 | 31 |
| Sex (M/F) | M | F | F | F | F | F |
| Height (cm) | 172 | 174 | 165 | 156 | 162 | 150 |
| Weight (kg) | 66.3 | 75.9 | 63.1 | 68.5 | 73.1 | 44.8 |
| BMI (kg/m$^2$) | 22.3 | 25.1 | 23.1 | 28.4 | 27.8 | 20.0 |
| Anatomical diagnosis | DORV | TOF | TOF | TOF | TOF | TOF |
| Type of surgery | Allograft implantation | RVOTO resection | Conduit change | Conduit change | RVOTO resection, conduit change | Conduit change |
| Age at corrective repair (years) | 4 | 3.5 | 0.4 | 4 | 11 | 5 |

BMI, body mass index; DORV, double outlet right ventricle; RVOTO, right ventricular outflow tract obstruction; TOF, tetralogy of Fallot.

**Table 2** Patients characteristics of the infant patient group

| Infant patients | Identification number | | | | | | |
|---|---|---|---|---|---|---|---|
| | 111093 | 111095 | 111096 | 111097 | 111099 | 111100 | 111198 |
| Age (months) | 5 | 5 | 5 | 5 | 5 | 5 | 4 |
| Sex (M/F) | M | M | F | M | M | M | M |
| Height (cm) | 64 | 62 | 63 | 62.5 | 63 | 68 | 63 |
| Weight (g) | 7,605 | 5,950 | 5,960 | 7,405 | 5,900 | 6,960 | 5,700 |
| Anatomical diagnosis | TOF | TOF | TOF | TOF | TOF | TOF | TOF |
| Type of surgery | Corrective repair | Corrective repair | Corrective repair | Corrective repair | Corrective Repair | Corrective repair | Corrective repair |

TOF, tetralogy of Fallot.

**Table 3** Clinical parameters, cardiorenal and cardiohepatic serum markers, electrocardiogram as well as imaging parameters and preoperative medication

| Adult patients | Patient identification number | | | | | |
|---|---|---|---|---|---|---|
| | 111087 | 111088 | 111089 | 111091 | 111092 | 111094 |
| Clinical parameters | | | | | | |
| Functional capacity (NYHA classification) | II | III | II | III | IV | III |
| Peripheral edema | 0 | 0 | 0 | 0 | + | 0 |
| Engorgement of jugular veins | 0 | + | + | + | 0 | 0 |
| Enlargement of liver | 0 | 0 | + | + | + | 0 |
| Dyspnea | 0 | + | + | + | + | + |
| Cyanosis | 0 | 0 | 0 | 0 | 0 | 0 |
| Cardiorenal serum markers | | | | | | |
| GFR (mL/min) | – | 69 | 120 | 106 | 44 | 118 |
| Creatinine (mg/dL) | 1.60 | 1.00 | 0.67 | 0.66 | 1.24 | 0.61 |
| Blood urine nitrogen (mg/dL) | 85.5 | 31.0 | 20.7 | 33.9 | 68.4 | 27.9 |
| Cardiohepatic serum markers | | | | | | |
| Bilirubin (mg/dL) | 0.591 | 1.25 | 0.430 | 0.25 | 2.49 | 0.33 |
| γ-GT (U/L) | 53.6 | 21.6 | 57.5 | 24.7 | 480 | 38.4 |
| AP (U/L) | 38.2 | 56.7 | 84.4 | 46.0 | 128 | 56.5 |
| Electrocardiogram | | | | | | |
| Right-axis deviation | 0 | + | + | 0 | 0 | 0 |
| Incomplete/complete right bundle branch block | + | + | + | + | + | + |
| Length of QRS interval (ms) | 200 | 100 | 110 | 100 | 120 | 120 |
| Abnormal repolarization | + | + | + | 0 | + | + |
| Atrial/ventricular tachyarrhythmias | +[a] | 0 | 0 | +[b] | +[c] | 0 |
| Implantable cardioverter-defibrillator implantation | 0 | 0 | 0 | 0 | +[d] | 0 |

**Table 3** (*continued*)

 

**Table 3** (*continued*)

| Adult patients | Patient identification number | | | | | |
|---|---|---|---|---|---|---|
| | 111087 | 111088 | 111089 | 111091 | 111092 | 111094 |
| Cardiac magnetic resonance imaging | | | | | | |
| RVEF (%) | 50 | 52 | 67 | 57 | – | 52 |
| LVEF (%) | 51 | 58 | 59 | 68 | – | 60 |
| Pulmonary regurgitation fraction (%)[A] | 52 | 10 | 21 | 11 | – | – |
| RVSV (mL) | 121 | 87 | 92 | 48 | – | 58 |
| RVEDVI (mL/m$^2$) | 138 | 89 | 84 | 51 | – | 80 |
| Increased RVEDV[B] | + | + | + | 0 | – | + |
| RVESVI (mL/m$^2$) | 68 | 42 | 28 | 22 | – | 39 |
| Increased RVESV[C] | + | 0 | 0 | 0 | – | 0 |
| Echocardiography | | | | | | |
| Decreased RVEF[D] | 0 | 0 | 0 | 0 | + | 0 |
| Pulmonary regurgitation[E] | + | 0 | + | 0 | + | 0 |
| PV Vmax (m/s) | 3.90 | – | 3.48 | 4.53 | 1.94 | 4.05 |
| PV mean PG (mmHg) | – | – | – | 54.52 | 11.24 | – |
| PV max PG (mmHg) | 60.78 | 85 | 48.5 | 82.07 | 15.07 | 65.73 |
| RV hypertrophy | + | + | + | + | + | + |
| Increased RVP[F] | 0 | + | + | – | + | + |
| Preoperative medication | | | | | | |
| Beta-blocker | + | 0 | 0 | 0 | + | 0 |
| Diuretic | + | 0 | 0 | 0 | + | 0 |
| Angiotensin converting enzyme inhibitor | + | 0 | 0 | 0 | 0 | 0 |

0, not present; +, present; –, data not available. The electrocardiogram criteria for right ventricular impairment were taken from the scientific statement of the American Heart Association (10). The imaging reference values used were taken from the Guidelines for the Echocardiographic Assessment of Right Heart in Adults reported from the American Society of Echocardiography (13). The cut of value for moderate to severe pulmonary regurgitation were based on the publication of the Department of Cardiology and the Toronto Congenital Cardiac Care Centre for Adults (14). [a], atrial fibrillation; [b], non-sustained ventricular tachycardia on exercise testing; [c], atrial flutter and non-sustained ventricular tachycardia in invasive electrophysiological examination; [d], primary prophylactic implantable cardioverter-defibrillator with adequate shocks later on; [A], moderate to severe pulmonary regurgitation fraction >20%; [B], right ventricular enddiastolic volume index >80 mL/m$^2$; [C], right ventricular end-systolic volume index >46 mL/m$^2$; [D], RVEF <44%; [E], moderate to severe pulmonary regurgitation; [F], increased RVP: right ventricular pressure >40 mmHg. NYHA, New York Heart Association Class; GFR, glomerular filtration rate; γ-GT, gamma-glutamyl transpeptidase; AP, alkaline phosphatase; RVEF, right ventricular ejection fraction; LVEF, left ventricular ejection fraction; RVSV, right ventricular stroke volume; RVEDVI, right ventricular enddiastolic volume index; RVESVI, right ventricular end-systolic volume index; PV Vmax, maximum velocity over pulmonic valve; PV mean PG, mean pressure gradient over pulmonic valve; PV max PG, maximum pressure gradient over pulmonic valve; RV, right ventricular.

23,398 genes to be differentially expressed in the case group of adult patients with RVI as compared to the control group of infant TOF patients. Thereof, 12,626 genes were upregulated, and 10,772 genes were downregulated. Considering a P adjusted value of less than 0.01 and a fold change value of greater than 2 and less than –2, a total of 3,010 genes were significantly differentially expressed. Of those, 1,703 genes were upregulated, and 1,307 genes were downregulated (*Figure 5*). Hierarchical clustering of the 100 most significantly differentially expressed genes is shown in *Figure 6*.

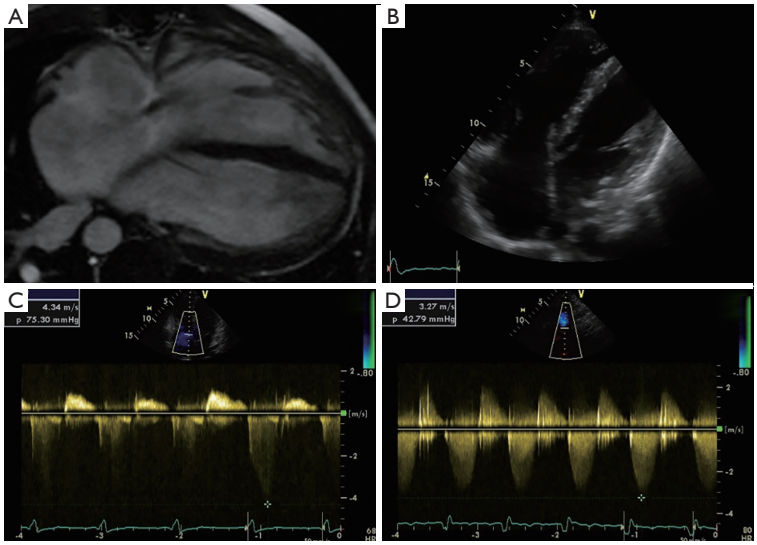


**Figure 3** Exemplary imaging from patient 111087 showing right ventricular enlargement on cardiac magnetic resonance imaging (A) and echocardiography (B) with elevated right ventricular pressure estimated by pressure gradient between the right ventricle and the right atrium on Doppler imaging (C). Echocardiographic continuous wave Doppler imaging illustrates severe stenosis and regurgitation of the right-ventricular-to-pulmonary-artery (RV-PA) allograft (D).

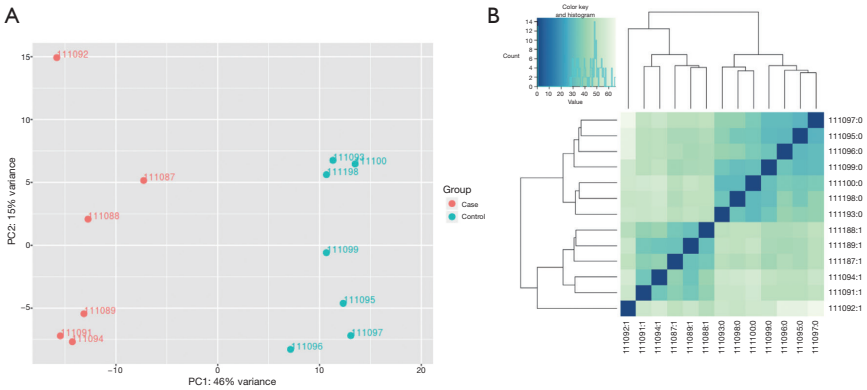


**Figure 4** Principal component analysis of adult TOF patients and RVI secondary to chronic RV volume and/or pressure overload compared to infant TOF patients. The diagram charted both groups as clusters, at which red dots symbolize the adult patients ("cases") and blue dots the infant patients ("controls") (A). The samples are well separated, demonstrating difference in the compared groups (B). Low variance and homogeneity within both clusters is the base for comparability. TOF, tetralogy of Fallot; RVI, right ventricular impairment; RV, right ventricular.

 

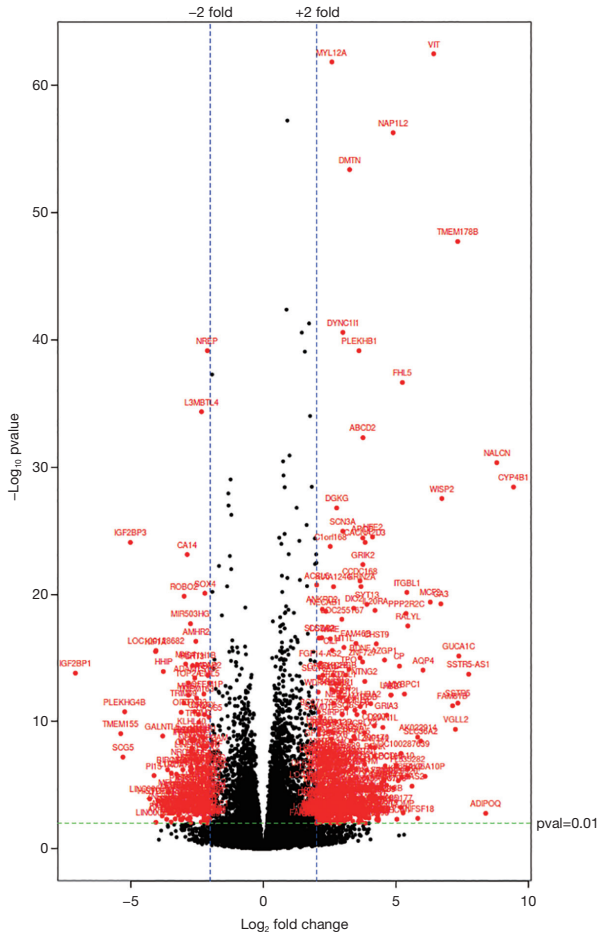


**Figure 5** Volcano Plot visualizes detected significantly dysregulated genes from gene expression analysis. The log 2-fold change is displayed on the x axis and the significance is shown on the y axis as the negative logarithm (log10scale) of the FDR corrected P value. The significance cutoff (P value <0.01) is highlighted with a green dashed line. Dysregulated genes which lie within the defined significant limits are shown in red.

Pathway and gene set enrichment analysis revealed 162 GO terms to be significantly upregulated by the expressed genes. The highly expressed GO terms for cellular components indicated cytoplasmic part and cytoplasm, including contractile fiber as well as contractile fiber part with myofibril, sarcomere, I Band and Z Disc, to be dysregulated in adult patients with CCHD. Additionally, extracellular space, extracellular organelle inclusive of extracellular vesicle and extracellular exosome were highlighted to be involved in dysregulated pathways. Cellular component genes, associated with vesicle, intracellular vesicle, cytoplasmic vesicle, secretory vesicle and secretory granule rank among the most significantly upregulated pathways (Tables S1,S7). Most significant

 

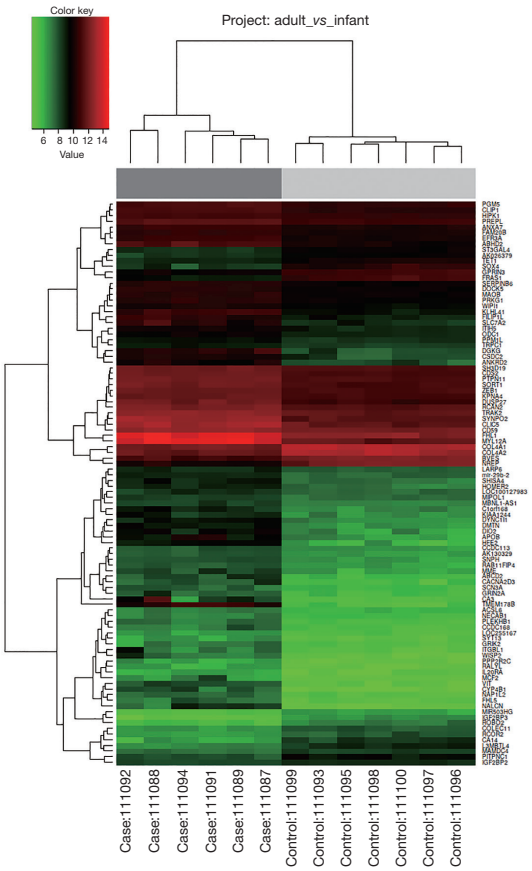


**Figure 6** Hierarchical clustering of the 100 most significantly dysregulated genes. Heatmap classified the samples into two major clusters of upregulated (red colored) and downregulated (green colored) genes according to the condition ("cases/adult" versus "controls/infant" patients) of the samples. By using the heatmap, raw gene expression data were visualized and grouped, based on the similarity of their gene expression pattern. Each column signifies different patient samples, and each row represents a significantly dysregulated gene. The variation in color and intensity of the boxes symbolizes the changes in gene expression, entailing red represents upregulated genes, green represents downregulated genes and black implies unchanged gene expression. The appositional heatmap features the 100 most significant dysregulated genes of the gene expression raw data.

increase of molecular function included protein binding as well as oxidoreductase, catalytic peptidase regulator, peptidase inhibitor activity (Tables S2,S7). Significantly increased biological processes included oxidation reduction process, vesicle mediated transport and secretion by cells, as well es regulation of exocytosis (Tables S3,S7).

A total of 218 GO terms were significantly downregulated in adult compared to infant patients on functional annotation of differentially expressed genes. The most significant GO terms for cellular components included intracellular organelles, chromosome and protein-DNA complexes (Tables S4,S8). Significantly downregulated genes of

 

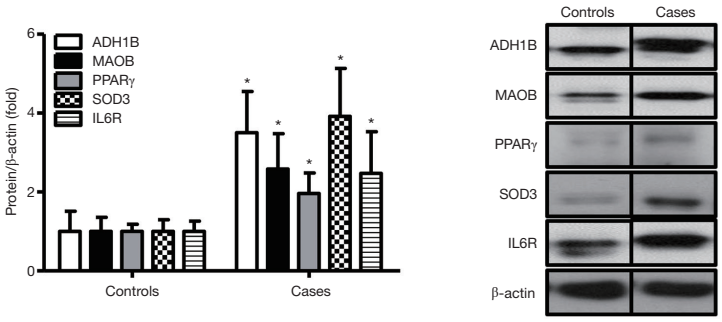


**Figure 7** Western blot validation of selected dysregulated target proteins. Total proteins were isolated from the right ventricular myocardium of infant- ("controls") and adult ("cases") patients. Western blot analyses were performed using antibodies against alcohol dehydrogenase 1B (ADH1B), monoamine oxidase B (MAOB), peroxisome proliferator-activated receptor gamma (PPARγ), superoxide dismutase 3 (SOD3), and interleukin 6 receptor (IL6R). β-actin served as a loading control. Representative blots are shown. Two-tailed Student's *t*-test was used (n=3; *, P<0.05 vs. corresponding control).

molecular function were mostly associated with DNA and transcription processes (Tables S5,S8). In line Biological processes involving cell cycling and regulation of gene expression were significantly downregulated (Tables S6,S8).

KEGG analysis showed retinol metabolism (hsa00830) and PPAR signaling pathway (hsa03320) to be significantly upregulated, considering a P value of less than 0.05 (Table S7). Cell cycle (hsa04110), DNA replication (hsa03030) and ribosome (hsa03010) were most significantly downregulated on KEGG analysis, based on a P value of less than 0.05 (Table S8).

Western blot analyses on proteins extracted from RV tissues were performed for validation of RNA sequencing results. Protein levels of *ADH1B*, *PPAR gamma*, *MAOB* and *SOD3* representing critical genes involved in the retinol metabolism, PPAR signaling, and response to oxidative stress pathways, respectively, were significantly increased (*Figure 7*).

## Discussion

Differential gene expression and molecular signaling pathways contributing to pathologic myocardial remodeling differ between the left (6,15) and the right ventricle (5,6,15,16). Knowledge about the molecular fundamentals and cellular changes which are involved in the development of RV remodeling secondary to chronic pressure and/or volume overload in CCHD patients is limited (7). The present study evaluated differentially expressed genes of RV tissue from adult patients with TOF and chronic pressure and/or volume overloaded right ventricle compared to infants with TOF by whole transcriptome profiling. Detailed bioinformatic and statistical analysis identified signaling pathways potentially contributing to pathologic RV remodeling in affected patients.

GO Enrichment Analysis and KEGG pathway analysis identified significant dysregulation of genes encoding for structural cellular component and of genes involved in certain biological and functional processes to be significantly dysregulated in RV tissues of adult patients with TOF and chronic hemodynamic RV stressors. Genes associated to the contractile fiber part and actin cytoskeleton of the cell, in particular genes of myofibril, I Band, Z Disc and the contractile units of myocytes, the sarcomere, were particularly upregulated. Increase of contractile protein expression was also described by others in infants with pressure overloaded right ventricles (17). Those findings might explain diastolic RV dysfunction reported by others in patients with TOF (18,19). Analogous findings of alterations in gene expression of cytoskeleton proteins and extracellular matrix proteins were also made in myocardium of failing left ventricles (3,7,15,16). Additionally, upregulation of oxidation-reduction processes was identified as critical biological process involved in RV remodeling of affected patients in the current study. Mechanical pressure is one of the main cardiac stress factors, leading to dysfunctional mitochondria, resulting in increased

 

reactive oxygen species (ROS) production. Dysfunctional mitochondria and imbalance of the redox system contribute to HF (20). Furthermore, upregulation of genes encoding for proteins involved in cell-cell-communication and several molecular pathways, implicating extracellular exosome, extracellular vesicle, vesicle mediated transport and secretory vesicle, were found to be upregulated in adults with RVI in the current study. There is evidence that ROS can stimulate the production of exosomes and affect the molecular content of exosomes, among various other external stimulation factors, of which are some still unknown (21,22). Exosomes are extracellular 40 to 150 nm diameter lipid vesicles secreted by cells (22,23) to facilitate intercellular communication by transferring miRNA, mRNA and proteins between cells (24). It is hypothesized that exosomes might play a role in cardiac remodeling in the response to cardiac stress by influencing inflammation, interstitial fibrosis, ventricular hypertrophy and changes in contractility (22). Due to the unique function of exosomes to promote intercellular communication, they are currently of interest as therapeutic agents and potential biomarkers for various diseases, including cardiovascular diseases (25,26).

The current study also showed the upregulation of the peroxisome proliferator-activated receptor (PPAR) signaling pathway. PPARs play a role as nuclear receptor transcription factors, activated by specific ligands such as long chain fatty acids (27). The expression of genes, regulated by the three PPAR isoforms, contribute to cardiac metabolic processes, in particular lipid, fatty acid and glucose homeostasis, cell differentiation and control of inflammation (27-30). Upregulation of the PPAR signaling pathway in the current study is consistent to results of previous studies which report dysregulated PPAR signaling pathway in the context of cardiac remodeling, myocardial hypertrophy and HF (27,31-33). PPARs regulate gene expression by forming a nuclear heterodimeric complex with retinoid X receptor (RXR) and binding to specific promoter regions of the target genes to be regulated (29,30). *RXR* is one of the major genes involved in retinol metabolism. In line with this, KEGG bioinformatical analysis revealed a highly significant involvement of the retinol pathway in RV tissues of affected patients. In addition to *RXR* (P value 0.0051), genes such as *RDH10* (P value 0.00076), *ADH1B* (P value 4.93E-11), *ADH1C* (P value 0.00037) and *CYP1A1* (P value 1.01E-05) were significantly upregulated. Activation of retinoic acid signaling in left ventricular tissue in conjunction with myocardial ischemia and remodeling after myocardial infarction in mice was reported by others (34). The retinol metabolism utilizes vitamin A and its derivates to ensure cardiac supply of various forms of all trans retinoic acids (atRA) (34). AtRA function as ligands via binding to nuclear receptors like RAR or RXR (34). The receptors form heterodimers and bind to DNA regulatory sequences, whereby regulating the frequency of gene expression, plus modifying interconnection with other signaling pathways (34). The results of the current study concur with previous studies, investigating dysregulation of retinol metabolism in ventricular remodeling and HF. We speculate that not only the left ventricle, but also the right ventricle shows a dysregulation of retinol metabolism in the development of RVI, which is also supported by our results. The activation of metabolites of retinol metabolism may play a previously unimagined role in RV remodeling and in response to cardiac damage and repair. The simultaneous overexpression of genes involved in fatty acid metabolism, PPAR signaling pathway as well as retinol metabolism indicate the interdependence of the individual signaling pathways (31) and promote the presumption of contributing to RV remodeling in stages of RVI in adult patients with CCHD.

During the development of RV remodeling and RVI, RNA sequencing revealed a marked adjustment of gene expression, regarding mitotic cell cycle, cell division and DNA replication. Downregulation of differentially expressed genes, associated with DNA binding transcription factor activity, transcription regulator activity as well as RNA polymerase 2 transcription factor activity, support the assumption of decreased cell cycling in stressed RV myocardium of adult patients with signs of RVI. In line with above findings, reduction of gene expression with respect to nucleus, chromosome, protein-DNA complex, spindle and replication fork strengthen suspicion of declined cell proliferation and cell maintenance in cardiac remodeling. Downregulation of cell cycle leads to a fatal lack of myocardial regeneration, due to failing proliferation of normal functioning cardiomyocytes (35). Experimental research determined cardiac loss of ability to reentry cell cycle, since differentiated cells such as adult cardiomyocytes typically become post-mitotic and exit the cell cycle postnatal (36,37). The present study indicates that stressed myocardium retains the incapability to re-enter cell cycle in patients with CCHD and RVI, facing adverse cardiac remodeling. Cardiac tissue consequently encounters difficulties in adaption to pathologic conditions such as pressure and volume overload, which results in impaired

 

cardiac injury repair and hypertrophy, leading to increased risk of cardiac morbidity and mortality (38,39). The incapacity to enter cell cycle, guaranteeing cell proliferation, injury regeneration and preservation of ventricular function, aggravate pathophysiologic conditions and encourage development of RV remodeling and RVI.

The major limitation of the current study is the lack of whole transcriptome profile of RV tissue gained from healthy patients without structural heart disease and without myocardial disease state as a control group. The use of a donor heart, which is discarded for organ transplantation, would have provided an opportunity to remove healthy tissue from the right ventricle and use it as a reference material. Nevertheless, it must be noted, that not-transplantable donor hearts are often not completely healthy with exposition to abundant factors, typically changing gene expression. Besides, the rejection of a donor heart is a rarity. Also, the falsification of the results of gene sequencing by temporal issue and processing complications should also not be underestimated. Because of altered myocardial gene expression after organ ischemia and organ processing outside the body it is not ideal to use not-transplantable donor hearts as healthy reference tissue (40). The focus of the current study was therefore the alteration of gene expression patterns contributing to RV remodeling in chronic volume and/or pressure overloaded RVs over lifetime in a cohort of CCHD patients with similar structural heart disease, namely TOF. The control group consistent of infants TOF where RV tissue was collected at the time of surgical repair at the age of 4–5 months. Limited data of developmentally regulated genes in myocardial tissue, showing a switch during aging process, hamper the identification and exclusion of all genes, being essential in heart development during early childhood. By comparing most significant single-cell RNA sequencing results of murine cells to our results, genes associated with cardiac development were identified and excluded for further interpretation. However, according to few studies there are indications that genes play a variety of roles in both heart development and pathological cardiac remodeling (41). It has also been described that embryonic patterns and fetal gene programs, which are crucial for heart development and maturation, get reactivated in pathological conditions, leading to HF and death (42). Studies have shown that members of the myocyte enhancer factor 2 (*MEF2*) family are expressed in both embryonic cardiac tissue and postnatal hypertrophic cardiac remodeling (43). MEF2 proteins such as MEF2C function as transcription factors, increasing expression of certain fetal and cardiac genes, including natriuretic peptide A (*NPPA*), skeletal alpha actin (*ACTA1*), desmin (*DES*) and dystrophin (*DMD*). Concomitant to previous observations expression of *MEF2C*, *NPPA*, *ACTA1*, *DES* and *DMD* were found to be upregulated in our study, reinforcing the suggestion of reactivation of fetal gene programs in pathological heart conditions (Table S10). Considering these emerging evidences, important information of gene expression changes during cardiac remodeling might get missed by excluding alleged developmentally regulated genes. Another limitation is the unequal distribution of female and male patients in the infant and adult patient group. While the infant patient group is dominated by males (6/7), the adult patient group is composed of more females (5/6). The gender disparity might have an impact on gender specific alterations in gene expression, which makes transferability more difficult. However, whereas there are contradictory data regarding gender differences in gene expression of the left ventricular myocardium in human (44) and animal (45) studies, to our knowledge the impact of gender on mRNA expression in human RV heart tissue has not been described so far in the literature. Two of the six adult patients were on cardiac medication at the time of study. The influence of this on gene expression in unknown and due to small sample size, we were not able to focus on this in the current analysis. Lastly, as this is a single-center study with retrospective data analysis, the results cannot simply be generalized and extrapolated to other centers.

## Conclusions

There is altered RV gene expression over lifetime in adult patients with RVI due to long-term sequelae of CCHD. Analysis of different gene expression profiles identified molecular pathways involved in cell-cell communication, exosomes, extracellular vesicles, oxidation reduction process, contractile fiber and retinol metabolism to be involved in pathologic RV remodeling secondary to chronic volume and/or pressure overload. The identification of molecular pathways leading to pathological RV remodeling and failure is crucial for the development of new therapies and upcoming biomarkers, screening assays for risk stratification and disease monitoring. The current study contributes to a better understanding of the molecular characterization of RV remodeling and RVI, which significantly increases morbidity and mortality in patients with CCHD, surviving into adulthood.

 

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## 4.2 Compound mutation in cardiac sarcomere proteins is associated with increased risk for major arrhythmic events in pediatric onset hypertrophic cardiomyopathy (Pollmann, Kaltenecker, et al., 2021)

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Summary:

HCM is the most common inherited cardiovascular disease, being one of the main reasons for increased morbidity and mortality caused by arrhythmic SCD and heart failure. Disease-causing mutations have been identified in mainly sarcomere protein-coding genes, affecting protein structure and function, activating signaling pathways and modifying biophysical properties of cardiomyocytes. Consequently, LV remodeling, including disturbed calcium handling, fibrosis, LV dysfunction and malignant arrhythmias, occur. HCM phenotypes and clinical course vary widely due to genetic heterogeneity, which is still poorly understood. Therefore, this study aimed to investigate genotype-phenotype association and disease progression in patients with pediatric onset HCM.

Medical chart review was carried out at first presentation and latest follow-up, including demographic, clinical, electrocardiographic, long-term ECG, echocardiographic, CPET and CMR parameter. Diagnosis of HCM conformed to generally accepted guidelines (Authors/Task Force et al., 2014) (Ommen et al., 2020). Based on the results of molecular genetic testing, groups were defined for comparison.

Molecular genetic testing identified that most mutations were present in the genes *MYH7* (48.6%) and *MYBPC3* (31.4%), followed by *TNNT2* (11.4%), *TPM1* (2.9%), *MYL2* (2.9%) and *TNNI3* (2.9%) in single-mutation carrier. These findings correspond to already described mutation distribution in study cohorts of adults diagnosed with HCM. Analysis of clinical data revealed no significant differences in terms of NYHA classification, LV morphology, hypertrophy, LV function, fibrosis and cardiac medication. However, SCD equivalents occur significantly more often in multiple-mutation carrier than in single-mutation carrier, regardless of mutation type. SCD equivalents were defined as at least one reanimation or ICD discharge or SCD. Additionally, a family history positive for familial HCM and SCD under 40 years of age were found more often in genotype positive patients than in genotype negative patients. There was no evidence that mutations in cardiac troponins cause worse phenotypes or severe clinical outcome. Differences in disease progression depending on the underlying genotype could not be detected.

Individual Contribution:

The principal investigator of the study was PD Dr. med. Cordula Wolf. Kathrin Pollmann was responsible for reviewing medical charts to collect patient data. Since a subset of patients who were also included in other studies, data acquisition was supported by Julia Schleihauf and Emanuel Kaltenecker. Kathrin Pollmann collected the results of molecular genetic testing and re-evaluated them using ClinVar. With the support of PD Dr. med. Cordula Wolf, data analysis and statistical calculation was performed by the doctoral candidate. She wrote the first manuscript draft and after all co-authors carefully reviewed the manuscript, she submitted it to the Journal of Clinical Medicine together with PD Dr. med. Cordula Wolf. Revisions she composed together with PD Dr. med. Cordula Wolf.

All actions were taken in consultation with the co-authors. PD Dr. med. Cordula Wolf was responsible for study conception and design. Prof. Dr. med. Agnes Görlach was the supervisor. Administrative support was provided by Prof. Dr. med. Peter Ewert. All co-authors reviewed and approved the manuscript, contributing to the manuscript improvement.

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Journal of
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# Compound Mutation in Cardiac Sarcomere Proteins Is Associated with Increased Risk for Major Arrhythmic Events in Pediatric Onset Hypertrophic Cardiomyopathy

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**Abstract:** Hypertrophic cardiomyopathy (HCM) is associated with adverse left ventricular (LV) remodeling causing dysfunction and malignant arrhythmias. Severely affected patients present with disease onset during childhood and sudden cardiac death risk (SCD) stratification is of the highest importance in this cohort. This study aimed to investigate genotype–phenotype association regarding clinical outcome and disease progression in pediatric onset HCM. Medical charts from forty-nine patients with pediatric HCM who had undergone genetic testing were reviewed for retrospective analysis. Demographic, clinical, transthoracic echocardiographic, electrocardiographic, long-term electrocardiogram, cardiopulmonary exercise test, cardiac magnetic resonance, and medication data were recorded. Childhood onset HCM was diagnosed in 29 males and 20 females. Median age at last follow-up was 18.7 years (range 2.6–51.7 years) with a median follow-up time since diagnosis of 8.5 years (range 0.2–38.0 years). Comparison of patients carrying mutations in distinct genes and comparison of genotype-negative with genotype-positive individuals, revealed no differences in functional classification, LV morphology, hypertrophy, systolic and diastolic function, fibrosis and cardiac medication. Patients with compound mutations had a significantly higher risk for major arrhythmic events than a single-mutation carrier. No association between affected genes and disease severity or progression was identified in this cohort.

**Keywords:** pediatric onset hypertrophic cardiomyopathy; major arrhythmic events; sudden cardiac death risk stratification; genotype–phenotype association



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## 1. Introduction

Hypertrophic cardiomyopathy (HCM) is the most common genetically inherited heart disease with a prevalence of about 0.2% [1,2]. It is defined by isolated hypertrophy and progressive pathologic remodeling of the left ventricular (LV) myocardium [2]. Clinical signs and symptoms include systolic and diastolic ventricular dysfunction and an increased risk for malignant arrhythmias. Disease course is highly variable, and onset usually occurs during adulthood. Severely affected patients present with childhood onset HCM, which is associated with significant lifetime morbidity and mortality.

Disease-causing mutations inherited in an autosomal dominant manner are currently identified in about 60% of HCM patients [2]. They are located predominantly in genes, encoding for essential cardiac sarcomere proteins [1] of cardiomyocytes, most frequently ß-myosin heavy chain (MYH7) and myosin binding protein C (MYBPC3) [3,4]. Other genes such as cardiac troponin T2 (TNNT2) [5], cardiac troponin I3 (TNNI3) [6], cardiac troponin

C (TNNC1) [7], myosin light chain 2 (MYL2) [3], and α tropomyosin (TPM1) [3] have also been identified as disease-causing.

The wide range of genes that can be affected by mutations makes HCM a genetically heterogeneous disease [8]. Genetic variability might be a reason for the large spectrum of diverse phenotypes with various clinical outcomes [9]. HCM is known as a disease with variable progression, which can be classified into different stages [8]. Pathological LV remodeling occurs over a lifetime with mostly severe asymmetric septal hypertrophy, contributing to serious left ventricular outflow tract obstruction (LVOTO), myocardial fibrosis, systolic dysfunction with altered LV ejection fraction (EF), diastolic dysfunction including atrial dilatation as well as atrial fibrillations and LV apical aneurysms [8]. Increased morbidity and mortality in the terminal stage of HCM is common due to life-threatening arrhythmias, heart failure, and an increased risk of sudden cardiac death (SCD), especially in youth and competitive athletes [2,10].

Specifically, in pediatric onset HCM, precise knowledge about disease progression, depending on different mutations, is still lacking. Insight of HCM genotype–phenotype association would facilitate counseling and management of affected patients.

This study aimed to investigate genotype–phenotype association of disease severity and progression in patients with childhood onset HCM.

## 2. Materials and Methods

A total of 49 patients diagnosed with pediatric onset HCM between November 1981 and November 2019 at the outpatient clinic of the German Heart Center Munich were included in the study. Pediatric onset HCM was defined as either positive molecular-genetic testing or evidence of disease phenotype before the age of 18 years. Phenotype-positive was defined by the presence of isolated hypertrophied left ventricle with a z-score of $\geq$2 on transthoracic echocardiographic evaluation based on the guidelines of the European Society of Cardiology (ESC) [11] and guidelines for the diagnosis and treatment of patients with HCM of the American Heart Association and the American College of Cardiology [12]. Accordingly, patients with secondary factors, leading to equivalent LV hypertrophy and the presence of other complex congenital heart disease, syndromic, metabolic, or neuromuscular disorders were excluded.

Data collection was performed retrospectively by medical chart review including demographic and clinical status, transthoracic echocardiography (TTE), electrocardiogram (ECG), 24-h Holter ECG, cardiopulmonary exercise testing (CPET), and cardiac magnetic resonance imaging (CMR). To assess disease progression, patient-related information was obtained at first presentation at the outpatient clinic and compared to information available from last follow-up in those patients, where information for both timepoints was available. CMR data were obtained from 31 patients (63.3%). Since CMR was performed at only one time point, data could not be used for the analysis of disease progression.

Clinical and imaging parameters available to assess the presence of pathological myocardial remodeling included end-diastolic and end-systolic LV diameters on CMR and TTE for general cardiac morphology, CMR mass (g/m$^2$) and TTE end-diastolic LV wall thicknesses (z-score) for myocardial hypertrophy, CMR late gadolinium enhancement (LGE) for patchy and CMR T1 map for interstitial myocardial fibrosis, echocardiographic pulse wave mitral valve (MV) E/A ratio and septal/lateral MV tissue Doppler measurements for LV diastolic function, TTE EF and strain analysis for LV systolic function, presence of arrhythmias on ECG, CPET, and 24-h Holter ECG, and clinical functional status assessed by the New York Heart Association (NYHA) class or modified age-adjusted Ross classification [13], medication use, implantable cardioverter defibrillator (ICD) implantation and appropriate discharge, and the need to hospitalization to assess morbidity (Figure 1) [8]. Arrhythmia on Holter or cardiopulmonary exercise test was classified into none, mild (premature ventricular or supraventricular beats), and severe (non-sustained or sustained ventricular or supraventricular tachycardia). Major arrhythmic events (MAEs) were defined as at least one reanimation or appropriate ICD discharge or SCD, in which

an appropriate ICD discharge was for ventricular fibrillation or ventricular tachycardia. According to the guidelines of the American College of Cardiology Foundation and the American Heart Association, LVOTO was defined [14].

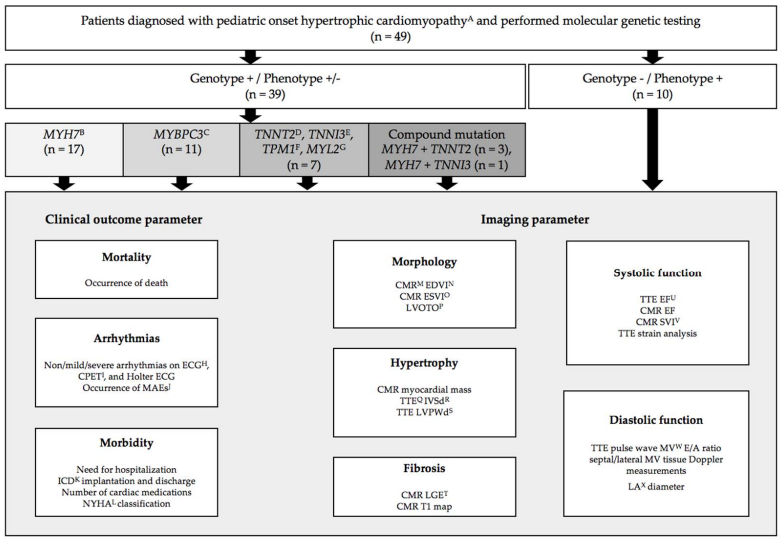



**Figure 1.** Study design and population with disease-causing mutations in affected genes encoding for sarcomere proteins in the myocardium. Shown are the flow chart of study design and population as well as the distribution of disease-causing mutations in genes encoding for sarcomere proteins in the myocardium as schematic illustrations of the affected proteins of the contractile apparatus of the cardiomyocyte in relation to their location of the thick and thin filament: β myosin heavy chain (MYH7), myosin binding protein C (MYBPC3), cardiac troponin T2 (TNNT2), cardiac troponin I3 (TNNI3), α tropomyosin (TPM1), myosin light chain 2 (MYL2). [A], isolated hypertrophic cardiomyopathy according to current European Society of Cardiology and American Heart Association definitions; [B], β myosin heavy chain; [C], myosin binding protein C; [D], cardiac troponin T2; [E], cardiac troponin I3; [F], α tropomyosin; [G], myosin light chain 2; [H], electrocardiogram; [I], cardiopulmonary exercise test; [J], major arrhythmic events: reanimation and/or appropriate discharge of cardioverter-defibrillator and/or sudden cardiac death; [K], implantable cardioverter defibrillator; [L], New York Heart Association; [M], cardiac magnetic resonance imaging; [N], end-diastolic volume index; [O], end-systolic volume index; [P], left ventricular outflow tract obstruction; [Q], transthoracic echocardiography; [R], end-diastolic septal wall thickness; [S], end-diastolic left ventricular posterior wall thickness; [T], late gadolinium enhancement; [U], ejection fraction; [V], stroke volume index; [W], mitral valve; [X], left atrial.

Routine molecular genetic testing was performed for the identification of causative genes [4,15]. One mL of the EDTA blood samples were obtained from all patients for genetic testing at certified laboratories for human genetics, following the recommendations of the European and North American guidelines [11,16–18]. Interpretation of mutation pathogenicity was based on the classification system and guidelines of the American College of Medical Genetics (ACMG) and Genomics and the Association for Molecular Pathology (AMP) [19,20]. Patients were classified into subgroups based on the gene affected on genetic testing (Figures 1 and 2). Please see the Supplementary Materials for further information (Methods S1).

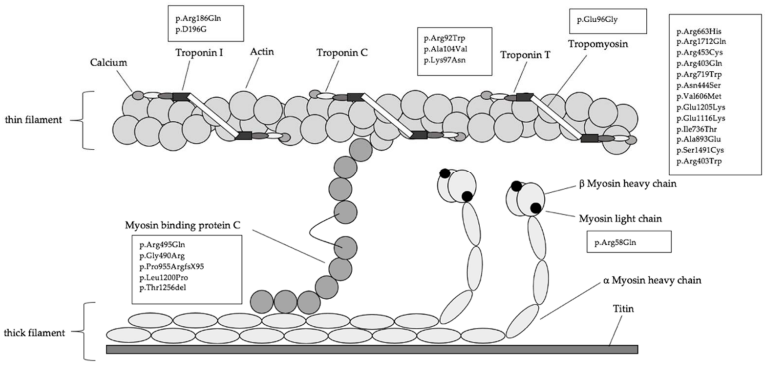



**Figure 2.** Specific protein modifications of the study population.

Statistical analysis was performed with the SPSS software program version 25.0.0 (SPSS Inc., IBM Company, Chicago, IL, USA). Differences between all groups defined at latest follow-up were analyzed by the Kruskal–Wallis test. For comparison of two individual selected groups, the Mann–Whitney U test was utilized. Categorical variables were analyzed by Pearson Chi-square test. With the help of the Kaplan–Meier calculation, the survival probability was estimated and compared between the groups by log-rank test. Continuous variables were expressed as median (minimum–maximum). For assessment of disease progression, the delta of respective parameters between the date of first presentation and last follow-up was compared between groups using the dependent non-parametric Kruskal–Wallis test. Statistical tests were two sided and a $p$-value of <0.05 was considered statistically significant.

## 3. Results

### 3.1. Patients Characteristics

The study population consisted of 29 male (59.2%) and 20 female (40.8%) patients with a diagnosis of pediatric onset HCM based on either clinical findings and/or positive molecular genetic testing. Genetic testing identified 35 consecutive patients as genotype-positive/phenotype-positive (71.4%), four patients as genotype-positive/phenotype-negative (8.2%), and 10 patients as genotype-negative/phenotype-positive (20.4%) at first presentation. Distribution of the detected mutations are depicted in Figures 1 and 2. In 35 pediatric HCM patients, the identified variant was inherited from one parent (89.7%) and four patients had de novo mutation (10.3%) (Table S1). Additional variants of unknown significance (VUS) and benign variations were present in four patients. Of these, singular VUS was detected in two patients, so they were classified as genotype-negative/phenotype positive. One patient carried one VUS in addition to a MYH7 mutation and one patient had one VUS with two further mutations, both defined as genotype positive. Upon closer verification by ClinVar, hosted by the National Center for Biotechnology Information (NCBI) and founded by the National Institutes of Health (NIH), we identified that most mutations were missense variants, followed by frameshift, deletion, and splice mutations (Table S1) [21]. According to the classification systems, all identified mutations were classified as likely pathogenic and/or pathogenic (Table S1).

Patient characteristics are depicted in Table 1. The median age of the entire cohort at the time of diagnosis was 7.0 years (range 0.0–18.95 years) and at last follow-up 18.7 years (range 2.6–51.7 years). No difference was found in disease onset between pediatric HCM patients carrying mutations in thin filament genes compared to patients with mutations in thick filament genes ($p$-value 0.982). Family history was negative in 15 patients (30.6%), positive for the presence of HCM in 23 patients (46.9%) and for SCD in 11 patients (22.4%) (Table 1).

**Table 1.** Patient characteristics and clinical outcome parameters.

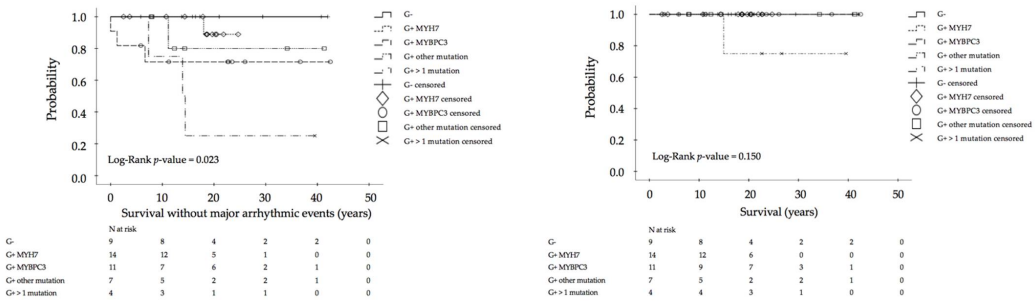
| Patients Characteristics and Clinical Outcome Parameters | Genotype-Positive (MYH7) | Genotype-Positive (MYBPC3) | Genotype-Positive (Others) | Genotype-Positive (>1 Mutation) | Genotype-Negative/Phenotype-Positive | *p*-Value |
|---|---|---|---|---|---|---|
| Patients, *n* [1] (% of total) | 17 (34.7) | 11 (22.4) | 7 (14.3) | 4 (8.2) | 10 (20.4) | |
| Male, *n* (%) | 9 (52.9) | 10 (90.9) | 1 (14.3) | 3 (75.0) | 6 (60.0) | 0.025 [10] |
| Age at first diagnosis (years) | 13.0 (0.0–19.0) | 9.0 (0.0–18.0) | 6.8 (0.0–15.0) | 3.5 (0.0–7.0) | 8.3 (0.0–15.6) | 0.674 [11] |
| Age at last follow-up (years) | 18.7 (2.6–51.7) | 22.7 (3.0–42.5) | 12.4 (7.9–41.4) | 24.6 (14.6–39.6) | 18.0 (5.9–42.0) | 0.521 [11] |
| Follow-up time (years) | 5.8 (0.2–22.6) | 8.8 (2.3–31.7) | 10.3 (1.2–27.3) | 21.1 (13.5–33.5) | 7.0 (0.2–38.0) | 0.188 [11] |
| **Family History** | | | | | | |
| Negative, *n* (%) | 3/17 (17.6) | 4/11 (22.4) | 1/7 (14.3) | 0/4 (0.0) | 7/10 (70.0) | |
| HCM [2], *n* (%) | 12/17 (70.6) | 5/11 (45.5) | 2/7 (28.6) | 1/4 (25.0) | 3/10 (30.0) | |
| SCD [3], *n* (%) | 2/17 (11.8) | 2/11 (18.2) | 4/7 (57.1) | 3/4 (75.0) | 0/10 (0.0) | 0.003 [10] |
| **Mortality** | | | | | | |
| Death, *n* (%) | 0/17 (0.0) | 0/11 (0.0) | 0/7 (0.0) | 1/4 (25.0) | 0/10 (0.0) | 0.022 [10] |
| **Arrhythmia** [4] | | | | | | |
| None, *n* (%) | 7/14 (50.0) | 3/11 (27.3) | 4/7 (57.1) | 1/4 (25.0) | 6/9 (66.7) | |
| Mild [5], *n* (%) | 5/14 (35.7) | 4/11 (36.4) | 1/7 (14.3) | 0/4 (0.0) | 3/9 (33.3) | |
| Severe [6], *n* (%) | 2/14 (14.3) | 4/11 (36.4) | 2/7 (28.6) | 3/4 (75.0) | 0/9 (0.0) | |
| MAEs [7], *n* (%) | 1/17 (5.9) | 3/11 (27.3) | 1/7 (14.3) | 3/4 (75.0) | 0/10 (0.0) | 0.006 [10] |
| **Morbidity** | | | | | | |
| Hospitalization, *n* (%) | 6/17 (35.3) | 8/11 (72.7) | 2/7 (28.6) | 3/4 (75.0) | 4/10 (40.0) | 0.180 [10] |
| Age (years), median (range) | 6.5 (1.1–17.3) | 1.0 (0.0–27.9) | 10.5 (4.0–17.0) | 10.9 (0.0–31.8) | 9.0 (0.0–34.5) | 0.722 [11] |
| ICD [8], *n* (%) | 2/17 (11.8) | 4/11 (36.4) | 2/7 (28.6) | 3/4 (75.0) | 2/10 (20.0) | 0.112 [10] |
| Age (years), median (range) | 15.2 (14.8–15.5) | 23.9 (9.9–40.5) | 17.7 (4.3–31.0) | 14.3 (10.9–37.8) | 28.5 (16.5–40.4) | 0.700 [11] |
| Primary prevention, *n* (%) | 2/2 (100.0) | 4/4 (100.0) | 2/2 (100.0) | 1/3 (33.3) | 2/2 (100.0) | 0.096 [10] |
| Secondary prevention, *n* (%) | 0/2 (0.0) | 0/4 (0.0) | 0/2 (0.0) | 2/3 (66.6) | 0/2 (0.0) | 0.096 [10] |
| Appropriate discharge, *n* (%) | 1/2 (50.0) | 2/4 (50.0) | 1/2 (50.0) | 2/3 (66.7) | 0/2 (0.0) | 0.686 [10] |
| Number of cardiac medication, *n* (%) | 1 (0–2) | 1 (0–2) | 1 (0–2) | 1 (0–2) | 1 (0–2) | 0.624 [10] |
| NYHA [9]/Ross class | | | | | | |
| I, *n* (%) | 7/14 (50.0) | 6/11 (54.5) | 5/7 (71.4) | 1/4 (25.0) | 6/9 (66.7) | 0.582 [10] |
| II, *n* (%) | 7/14 (50.0) | 5/11 (45.5) | 2/7 (28.6) | 3/4 (75.0) | 3/9 (33.3) | |
| III, *n* (%) | 0/14 (0.0) | 0/13 (0.0) | 0/7 (0.0) | 0/4 (0.0) | 0/9 (0.0) | |
| IV, *n* (%) | 0/14 (0.0) | 0/13 (0.0) | 0/7 (0.0) | 0/4 (0.0) | 0/9 (0.0) | |

[1], number of cases; [2], hypertrophic cardiomyopathy; [3], sudden cardiac death; [4], arrhythmia on Holter or cardiopulmonary exercise test; [5], premature ventricular or supraventricular beats; [6], non-sustained or sustained ventricular or supraventricular tachycardia; [7], resuscitation and/or appropriate implantable cardioverter defibrillator discharge and/or sudden cardiac death; [8], implantable cardioverter defibrillator; [9], New York Heart Association or Modified Ross classification according to age; [10], Pearson-Chi-squared-test; [11], Kruskal–Wallis-test.

### 3.2. Genotype–Phenotype Relation of Clinical Outcome and Imaging Parameters

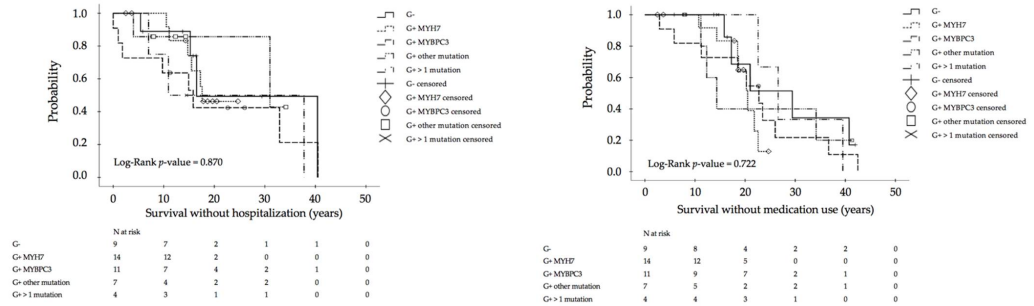
Age at first diagnosis and at last follow-up was not different between the distinct patient groups, separated by the respective results of moleculargenetic diagnosis (Table 1). Mortality in general was low (2.0%). One patient carrying a compound mutation of MYH7 and TNNT2 died suddenly at the age of 15 years. Male gender was less present

in genotypes other than MYH7 and MYBPC3. A negative family history occurred most often in genotype-negative/phenotype-positive children, a positive family history of HCM more often in children affected by mutations in the MYH7 gene, and a positive family history of SCD more often in patients carrying more than one mutation. Patients experiencing MAEs occurred significantly more often in multiple-mutation carriers than in genotype-negative patients or single-mutation carriers, regardless of mutation type (Table 1, Figure 3). Between defined groups, significant differences with respect to overall survival, survival without hospitalization, and survival without medication use could not be detected (Figures 3 and 4).




(**a**) (**b**)

**Figure 3.** Morbidity and clinical outcome Part I: (**a**) Kaplan–Meier calculation of survival without major arrhythmic events (MAEs) (reanimation or appropriate implantable cardioverter defibrillator discharge or sudden cardiac death) in hypertrophic cardiomyopathy (HCM) patients, depending on different mutations; (**b**) Kaplan–Meier calculation of overall survival; SCD: sudden cardiac death, G−: genotype-negative patients, G+ MYH7: genotype-positive patients with β-myosin heavy chain single-mutation, G+ MYBPC3: genotype-positive patients with myosin binding protein C single-mutation, G+ others: genotype-positive patients with cardiac troponin T2, cardiac troponin I3, α tropomyosin and myosin light chain 2 single-mutations, G+ multiple mutation: genotype-positive patients with compound mutations.




(**a**) (**b**)

**Figure 4.** Morbidity and clinical outcome Part II: (**a**) Kaplan–Meier calculation of survival without hospitalization in hypertrophic cardiomyopathy (HCM) patients, depending on different mutations; (**b**) Kaplan–Meier calculation of survival without medication use. SCD: sudden cardiac death, G−: genotype-negative patients, G+ MYH7: genotype-positive patients with β-myosin heavy chain single-mutation, G+ MYBPC3: genotype-positive patients with myosin binding protein C single-mutation, G+ others: genotype-positive patients with cardiac troponin T2, cardiac troponin I3, α tropomyosin and myosin light chain 2 single-mutations, G+ multiple mutation: genotype-positive patients with compound mutations.

There was no difference in the need for hospitalizations and surgery, medication use was similar between groups, and the majority of patients were in NYHA/Ross class I or II at latest follow-up without a difference between groups. Additionally, no differences were found with regard to LV morphology (end-diastolic and end-systolic diameters or presence of LVOTO), myocardial hypertrophy and fibrosis, LV systolic and diastolic function parameters, and the occurrence of arrhythmias on ECG, 24-h Holter ECG or CPET, when comparing patients grouped according to the affected gene (Tables 1 and 2).

**Table 2.** Disease phenotype at latest follow-up.

| Imaging Parameter | Genotype-Positive (MYH7) *n* = 17 | Genotype-Positive (MYBPC3) *n* = 11 | Genotype-Positive (Others) *n* = 7 | Genotype-Positive (>1 Mutation) *n* = 4 | Genotype-Negative/Phenotype-Positive *n* = 10 | *p*-Value |
|---|---|---|---|---|---|---|
| **Morphology** | | | | | | |
| CMR [1] EDVI [2] (mL/m$^2$), median (range) | 62.0 (50.0–89.0) | 57.0 (36.0–91.0) | 60.0 (53.0–65.0) | 63.5 (63.0–64.0) | 65.0 (48.0–76.0) | 0.949 [18] |
| CMR ESVI [3] (mL/m$^2$), median (range) | 19.5 (8.0–27.0) | 17.0 (7.0–35.0) | 19.0 (11.0–22.0) | 16.0 (16.0–16.0) | 17.0 (8.0–27.0) | 0.978 [18] |
| LVOTO [4], *n* [5] (%) | 7/14 (50.0) | 3/11 (27.3) | 0/7(0.0) | 0/4(0.0) | 2/9 (22.2) | 0.092 [19] |
| **Hypertrophy** | | | | | | |
| CMR myocardial mass (g/m$^2$), median (range) | 100.5 (39.0–168.0) | 83.0 (39.0–213.0) | 58.0 (48.0–95.0) | 80.0 (43.0–117.0) | 90.0 (56.0–126.0) | 0.602 [18] |
| TTE [6] IVSd [7] z-score, median (range) | 3.7 (0.6–7.6) | 3.4 (0.2–7.8) | 2.2 (0.1–7.6) | 3.7 (3.1–4.9) | 4.8 (2.2–6.1) | 0.536 [18] |
| TTE LVPWd [8] z-score, median (range) | 2.5 (0.7–3.8) | 1.7 (-1.0–5.1) | 2.1 (1.2–5.3) | 2.9 (1.7–3.3) | 2.6 (1.1–4.5) | 0.891 [18] |
| **Fibrosis** | | | | | | |
| CMR LGE [9], *n* (%) | 8/10 (80.0) | 8/9 (88.9) | 3/3 (100.0) | 2/2 (100.0) | 4/7 (57.1) | 0.396 [19] |
| CMR LGE localization | | | | | | 0.204 [19] |
| Negative, *n* (%) | 3/10 (30.0) | 3/9 (33.3) | 1/3 (33.3) | 0/2 (0.0) | 3/7 (42.9) | |
| Uncertain detection, *n* (%) | 3/10 (30.0) | 0/9 (0.0) | 1/3 (33.3) | 1/2 (50.0) | 1/7 (14.3) | |
| Septum, *n* (%) | 4/10 (40.0) | 4/9 (44.4) | 1/3 (33.3) | 0/2 (0.0) | 2/7 (28.6) | |
| Entire myocardium, *n* (%) | 0/10 (0.0) | 0/9 (0.0) | 0/3 (0.0) | 1/2 (50.0) | 0/7 (0.0) | |
| Papillary muscle + RVOT [10], *n* (%) | 0/10 (0.0) | 1/9 (11.1) | 0/3 (0.0) | 0/2 (0.0) | 0/7 (0.0) | |
| LV [11] front wall + septum, *n* (%) | 0/10 (0.0) | 0/9 (0.0) | 0/3 (0.0) | 0/2 (0.0) | 1/7 (14.3) | |
| Diffuse distribution, *n* (%) | 0/10 (0.0) | 1/9 (11.1) | 0/3 (0.0) | 0/2 (0.0) | 0/7 (0.0) | |
| CMR LGE mean, median (range) | 7.3 (0.6–14.0) | 20.2 (0.4–23.8) | 7.8 (7.8–7.8) | 4.8 (4.8–4.8) | 1.2 (1.2–1.2) | 0.278 [18] |
| CMR ECV [12] total mean, median (range) | 26.8 (25.9–30.6) | 28.6 (24.9–34.4) | 28.5 (28.5–28.5) | 23.1 (23.1–23.1) | 24.8 (24.8–24.8) | 0.289 [18] |
| CMR ECV septal mean, median (range) | 31.1 (27.8–34.0) | 28.7 (26.9–34.9) | 31.3 (31.3–31.3) | 22.1 (22.1–22.1) | 26.3 (26.3–26.3) | 0.926 [18] |
| **Systolic function** | | | | | | |
| TTE EF [13] (%), median (range) | 72.0 (44.0–88.0) | 73.0 (55.0–95.0) | 76.0 (61.0–88.0) | 59.0 (41.0–78.0) | 83.0 (59.0–89.0) | 0.156 [18] |
| CMR EF (%), median (range) | 72.5 (62.0.–83.0) | 73.0 (61.0–80.0) | 69.0 (66.0–80.0) | 75.0 (75.0–75.0) | 73.0 (65.0–88.0) | 0.987 [18] |
| CMR SVI [14] (mL/m$^2$), median (range) | 42.5 (35.0–67.0) | 45.0 (29.0–59.0) | 43.0 (41.0–44.0) | 47.0 (47.0–47.0) | 46.5 (37.0–59.0) | 0.918 [18] |
| GLS [15] average, median (range) | −16.3 (−22.5–−8.8) | −14.7 (−27.8–−7.4) | −10.1 (−24.1–−7.6) | −8.2 (−8.2–−8.2) | −16.25 −22.2–−11.9) | 0.535 [18] |
| GLS dispersion, median (range) | −14.5 (−36.0–−4.0) | −11.0 (−24.0–−6.0) | −8.0 (−13.0–−5.0) | −4.0 (−18.0–−2.0) | −11.0 (−19.0–−5.0) | 0.299 [18] |
| GLS minimum, median (range) | −24.0 (−38.0–−8.0) | −15.0 (−24.0– −6.0) | −18.0 (-33.0–−10.0) | −13.0 (−17.0–−9.0) | −20.0 (−33.0–−9.0 | 0.345[18] |

**Table 2.** *Cont.*

| Imaging Parameter | Genotype-Positive (MYH7) *n* = 17 | Genotype-Positive (MYBPC3) *n* = 11 | Genotype-Positive (Others) *n* = 7 | Genotype-Positive (>1 Mutation) *n* = 4 | Genotype-Negative/Phenotype-Positive *n* = 10 | *p*-Value |
|---|---|---|---|---|---|---|
| GLS maximum, median (range) | −8.5 (−20.0–7.0) | −7.0 (−17.0–3.0) | −6.0 (−22.0–−4.0) | −7.0 (−13.0–5.0) | −9.0 (−15.0–10.0) | 0.576 [18] |
| GLS septal basal, median (range) | −9.5 (−26.0–−4.0) | −7.0 (−17.0–3.0) | −11.0 (−22.0–−5.0) | −8.0 (−16.0–−3.0) | −10.0 (−17.0–−5.0) | 0.319 [18] |
| GLS septal middle, median (range) | −13.0 (−29.0–−4.0) | −10.0 (−27.0–1.0) | −8.0 (−27.0–−4.0) | −7.0 (−17.0–−4.0) | −12.0 (−25.0–−4.0) | 0.687 [18] |
| GLS septal apex, median (range) | −18.0 (−32.0–−5.0) | −15.0(−39.0–−8.0) | −14.0 (−33.0–−7.0) | −13.0 (−13.0–−9.0) | −16.0 (−33.0–10.0) | 0.849 [18] |
| GLS lateral basal, median (range) | −20.5 (−38.0–7.0) | −13.0 (−27.0–−5.0) | −18.0 (−33.0–−7.0) | −1.5 (−8.0–5.0) | −14.0 (−27.0–−6.0) | 0.304 [18] |
| GLS lateral middle, median (range) | −15.0 (−28.0–3.0) | −18.5 (−32.0–1.0) | −9.5 (−21.0–−6.0) | N/A [20] | −16.0 (−18.0–−12.0) | 0.626 [18] |
| GLS lateral apex, median (range) | −15.0 (−30.0–−5.0) | −34.0 (−34–−34.0) | −12.0 (−29.0–−10.0) | −8.0 (−8.0–−8.0) | −16.0 (−20.0–−12.0) | 0.360 [18] |
| **Diastolic function** | | | | | | |
| MV [16] E/A Ratio, median (range) | 1.4 (0.7–2.2) | 1.8 (1.4–3.0) | 1.7 (1.0–2.5) | 2.0 (1.1–2.0) | 1.5 (1.0–12.8) | 0.420 [18] |
| MV E Deceleration (m/s), median (range) | 2.0 (1.1–2.5) | 2.0 (1.5–2.9) | 2.1 (1.7–2.8) | 2.2 (2.0–2.6) | 2.0 (1.6–2.9) | 0.610 [18] |
| MV E maximum (m/s), median (range) | 0.8 (0.4–1.3) | 0.8 (0.7–1.5) | 0.7 (0.6–1.1) | 0.6 (0.5–0.8) | 0.8 (0.6–1.8) | 0.504 [18] |
| MV E′ septal (m/s), median (range) | 9.5 (5.0–13.0) | 5.7 (5.0–7.0) | 11.0 (3.0–15.0) | 5.5 (4.0–7.0) | 7.5 (3.0–12.0) | 0.176 [18] |
| MV E′ lateral (m/s), median (range) | 10.0 (5.0–12.0) | 9.0 (7.0–11.0) | 7.0 (5.0–18.0) | 7.0 (5.0–9.0) | 11.5 (7.0–14.0) | 0.459 [18] |
| E/E′ septal, median (range) | 7.9 (5.2–21.8) | 12.3 (1.7–14.7) | 7.5 (6.1–20.0) | 10.5 (8.6–12.5) | 11.6 (6.9–19.8) | 0.507 [18] |
| E/E′ lateral, median (range) | 8.0 (5.8–11.9) | 8.1 (7.0–9.4) | 8.1 (−5.1–12.0) | 8.3 (6.7–10.0) | 7.3 (5.2–14.1) | 0.895 [18] |
| LA [17] Diameter (cm), median (range) | 3.3 (1.7–5.2) | 3.2 (2.0–4.8) | 2.5 (1.9–4.9) | 4.6 (4.3–5.1) | 2.6 (2.0–3.8) | 0.106 [18] |

[1], Cardiac magnetic resonance imaging; [2], end-diastolic volume index; [3], end-systolic volume index; [4], left ventricular outflow tract obstruction; [5], number of cases; [6], transthoracic echocardiography; [7], end-diastolic inter-ventricular septal; [8], end-diastolic left ventricular posterior wall thickness; [9], late gadolinium enhancement; [10], right ventricular outflow tract; [11], left ventricular; [12], extracellular volume fraction; [13], ejection fraction; [14], stroke volume index; [15], global longitudinal strain; [16], mitral valve; [17], left atrium; [18], Kruskal Wallis test; [19], Pearson Chi-square test; [20], N/A: not available.

To evaluate gender-difference in disease presentation of pediatric onset HCM patients, a comparison of female (*n* = 20) and male (*n* = 29) patients was performed. Analysis of clinical outcome and imaging parameter did not reach significance, which might be due to the small sample size. Furthermore, investigation of whether there are sex-differences between females and males carrying mutations in the same gene did not yield significant results.

Since carriers with compound mutations were identified at increased risk for MAEs, a subgroup analysis was carried out comparing clinical and imaging findings between patients with one compared to patients with more than one pathogenic/likely pathogenic mutation. Patients with compound mutations had increased maximal wall thickness with higher end-diastolic interventricular septal z-scores at latest follow-up compared to single-mutation carrier (*p*-value 0.05). Additionally, there was a difference in LGE localization measured by CMR (*p*-value 0.019). Patients with single mutations were LGE-positive mainly in the septal area, whereas LGE was positive in the entire myocardium in compound-mutation carrier. There was a trend toward increased left atrial parameters and decreased EF in TTE in patients with compound mutations compared to single-mutation carriers, but the findings did not reach statistical significance, possibly due to small patient numbers (*p*-value 0.056). Detailed descriptive information regarding clinical characteristics, outcome, and imaging parameter of patients with compound mutation are displayed in Table S2.

All multiple-mutation carriers comprised one mutation in the troponin gene in addition to a MYH7 mutation (Figure 1). To assess the contributing influence of a troponin mutation, an additional analysis was performed comparing patients with single or compound mutation in at least one gene encoding a cardiac troponin with patients not carrying a troponin mutation. Patients with a troponin mutation had a positive family history for SCD under the age of 40 more often than patients carrying mutations in other cardiac sarcomere proteins ($p$-value 0.010). Less patients with troponin mutation displayed LVOTO compared to patients with non-troponin mutations ($p$-value 0.032).

In order to differentiate between the effect of a single troponin mutation compared to a troponin mutation in addition to a second mutation, we compared the patients with a single troponin mutation with those carrying multiple mutations. Only patients with compound mutations including a troponin mutation and no patient with a single troponin mutation experienced MAEs ($p$-value 0.018).

### 3.3. Genotype–Phenotype Assessment for Disease Progression

After examination of individual groups carrying diverse mutations, indicating no genotype–phenotype association, we additionally examined whether disease progression (i.e., myocardial hypertrophy, fibrosis, systolic and diastolic dysfunction, or presence of arrhythmias) differed depending on mutation type and count. For this, differences between clinical and imaging parameters at follow-up and first presentation were calculated in those patients, where data were present for both timepoints. Based on the results of the statistical analysis, no evidence of statistically significant differences in disease progression of different patient groups could be found (Figure 5, Figures S1 and S2).

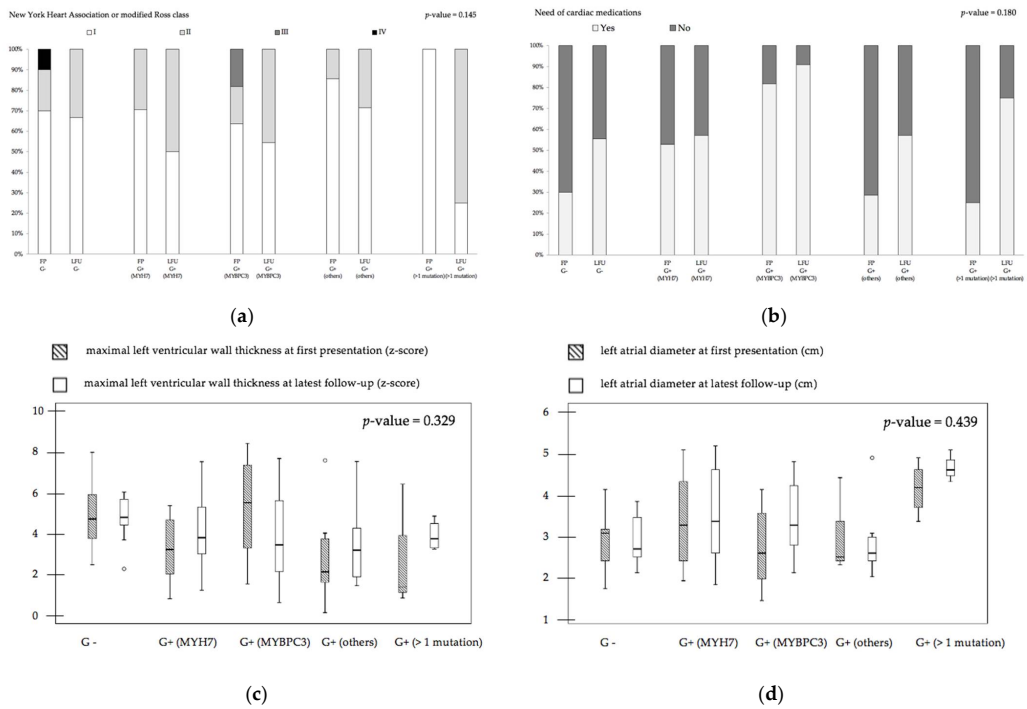



**Figure 5.** Disease progression Part I. Clinical outcome parameter of pediatric onset HCM patients are presented by the (**a**) New York Heart Association or modified Ross class and by (**b**) the need of cardiac medications. Imaging parameters are presented

by (**c**) maximum myocardial wall thickness and by (**d**) the left atrial diameter measured by transthoracic echocardiography. *p*-values were calculated with the non-parametric Kruskal–Wallis test. The delta of the respective parameter between first presentation (FP) and last follow up (LFU) did not differ between consecutive groups. G−: genotype-negative patients, G+ MYH7: genotype-positive patients with β-myosin heavy chain single-mutation, G+ MYBPC3: genotype-positive patients with myosin binding protein C single-mutation, G+ others: genotype-positive patients with cardiac troponin T2, cardiac troponin I3, α tropomyosin and myosin light chain 2 single-mutations, G+ multiple mutation: genotype-positive patients with compound mutations, °: outliers.

## 4. Discussion

The purpose of this study was to investigate genotype–phenotype association in patients with childhood onset HCM, depending on mutation type and count. Furthermore, we examined disease progression in these individuals, especially the dependency of different mutations on disease severity. Genotype–phenotype relationship and predictors for disease progression in adult patients have been previously described, but precise data of children with HCM are rare. This study adds to the body of evidence about genotype–phenotype association in patients diagnosed with HCM during childhood.

The current study revealed no differences with regard to clinical disease course and myocardial pathology comparing patients carrying a single mutation in distinct genes. These findings are in line with other studies mostly including adult HCM patients stating that mutation type is negligible for clinical phenotype and prognosis prediction [22–24]. Van Driest et al. categorized genotype-positive tested patients in myofilament-based subgroups for comparison [25]. Concomitant to the results of the current study, researchers faced difficulties to detect clear differences in clinical phenotype. Similar findings of phenotypic independency and tremendous genetic heterogeneity were also determined by comparing MYH7 with MYBPC3 mutation carrier in a study cohort of adults with HCM [26]. In contrast to those findings, authors of a pediatric HCM investigation suggest that pathogenic variants in the MYH7 gene might have a greater impact on phenotypic severity and worse clinical outcome [27]. Earlier disease onset and greater severity of HCM phenotype was identified more frequently in patients with MYH7 variants [27]. One reason for the absence of genotype–phenotype association in our study could be the small sample size of pediatric HCM patients. However, HCM is, in general, caused by rare mutations and the prevalence of variability of mutations in particular gene domains is low, which remains the challenge of detecting clear genotype–phenotype correlation [18]. Taken together, no clear genotype–phenotype relationship in pediatric HCM patients has been established based on current data. More so, data suggest that multiple other non-genetic factors and gene modifications are playing a pivotal role in phenotypic expression [22].

A major finding of the current study is that pediatric patients carrying more than one disease-causing mutation, one of which affects the troponin proteins, are at substantially increased risk for experiencing MAEs. This finding is in line with a large body of evidence derived mostly from adult studies, where HCM patients carrying compound mutations or mutations in the cardiac troponin T are expected to be at higher risk for life-threating arrythmias and SCD [28–32]. One study including adult and adolescent HCM patients reported that multiple-mutation carriers suffered more than twice as many SCDs as single-mutation carriers [28]. A pediatric study including 16 children with multiple variants also reported a higher rate of major arrhythmic cardiac events when compared to single-variant carriers [27]. In summary, data from the literature and the current study results are consistent, supporting the presumption that multiple variants are worse and affect phenotypic severity more strongly in both the adult as well as childhood onset HCM patients. This further strengthens the theory of a "gene dose effect" even in pediatric HCM. Currently, family screening for first-degree relatives of affected patients with HCM is recommended at the age of 12. The results of the current study, together with the fact that early onset HCM was described in children experiencing MAEs [33], underline the necessity of early clinical and genetic screening in young first-degree relatives to initiate risk stratification, preventing SCD in this high risk pediatric HCM cohort [34].

Patients carrying single-gene mutations encoding troponins in the absence of a second mutation did not display a higher risk for SCD and MAEs in the current study. In addition, troponin-mutation carriers were not affected by a worse myocardial phenotype or more severe disease progression compared to patients with other mutations. One reason for this could be the dilemma with a limited number of troponin T mutation carriers, similar to other studies [35], given the fact that troponin mutations are among the rather rare mutations in HCM [25]. The majority of investigations focused on HCM patients diagnosed in adulthood, complicating generalization and exploration to children with HCM. Nevertheless, the initial studies also described no malignant effect on phenotypes of troponin-mutation carriers and clinical phenotypic variability, supporting our findings [36,37]. Taken together, there is still insufficient data concerning a direct association with a worse phenotype in pediatric HCM patients carrying troponin mutations.

The cause of potentially lethal arrhythmic events such as sustained and non-sustained ventricular tachycardia is still unclear [38]. Adverse LV remodeling including myocardial fibrosis [39,40], cardiomyocyte disarray [35], myocyte automaticity [41], and increased calcium sensitivity [42] are suspected to be key factors. Additionally, rapid progression of childhood onset HCM associated with early adverse cardiac events and invasive interventions are already known [33]. We therefore also investigated whether disease progression was different in the respective groups carrying mutations affecting distinct genes. There was no difference in disease progression when comparing the study patients by their mutations. Even subgroup analysis could not reveal that certain mutations influence the course of the disease differently or worse than other mutations. Interestingly, we did not find a worse disease severity over the follow-up period neither in multiple-mutation carriers nor in patients with troponin mutation. As already known, HCM patients show variable clinical progression. Thereby, several patients remain asymptomatic or with mild symptoms over their lifetimes while others face extensive cardiac hypertrophy and further burdens, along with HCM typical anatomic features as well as remodeling processes [8,27]. Among the other components (e.g., further genetic, epigenetic, and environmental factors) influencing substantial variation, this contributes to clinically heterogeneous presentation and disease-related variability in its individual course, being an explanation for our findings. Taken together, the results of the current study could not show a detectable impact of specific genotypes on the severity of disease progression.

The major limitation of the present study is the small number of studied individuals. A general low prevalence of patients with pediatric onset HCM makes it difficult to obtain a large study cohort and to achieve an adequate informative value. A single-center design was chosen given the heterogeneity in imaging modalities, inter-observer variability in imaging data analysis, and the lack of moleculargenetic diagnosis in a multicenter setting. Additionally, the limitations of a retrospective study in general apply. Comparison to other studies was hampered by the fact that most studies investigating genotype–phenotype relationship in HCM to date have been conducted in adult patients who were not diagnosed with HCM until the age of 18. This affects the comparability of individual study results between childhood and adulthood onset of HCM. Finally, the study results cannot simply be transferred to other centers as this was a single-center study at a tertiary care university hospital.

Despite the limitations, the current study enhances a better understanding of the multifactorial nature of HCM in pediatric patients. Clinical phenotypes of patients with childhood onset HCM is heterogenous and mainly independent of distinct single mutations. As mutation-specific risk stratification still remains a challenge, further research is needed to identify predictors for severe arrhythmias, causing SCD in pediatric patients with HCM.

## 5. Conclusions

In this single center observational study, pediatric patients carrying compound mutations were at increased risk of suffering from MAEs, which drastically increased morbidity and mortality. Molecular genetic testing during childhood can identify those high-risk

children and allows for early initiation of preventive measures to avoid the occurrence of life-threatening arrhythmias and SCD.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/jcm10225256/s1, Methods S1: Molecular genetic testing, Table S1: Mutation description of molecular genetic testing of genotype-positive patients with hypertrophic cardiomyopathy, Table S2: Clinical characteristics and imaging parameter of pediatric hypertrophic cardiomyopathy patients with compound mutations, Figure S1: Disease progression Part II, Figure S2: Progress of arrhythmia in patients with pediatric onset hypertrophic cardiomyopathy.

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**Informed Consent Statement:** All patients gave written consent for the anonymous publication of their data.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# 5 Discussion

The purpose of this dissertation was to investigate VR in children and adults with CHD. There is common consensus that VR is a cardiac response to various pathological insults, such as altered loading conditions and ischemia which result in rearrangement of normally existing genetic, molecular, cellular and interstitial structures (Burchfield et al., 2013). In both ventricles, VR leads to the appearance of a complex sequence of heterogenous and multiple interrelated alterations. These alterations relate to gene expression, molecular composition, cellular signaling as well as clinically manifested changes in myocardial structure, electrophysiological conditions and cardiac function, leading to increased morbidity and mortality in affected patients (Burchfield et al., 2013). Particularly in patients with CHD a better understanding of pathomechanisms of VR is crucial to further advance therapeutics options in this vulnerable patient population.

Knowledge is limited about molecular fundamentals and cellular changes which are involved in the development of RV remodeling secondary to chronic pressure and/or volume overload (Reddy & Bernstein, 2015b). Therefore, study I focused on the identification of genetic and molecular mechanism of RV remodeling in adults with TOF and chronic pressure and/or volume overloaded right ventricles. Comparing whole transcriptome profiling of RV tissue from affected adult patients with RV tissue from infants with TOF not affected by long-term pressure and/or volume overload, bioinformatic and statistical analysis identified in total 3,010 differentially expressed genes in adults after TOF repair, which are involved in molecular signaling pathways, possibly contributing to RV remodeling. Pathway analysis highlighted cell metabolism, cell-cell communication, cell cycling, and cellular contractility being dysregulated during VR. Retinol metabolism and peroxisome proliferator-activated receptor (PPAR) $\gamma$ signaling were also identified to play a role in VR. GO Enrichment Analysis and KEGG pathway analysis showed genes associated to the contractile fiber part and actin cytoskeleton of the cell, in particular genes of myofibril, I Band, Z Disc and the contractile units of myocytes, to be significantly upregulated in adult patients with TOF and chronic hemodynamic RV stressors. Increased expression of contractile proteins in patients with pressure overloaded right ventricles was also described by others and might explain diastolic RV dysfunction in those patients (Bond et al., 2018) (Gatzoulis, Clark, Cullen, Newman, & Redington, 1995). Studies investigating myocardium of

failing left ventricles also show analogous results of changes in gene expression regarding cytoskeleton proteins and extracellular matrix proteins (Reddy & Bernstein, 2015a) (Reddy & Bernstein, 2015b) (Urashima et al., 2008). Although, there are similarities in cellular and molecular signaling pathways between the left and right ventricle, there are also numerous differences, especially in the production of ROS, energy generation and mitochondrial function (Reddy & Bernstein, 2015a) (Reddy & Bernstein, 2015b). Mitochondria play an essential role in energy generation, fatty acid metabolism, regulation of apoptosis, cell redox potential and ROS production (Palaniyandi, Qi, Yogalingam, Ferreira, & Mochly-Rosen, 2010). Dysfunctional mitochondria and imbalance in redox system with increased ROS production occur due to mechanical pressure, which is one of the main cardiac stressors, and contributes to heart failure (Palaniyandi et al., 2010). In line with this, bioinformatic analysis of significant signaling pathways showed upregulation of mitochondrion, oxidation-reduction process and ROS metabolic process. It is assumed that ROS act as stimulation factors in exosome production and affects the molecular content of exosomes (Malik et al., 2013) (Cervio, Barile, Moccetti, & Vassalli, 2015).

Exosomes are described as extracellular 40 to 150 nm diameter lipid vesicles which are secreted by cells, facilitating intercellular communication by transferring DNA, micro RNA, messenger RNA and proteins (Cervio et al., 2015) (Mathivanan, Ji, & Simpson, 2010) (Yellon & Davidson, 2014). In medical research, exosomes are of particular interest as therapeutic agents and potential biomarkers due to their unique function (Fevrier & Raposo, 2004) (Sluijter, Verhage, Deddens, van den Akker, & Doevendans, 2014). Currently it is assumed that exosomes are specifically involved in VR, influencing inflammation, interstitial fibrosis, ventricular hypertrophy and changes in contractility (Cervio et al., 2015). In study I, several molecular pathways, implicating extracellular exosome, extracellular vesicle, vesicle mediated transport and secretory vesicle, were found to be upregulated. Furthermore, gene expression analysis identified the upregulation of genes which are associated with vesicle and vesicle mediated transport. Most notably gene expression analysis also showed upregulation of typical exosome markers, including CD63 antigen (*CD63)* (p-value 0.0011) and heat shock protein 90 $\alpha$ family class A member 1 (*HSP90AA1)* (p-value 0.0018) (Yellon & Davidson, 2014). From this, it can be concluded, that vesicle and exosome, promoting intercellular communication, play a pivotal role in VR of patients with CHD. By performing proteomics, previous studies examined the content of exosomes from adult

cardiomyocytes, which contained cytosolic, mitochondrial and sarcomere proteins (Malik et al., 2013). These included α Actin, α Crystallin B chain, mitochondrial Malate Dehydrogenase and Tropomyosin (Malik et al., 2013). Concomitant to these results, we found matching genes including *ACTC1* (p-value 1.67E-06), crystallin α B (*CRYAB)* (p-value 5.81E-05), malate dehydrogenase 2 (*MDH2)* (p-value 0.0055), tropomyosin 3 (*TPM3)* (p-value 2.13E-06) and tropomyosin 2 (*TPM2)* (p-value 0.00053) to be upregulated. In line with reports stating that exosomes can also contain Annexin, Rab proteins for exosome release as well as Syndecan, which is involved in exosome formation, we found the specific genes annexin A7 (*ANXA7)* (p-value 8.92E-18), ras-related protein Rab-5A (*RAB5A)* (p-value 3.32E-09) and syndecan 2 (*SDC2)* (p-value 0.0024) to be upregulated in patients with CHD and RV remodeling (Starr, 2010) (Apitz et al., 2009).

Alterations of cell metabolism in pathological VR have been reported with variable findings regarding cardiac metabolism (Gibb & Hill, 2018) (Koop et al., 2019). Most studies have described a metabolic shift from fatty acid metabolism to a greater dependence in glucose uptake and increased glycolysis to meet cardiac energy demand in the stage of acute and chronic heart failure (Reddy & Bernstein, 2015a) (Gibb & Hill, 2018). However, others could demonstrate a decrease in glucose metabolism and increase in free fatty acid uptake and oxidation (Paolisso et al., 1994) (Taylor et al., 2001) (Sabbah & Stanley, 2002). This might be explainable by the fact that first, more free fatty acids are available as substrate for energy metabolism in cardiac tissue and second, that the myocardium uses free fatty acids to compensate deficiency in energy metabolism. Experiments with trimetazidine and ranolazine, aiming to inhibit fatty acid oxidation and thus reduce RV remodeling, were successful in animal models (Fang et al., 2012). Gene expression analysis of study I also detected upregulation of fatty acid metabolism in adults with TOF and RV remodeling. One reason for this could be the differences in disease severity, since most of the studies conducted, investigated failing left and right ventricles instead of impaired ventricles without heart failure as in our study. Reinforcing, studies outlined an existing correlation between functional impairment and fat utilization, indicating decreased fatty acid metabolism as a phenomenon of end-stage heart failure (Gibb & Hill, 2018). Hence, further research is needed to gain closer insight in changes of metabolic profiles during VR in patients with CHD.

Dysregulation of PPAR signaling pathways in the connection of VR, myocardial hypertrophy and heart failure was described before (van Bilsen, Smeets, Gilde, & van der Vusse, 2004). Consistent to previous studies, we found PPAR signaling pathways, mostly PPAR γ (p-value 2.03E-08), being upregulated (Asakawa et al., 2002). PPARs are known as nuclear receptor transcription factors which are activated by specific ligands, such as long chain fatty acids (Finck, 2007). Three PPAR isoforms (α, β/δ and γ) contribute to cardiac metabolic processes, in particular lipid, fatty acid and glucose homeostasis, cell differentiation and control of inflammation by regulating gene expression (Finck, 2007) (Osorio et al., 2002) (Warren, Oka, Zablocki, & Sadoshima, 2017) (Ahmadian et al., 2013). Research on cardiac metabolic remodeling so far has focused primarily on PPAR α, but few studies delineated a potential anti-inflammatory effect of PPAR γ, related to systemic impact (Ahmadian et al., 2013). Taken together, the function of PPAR γ and its role in VR of patients with CHD require further research.

By forming a nuclear heterodimeric complex with retinoid X receptor (RXR) and binding to specific promoter regions of the target genes, PPARs regulate gene expression (Warren et al., 2017) (Ahmadian et al., 2013). It also has turned out that RXR is one of the major genes involved in retinol metabolism. Apart from *RXR* (p-value 0.0051), KEGG pathway analysis revealed further genes, such as retinol dehydrogenase 10 (*RDH10)* (p-value 0.00076), *ADH1B* (p-value 4.93E-11), alcohol dehydrogenase 1C (*ADH1C)* (p-value 0.00037) and cytochrome P450 family 1 subfamily A member 1 (*CYP1A1)* (p-value 1.01E-05), which are equally involved in retinol metabolism, to be significantly upregulated. One function of retinol metabolism is to ensure cardiac supply of various forms of all trans retinoic acids, utilizing vitamin A and its derivates (Bilbija et al., 2012). As ligands all trans retinoic acids bind to nuclear receptors like RXR or retinol acid receptor, promoting the formation of receptor heterodimers. Receptor heterodimers bind to specific regulator sequences of the DNA, thus regulating the frequency of gene expression and the modification of the interconnection with other signaling pathways. There is emerged evidence that retinoic acid levels are diminished in the failing animal heart (Yang et al., 2021). But interpretation of these results remains difficult since adult patients after TOF repair were not in stage of heart failure. Others reported an association between reduction of retinoids and diastolic dysfunction as well as increased oxidative stress in mouse myocardium (Zhu et al., 2016). However, activation of retinoic acid signaling in LV remodeling and ischemia

after myocardial infarction in mice has also been described, hypothesizing implication of retinoids in cardiac regeneration (Bilbija et al., 2012). Based on findings of previous studies and our results, speculations exist that dysregulation of retinol metabolism occurs in VR and plays a previously unimagined role in both ventricles as response to cardiac damage and repair. More so, simultaneous overexpression of genes involved in fatty acid metabolism, PPAR signaling pathway and retinol metabolism strength the presumption of interdependency of detected signaling pathways in patients with VR (van Bilsen et al., 2004).

Study I also revealed downregulation of mitotic cell cycle, cell division and DNA replication. In particular, we found reduced expression of genes involved in DNA binding transcription factor activity, transcription regulator activity and RNA polymerase 2 transcription factor activity, representing decreased cell cycling in adult patients with VR. The presumption of a declined cell proliferation and maintenance was supported by significant downregulation of gene expression regarding nucleus, chromosome, protein-DNA complex, spindle and replication fork. Downregulation of cell cycling and the relative loss of cell proliferation of normal functioning cardiomyocytes lead to a disastrous lack of myocardial regeneration (Mohamed et al., 2018). In addition, it has been found that cardiac tissue loses postnatal the ability to reentry cell cycle for cell proliferation and differentiation, due to the fact that differentiated cells, such as cardiomyocytes, become post-mitotic and exit the cell cycle (Mohamed et al., 2018) (Bicknell, Coxon, & Brooks, 2007). As a consequence, the myocardium encounters difficulties to adapt to pathological pressure and/or volume overload. Resulting impairment of cardiac injury repair and persisting cardiac hypertrophy increases the risk for morbidity and mortality in those patients (Woo et al., 2006) (Valente et al., 2014). The incapacity to enter cell cycle, guaranteeing cell proliferation, injury regeneration as well as preservation of ventricular function, aggravate pathophysiologic conditions and encourage development of VR. Thus, prevention of cardiomyocytes leaving cell cycle and becoming post-mitotic, could be a promising approach for innovative therapeutic strategies to improve cardiac regeneration and attenuate VR (Woo et al., 2006). Studies already examined potential key players in cardiac cell cycle, demonstrating cyclin dependent kinase 1 (*CDK1)* and cyclin dependent kinase 4 (*CDK4)* which regulate the transition through G1/S phase and efficient progression through G2/M phase (Mohamed et al., 2018). Concomitant with this, study I showed cell cycle G1/S phase transition, regulation of cell cycle phase

transition, cell cycle G2/M phase transition and the genes *CDK1* (p-value 5.05E-05), cyclin dependent kinase 6 (*CDK6)* (p-value 2.20E-09) and cyclin dependent kinase 14 (*CDK14)* (p-value 0.00021) to be downregulated. Significant downregulation of the gene CDK4 could not be detected in our study. By induced expression of Cyclin A2 (*CCNA2*), a cell cycle regulator activating cardiomyocyte proliferation, induction in myocardial regeneration and enhancement of cardiac function in failing rat hearts has been shown (Woo et al., 2006). We also found gene expression of *CCNA2* to be significantly downregulated (p-value 0.0065), which accordingly could be an interesting approach for therapeutic strategies. Overall, gene expression and signaling pathway analysis of study I successfully identified molecular characteristics of VR in patients with CHD.

In recent years, it has been found out that remodeling of the left ventricle occurs in patients with HCM (Musumeci et al., 2021). It is characterized by an interplay of multiple pathophysiological mechanisms, including genetic background, alteration in LV architecture, myocardial fibrosis and ischemia in the presence of severe LV hypertrophy (Musumeci et al., 2021). There is consensus that there is an association between the patterns of VR and their specific impact on the clinical course of affected patients (Musumeci et al., 2021). In individuals with pediatric onset HCM and LV remodeling, data concerning phenotypic manifestation and clinical impact of VR is still insufficient. So far, only few studies investigating genotype-phenotype association and predictors of clinical course in patients with HCM and VR exist, but precise data of children are rare. Therefore, study II examined genotype-phenotype association and clinical disease course as VR manifestation in the form of structural and electrophysiological changes. To the best of our knowledge, study II is the first, evaluating ECG, TTE and CMR data to this extent as well as investigating disease progression in pediatric patients with early onset HCM and VR, depending on different genetic variants.

The results of study II demonstrated no differences regarding clinical disease course and myocardial pathology in dependency of different genetic variants in patients with single mutations. These findings are in accordance with current studies, mostly including HCM patients diagnosed in adolescence. For example, one study also failed to demonstrate a significant difference in clinical parameter among adult patients carrying mutations belonging to the intermediate filament (*MYBPC3*), the thin filament

(*ACTC, TNNT2, TNNI2, TPM1*), the thick filament (*MYH7, MYL2, MYL3*) and genotype-negatives (Van Driest et al., 2004). When comparing individuals with mutations in proteins of the thin filament and patients with mutations in proteins of the thick filament, we also could not detect any. Furthermore, others were unable to discern clear clinical phenotype by comparing symptomatic individuals carrying *MYH7* to symptomatic individuals carrying *MYBPC3* mutations (Viswanathan et al., 2017). Even the comparison of symptomatic carrier with asymptomatic carrier failed to detect significant phenotypic differences (Viswanathan et al., 2017). Concomitant to our results, authors of a systematic review and meta-analysis of genotype-phenotype association faced difficulties to report clinical differences between *MYH7* and *MYBP3* mutation carrier (Lopes, Rahman, & Elliott, 2013). In contrast, one study including children with HCM reported an association between pathological variants in the *MYH7* gene and earlier disease onset as well as greater phenotypic severity (Mathew et al., 2018). The small sample size of pediatric patients in our study cohort could be one reason for lacking significance in genotype-phenotype association. However, clear detecting of genotype-phenotype association is still challenging for pediatric onset HCM patients as it is a seldom disease, caused by only rare mutations (Marian & Braunwald, 2017). High variability of mutations in different domains of the causative genes could also result in HCM being a genetically or phenotypically heterogeneous disease (Marian & Braunwald, 2017). Additionally, significant phenotypic variability and utmost heterogenous clinical courses of HCM patients have been discovered, indicating that multiple other non-genetic factors and gene modifications are also responsible to influence phenotypic expression of VR (Musumeci et al., 2021) (Landstrom & Ackerman, 2010) .

One of the major findings of study II is the fact that patients with pediatric onset HCM and compound mutation are at substantially increased risk to develop MAE compared to single mutation carrier. Interestingly, all of these multiple mutation carriers had one disease causing variant, which affected the troponin proteins. Based on that, we assume that electrical remodeling of the left ventricle might be worse in patients with more than one mutation. In line with previous studies, a more frequent occurrence of life-threatening arrhythmic events and experience of SCD in patients carrying multiple mutations and genetic variants in the cardiac troponin T, has been reported (Ingles et al., 2005) (Moolman et al., 1997) (Maass, Ikeda, Oberdorf-Maass, Maier, & Leinwand, 2004) (Girolami et al., 2010). In an Australian study cohort, investigator found out that

multiple mutation carrier suffered more than twice as many SCD events as individuals with single variants (Ingles et al., 2005). From this, researcher concluded the presence of a more sever clinical phenotype in individuals with more than one mutation, due to the “double dose effect”. These findings also highlight the importance of performing an extended panel diagnostic in HCM patients to identify possible multiple mutation carrier. A higher risk of SCD due to malignant ventricular arrythmias and a predisposition for developing a more severe phenotype has also been described by others (B. J. Maron, Maron, & Semsarian, 2012). Apart from earlier positive phenotypic presentation, children with multiple mutation indicated earlier and more frequent occurrence of MAEs compared to individuals with single mutation (Mathew et al., 2018). Taken together, results of the literature and study II support the presumption that the presence of multiple variants leads to a higher disease burden in both, patients with HCM onset in childhood and adulthood. Results of study II also strengthen the theory of a “gene dose effect” not only in adults but also in children with HCM, probably impacting VR (Keren et al., 2008). Currently, there is the recommendation for molecular genetic testing as family screening for first degree relatives of affected HCM patients at the age of 12 (Gersh et al., 2011b). In contrast, results of study II and the current literature draw attention to the necessity of rather earlier clinical and molecular genetic testing of these young high-risk patients to initiate risk stratification, prevent MAEs and reduce SCD (Miron et al., 2020). The need for early as possible family screening for first-degree relatives is supported by others (Lafreniere-Roula et al., 2019). It has been reported that current screening guidelines miss early onset HCM since one third of affected patients did not met the criteria for early screening as well as the fact that these patients experience MAEs already in young age, support our findings (Lafreniere-Roula et al., 2019).

In general, mutation distribution of affected genes was similar to previously reported studies (Seidman & Seidman, 2011) (Richard et al., 2003). Pathogenic variants were mostly found in the genes for *MYH7* and *MYBPC3*, followed by genetic variants in *TNNT2*, *TPM1*, *MYL2* and *TNNI3*. Particularly mutations in genes encoding for cardiac troponins are reported to be rare and are assumed to influence the individual phenotype more strongly and worse than other mutations (Coppini et al., 2014). Indications exist that genetic variants in the cardiac troponin T might be associated with mild LV hypertrophy but enhanced potential of malignant arrhythmias with higher risk of SCD (Moolman et al., 1997). One study could demonstrate that the presence of

a mutation in the cardiac troponin T leads to enhanced arrhythmia susceptibility through increased calcium sensitivity in the myofilament of mice, even in the absence of hypertrophy and fibrosis (Baudenbacher et al., 2008). Mutations in *TNNI3* are described with the development of LV restrictive phenotypes and poor prognosis (Kubo et al., 2007). In general, mutations in genes coding for the thin filament indicate an earlier onset of disease, significant diastolic dysfunction and atypical localization of LV hypertrophy (Watkins et al., 1995). In contrast, no detectable difference on myocardial phenotype or more severe disease progression was found in our study by comparing troponin mutation carrier with patients carrying non-troponin mutations. Especially in terms of MAEs, troponin mutation carrier did not display a higher risk of arrhythmic events but compared to multiple mutation carrier they had less arrhythmias. Again, this result further underlines the assumption that mutation count worsens myocardial phenotype and affects VR, becoming more adverse. Since troponin mutations rank among the rather rare mutations in HCM (Van Driest et al., 2004), the dilemma of a limited number of patients carrying troponin mutations could be one reason for the contradictory results, similar to other studies (Varnava et al., 2001). Comparison, generalization and exploration of current results to children remain complicated due to the fact that major studies investigated HCM patients, diagnosed in adulthood. Nevertheless, no malignant effect on phenotypes of troponin mutation carrier and clinical phenotypic variability was also described by others, supporting our findings (Menon et al., 2008) (Kokado et al., 2000). In summary, further research is needed to make a clear statement on direct association between clinical phenotype of pediatric patients with mutations in cardiac troponins.

Sustained and non-sustained ventricular tachycardia account to life-threatening arrhythmias increasing morbidity and mortality in HCM patients (Monserrat et al., 2003) (McKenna et al., 1981). Especially in the young, SCD is the main cause of mortality, triggered by malignant arrythmias (Musumeci et al., 2021). So far, causes of potentially lethal arrhythmic events remain unclear (Sedaghat-Hamedani et al., 2018). Adverse VR is suspected playing a crucial role in the development of malignant arrhythmias due to myocardial fibrosis (Wolf & Berul, 2008) (Ho et al., 2010), cardiomyocyte array (Varnava et al., 2001), myocyte automaticity (Saeed et al., 2000) and increased calcium sensitivity (Schober et al., 2012). In addition, early adverse cardiac events and the need for invasive interventions are assumed to be associated with rapid progression of childhood onset HCM (Lafreniere-Roula et al., 2019). Therefore, study

II additionally investigated whether clinical course changes over the follow up period in pediatric HCM patients, depending on mutation type and count. We found no significant differences in disease progression or evidence that certain affected genes influence the clinical course differently when comparing the study patients by their distinct mutations. Even subgroup analysis did not reveal a worse disease severity in multiple mutation or troponin mutation carrier. The fact of a wide spectrum of clinical presentation and individual variability of disease severity in HCM patients is well known (Musumeci et al., 2021). So, clinical course ranges from asymptomatic patients to patients with mild symptoms over lifetime and reaches up to individuals developing severe phenotypes with malignant arrhythmias, SCD and progressive heart failure (Wolf, 2019) (Mathew et al., 2018). While most patients show stable morphologic features and clinical phenotypes with relatively favorable life expectancy, some others face progressive changes regarding LV wall thickness, cavity size, fibrotic replacement, formation of aneurysm and LV dysfunction (Musumeci et al., 2021). An explanation for our results might be the interplay of further genetic, epigenetic and environmental factors, which contribute to clinical heterogeneity and influence substantially disease-related variability. Furthermore, typical anatomical features of HCM and VR processes may occur imperceptibly and develop slowly and gradually over years or decades, suggesting that our observation period might not have been sufficient. Disease-causing mutations have been reported to have multiple adverse effects on cardiomyocytes, further promoting variability in clinical phenotype (Musumeci et al., 2021). Apart from complex genetic background influencing LV hypertrophy and electrical pathophysiology, vascular and myocardial modifications, causing ischemia and fibrosis, are additionally responsible for VR (Musumeci et al., 2021). From this it can be concluded that VR and its clinical course is impacted by the interaction of multiple mechanism.

## 5.1 Limitations

TOF is the most common cyanotic heart defect and HCM ranks among the most common inheritable cardiovascular diseases. Nevertheless, the prevalence of CHD, including TOF and pediatric-onset HCM, is generally low, making it difficult to obtain large study cohorts. Therefore, both studies are limited due to small sample sizes, leading to limited statistical power, adequate informative value and the feasibility of further subgroup analysis. Insufficient amount of RV tissue and the lack of clinical patient-related data further compromised inclusion of eligible patients. For instance, examinations such as CPET and CMR were not performed immediately before hospitalization or generally in each patient and were not repeated during the follow up period if not indicated. Both studies carried out are single center studies, being another limitation. In view of heterogeneity of imaging modalities, interobserver variability in imaging data analysis and the lack of molecular genetic diagnosis in a multicenter setting, a single center was chosen. Retrospective study design was chosen as both studies involved rather rare diseases. Therefore, limitations of retrospective study design apply to both studies, complicating interpretation and generalization of study results to larger populations.

A major limitation of study I is the unavailability of RV tissue samples from healthy patients without VR due to structural heart disease and myocardial burden as a control group. One possibility to gain reference material would have been the use of a non-transplantable donor heart. RV tissue samples could have been removed and whole transcriptome profiling performed, but there are essential disadvantages which have to be considered. First, exposure of donor hearts discarded for organ transplantation to ample factors, affecting gene expression results in donor hearts, that are not completely healthy (Lowes et al., 1997). Thus, there is no guarantee that non-transplantable donor hearts ensure healthy tissue samples. Second, it is a rarity that a donor heart is rejected nowadays. Finally, it should be clearly noted that timing issues and operational complications could further negatively impact whole exome transcription and its results. For this reason, study I focused on altered gene expression patterns contributing to VR in chronic volume and/or pressure overloaded right ventricles over lifetime in patients with CHD. Infant patients with the same underlying structural heart disease were selected as a control group, since the right ventricle was exposed to minimal hemodynamic stressors for only a short time. Due to age differences in the compared groups, we identified alleged genes playing a role in

aging with the help of GEO. Thereby, ventricular cardiac tissue samples of two 20-week-old patients were compared to samples of a 59 years and 53 years old patient. Genes showing a switch during aging process, identic to our gene expression data were excluded for further analysis. However, it should be noted that there is evidence for reactivation of fetal gene programs in pathological cardiac conditions (Verma et al., 2013). Consequently, the exclusion of genes seemed to be involved in regular aging processes might lead to the loss of essential information regarding gene expression changes during VR.

Another limitation relates to study II. The fact that studies investigating genotype-phenotype association in HCM are mostly from patients diagnosed after the age of 18 years, complicates the comparability of individual study results, particularly of patients with childhood onset HCM.

# 6 Conclusion and research perspective

This dissertation analyzed molecular patterns and clinical manifestation of VR in children and adults with CHD. In conclusion, the first study characterized molecular and cellular patterns of RV remodeling by whole transcriptome profiling. Total RNA sequencing effectively identified differentially expressed genes and molecular signaling pathways in adult patients with RVI and VR due to long-term sequelae of CHD. Especially cell-cell communication, extracellular vesicle including exosomes, and retinol metabolism were shown to be involved in pathological VR. In order to discover upcoming biomarkers and develop new therapies, the identification of differentially expressed genes and molecular signaling pathways is crucial. In the future, this could enable the development of targeted therapies to prevent VR in patients with CHD, supporting individualized, signaling-targeted patient treatment. In particular, further investigation of cell-cell communication and exosomes is necessary and recommended as it could provide essential information to facilitate risk stratification, to improve disease monitoring and to attenuate VR.

The impact of underlying genetic mutations on LV remodeling and clinical outcome in patients with pediatric onset HCM was investigated in the second study. Analogous to previous studies with mostly derived data from adults with HCM, we faced difficulties to detect clear relationship between clinical presentation as a function of distinct disease-causing and associated VR-causing mutation. Due to the small sample size, it is recommended to analyze the clinical manifestation of VR and disease progression in a larger cohort of patients with pediatric onset HCM at multiple centers. However, it has been demonstrated that multiple-mutation carriers show a higher risk for the occurrence of MAEs compared to patients with one disease-causing mutation. This finding assumes that carrier of multiple mutations may be more affected by pathological electrical remodeling than carrier of a single mutation, further supporting the hypothesis of a “gene dose effect” on VR and clinical presentation. Based on this, standardly performed early molecular genetic testing of children with HCM could help to identify these high-risk patients, allowing early risk-stratification and initiation of preventive measures to avoid the occurrence of life-threatening arrhythmias and SCD. Furthermore, it would be of great interest to investigate a large cohort of individuals with multiple mutations with respect to clinical outcome parameters and imaging parameters. In particular, with regard to the question whether certain mutation combinations lead to increased arrhythmia susceptibility.

In conclusion, this dissertation enhances the current knowledge of VR in children and adults with CHD. The characterization of the molecular and cellular patterns as well as the clinical manifestations of affected patients contribute to a better understanding of the complex interplay of the various factors involved in VR. Further research and ongoing clinical investigations of children and adults with CHD and VR is needed to identify and characterize the molecular basis and clinical impact of cardiac remodeling to reduce morbidity and mortality in these patients.

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# 8 Appendix

## 8.1 Appendix 1

### 8.1.1 Supplementary of the original article „Molecular signaling pathways in right ventricular impairment in adult patients after tetralogy of Fallot repair”

Supplementary

## Appendix 1

**Table of contents:**




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## Methods

### *Patients Characteristics*

Of all patients with a primary diagnosis of tetralogy of Fallot (TOF) undergoing surgical treatment at the German Heart Center Munich between April 2009 and May 2016, RV tissue and a signed informed consent were available in the institutional biomaterial bank from 31 patients (Figure 1). Surgeries included corrective repair for infants or elective replacement of the right ventricular to pulmonary artery conduit for adult patients, due to long-term consequences of hemodynamic abnormalities. Demographic, clinical, electrocardiographic data, transthoracic echocardiography (TTE), chest x-ray, cardiovascular magnetic resonance imaging (CMR), computed tomography (CT) scans and catheterization data were collected by retrospective chart review and were analyzed. Based on clinical information, clinical status with distinct signs of RVI and RV function was assessed, following the definition of adult and infant patient groups.

The criteria for RVI were selected based on the International Right Heart Foundation Working Group recommendations (1) and the scientific statement of the American Heart Association (2). These included clinical parameters such as functional capacity (New York Heart Association (NYHA) classification), peripheral edema, and engorgement of jugular veins, enlargement of liver, dyspnea and cyanosis. Serum markers like reduced GFR, increased creatinine and blood urea nitrogen were selected, in order to evaluate the cardiorenal abnormalities. For cardiohepatic abnormalities, elevated blood levels of bilirubin, ɣ-glutamyl transpeptidase (ɣ-GT) and alkaline phosphatase (AP) were chosen. The assessment of RV size and function was examined with (TTE) and CMR. Parameters included right ventricular ejection fraction (RVEF), left ventricular ejection fraction (LVEF), pulmonary trunk regurgitation fraction, right ventricular stroke volume (RVSV), right ventricular enddiastolic volume index (RVEDVI), right ventricular endsystolic volume index (RVESVI), evidence of moderate to severe pulmonary regurgitation, maximum velocity over pulmonic valve (PV V max), mean pressure gradient over pulmonic valve (PV mean PG), maximum pressure gradient over pulmonic valve (PV max PG), right ventricular hypertrophy and increased right ventricular pressure (RVP).

RV tissue from infants with TOF obtained at the time of corrective surgery during infancy was selected for comparison due to the similar underlying structural heart defect in this group and due to the lack of long-standing right ventricular hemodynamic stressors. Patients with confirmed genetic diseases, additional syndromes and any other organ failure were excluded.

### *RNA Isolation and Quality Assessment*

RV tissue was collected during surgical procedures. Immediately after tissue removal, all samples were frozen in liquid nitrogen and stored at -80 °C until RNA isolation. Following manufacturer's instructions, total RNA was isolated using miRNeasy Mini Kit and QIAcube robotic workstation (Qiagen, Hilden, Germany) at the Institute of human genetics at Helmholtz Centre Munich, Germany. Quantity of RNA was assessed by measuring the concentration of isolated total RNA, using NanoDrop 2000 spectrophometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States). RNA purity was estimated by examining the 260/280 ratio, as recommended by the manufacturer (Thermo Fisher, Scientific, Waltham Massachusetts, Unites States). Quality of the isolated RNA was estimated by determination of RNA integrity number (RIN), according to manufacturer's specifications.

### *Total RNA Sequencing*

Whole transcriptome analysis was performed by total RNA sequencing of cardiac tissue samples as previously described (3). RNA library was prepared by using 1 µg of RNA, which was poly (A) selected, fragmented and reverse transcribed with Elute, Prime and fragment mix adhered to Illumina's information (Illumina, San Diego, California United Stated). Subsequently, tailing, adaptor ligation and library enrichment was done, following manufacturer's recommendation of TruSeq Stranded mRNA Sample Prep Guide (Illumina, San Diego, California, United States). Quality and Quantity of the RNA library were estimated by Agilent 2100 Bioanalyzer and Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). RNA sequencing of 100 bp paired-end runs was performed with Illumina HiSeq 4000 platform (Illumina, San Diego, California, United States). A proximate alignment against human genome assembly hg19 (GRCh37) and UCSC known gene annotation was done by using STAR aligner (v2.4.2a) (4). Quantification of the number of reads mapping to annotated genes was accomplished by using HT-seq count (v0.6.0), in which fragments per kilobase of transcript per million fragments mapped (FPKM) were selected as the unit


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of measurement (5). Utilizing R Bioconductor package DESeq2 differential gene expression analysis was completed (6), followed by pathway- and Gene Set Enrichment Analysis with R Bioconductor package gage (7), pathview (8) and goseq (9). Tools like Gene Ontology (GO) (Department of Genetics, Stanford University School of Medicine, Stanford, California, United States.) (10,11), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Institute for Chemical Research, Kyoto University, Uji, Kyoto, Japan) (12), ToppFun (Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, United States) (13) and PANTHER Classification System (14) were applied to validate pathway analysis, biological activity and allocation of individual genes to Gene Ontology categories (Figure 2 and Figure S2).

The total of 3,010 differentially expressed genes were compared to 50 most significant genes, regulating cardiac development and heart maturation, which were identified by RNA sequencing of murine cells during embryonic and postnatal period. Overlapping genes were not included in further interpretation of results (15) (Figure S1).

### *Validation of selected genes with Western blot*

Total proteins, isolated from RV myocardial tissue from patients, were separated by 8-12% SDS polyacrylamide gel electrophoresis using Mini-Protean 3 system (Biorad), as previously described (16,17). Following separation, proteins were transferred to nitro-cellulose membranes, and incubated with one of the following primary antibodies: alcohol dehydrogenase 1B (ADH1B, Abcam, Cambridge, UK; ab175515, RRID: N/A), monoamine oxidase B (MAOB, Merck, Darmstadt, Germany; ST1582, RRID: AB_10617089), peroxisome proliferator-activated receptor gamma (PPARγ, Cell Signaling, Frankfurt, Germany; S4946, RRID: AB_2166051), superoxide dismutase 3 (SOD3, Merck, Darmstadt, Germany; S4946, RRID: AB_532286), interleukin 6 receptor (IL6R, R&D Systems, Wiesbaden-Nordenstadt, Germany; MAB227, RRID: AB_2127908), or β-actin (Santa Cruz, Heidelberg, Germany, SC-1616, RRID: AB_630836). Goat anti-rabbit, goat anti-mouse or rabbit anti-goat secondary antibodies were used (Merck, 401253, RRID: AB_437779; 401393, RRID: AB_437797; 401515, RRID: AB_437816). Following enhanced chemiluminescence reaction, bands were quantified using ImageJ. Two-tailored student's test was used for statistical evaluation. Data are presented as a mean ± standard deviation.

## Ethical Approval

The study was approved by the institution's ethical committee at the Technical University of Munich (approval 10/16/2017, number 242/17S, and approval 01/11/2017, number 592,16S). The study protocol conforms to the ethical standards of the Declaration of Helsinki 1975. All patients or parents gave written informed consent before enrollment.

## Statistics

Statistic evaluation was implemented by applying R Bioconductor package DEseq2 to transcriptome profiles and tested for differential gene expression between adult and infant patients. The P values were corrected for the purpose of multiple testing by Benjamini and Hochberg procedure. The level of significance was set at a P value of less than 0.01 and a fold-change value of greater than 2 or less than –2. Based on that, all significant differentially expressed genes were selected for further analysis. The results of Gene Ontology and Pathway analysis with an adjusted P value less than 0.01 (GO) and a P value less than 0.05 (KEGG) were assigned as significant.


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**Online Figures:**

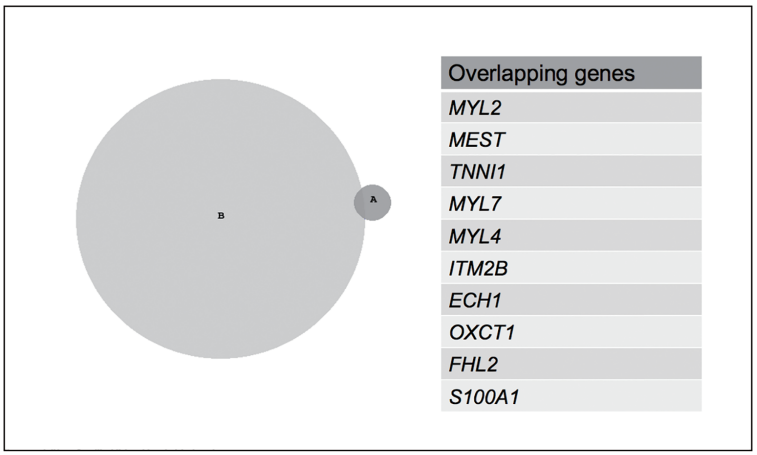


**Figure S1** Venn diagram demonstrating the intersection of differentially expressed genes. The total of 3,010 genes differentially expressed between infants and adults with right ventricular impairment are represented by B. The top 50 genes being potential markers for cardiac development and heart maturation are illustrated by A. Genes identical expressed in both studies are listed on the right. These 10 genes were identified as overlapping genes playing a role during heart development and were therefore excluded for further analysis. The complete list of the 50 developmental markers is shown in Table S9.


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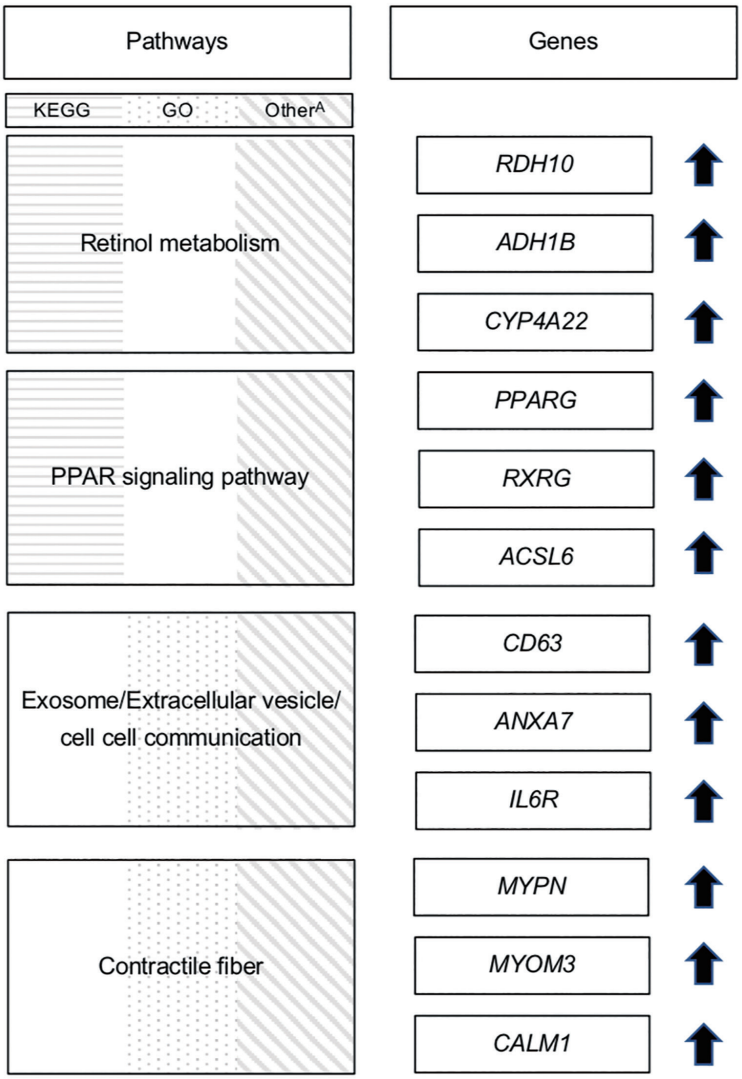


**Figure S2** Summary of the results in the form of an overview of the modified signaling pathways with associated genes and their regulation. A, used validation tools like PANTHER Classification, ToppFun. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; PPAR, peroxisome proliferator-activated receptor.


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## Online Tables:

**Table S1** Presentation of the 10 most significant upregulated GO terms for cellular component in adult patients with CCHD and RVI

| Category | Term | P value | Adjusted P value |
|---|---|---|---|
| GO:0044444 | Cytoplasmic part | 3.74E-33 | 8.36E-29 |
| GO:0005737 | Cytoplasm | 1.23E-29 | 1.37E-25 |
| GO:0030016 | Myofibril | 2.61E-18 | 1.95E-14 |
| GO:0043292 | Contractile fiber | 2.36E-17 | 1.32E-13 |
| GO:0030017 | Sarcomere | 1.24E-16 | 5.53E-13 |
| GO:0044449 | Contractile fiber part | 2.68E-16 | 1.00E-13 |
| GO:0005829 | Cytosol | 4.18E-15 | 1.34E-11 |
| GO:0070062 | Extracellular exosome | 3.81E-14 | 1.06E-10 |
| GO:1903561 | Extracellular vesicle | 8.00E-14 | 1.68E-10 |
| GO:0043230 | Extracellular organelle | 9.03E-14 | 1.68E-10 |

GO, Gene Ontology.

**Table S2** Presentation of the 10 most significant upregulated GO terms for molecular function in adult patients with CCHD and RVI

| Category | Term | P value | Adjusted P value |
|---|---|---|---|
| GO:0008092 | Cytoskeletal protein binding | 5.69E-14 | 1.41E-10 |
| GO:0016491 | Oxidoreductase activity | 8.96E-12 | 1.34E-08 |
| GO:0003824 | Catalytic activity | 3.99E-11 | 3.72E-08 |
| GO:0005515 | Protein binding | 2.10E-10 | 1.45E-07 |
| GO:0003674 | Molecular_function | 8.28E-09 | 3.63E-06 |
| GO:0061134 | Peptidase regulator activity | 4.40E-08 | 1.61E-05 |
| GO:0003779 | Actin binding | 1.18E-07 | 3.66E-05 |
| GO:0005488 | Binding | 1.36E-07 | 4.11E-05 |
| GO:0004857 | Enzyme inhibitor activity | 2.11E-07 | 6.05E-05 |
| GO:0061135 | Endopeptidase regulator activity | 2.39E-07 | 6.69E-05 |

GO, Gene Ontology.


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**Table S3** Presentation of the 10 most significant upregulated GO terms for biological processes in adult patients with CCHD and RVI

| Category | Term | P value | Adjusted P value |
|---|---|---|---|
| GO:1901564 | Organonitrogen compound metabolic process | 8.61E-14 | 1.68E-10 |
| GO:0044281 | Small molecule metabolic process | 7.13E-12 | 1.14E-08 |
| GO:0055114 | Oxidation-reduction process | 1.44E-11 | 2.02E-08 |
| GO:0002283 | Neutrophil activation involved in immune response | 1.72E-11 | 2.26E-08 |
| GO:0002446 | Neutrophil mediated immunity | 1.82E-11 | 2.26E-08 |
| GO:0043312 | Neutrophil degranulation | 2.33E-11 | 2.60E-08 |
| GO:0042119 | Neutrophil activation | 2.69E-11 | 2.86E-08 |
| GO:0036230 | Granulocyte activation | 3.56E-11 | 3.46E-08 |
| GO:0016192 | Vesicle-mediated transport | 6.82E-11 | 6.10E-08 |
| GO:0006887 | Exocytosis | 1.03E-10 | 8.85E-08 |

GO, Gene Ontology.

**Table S4** Presentation of the 10 most significant downregulated GO terms for cellular component in adult patients with CCHD and RVI

| Category | Term | P value | Adjusted P value |
|---|---|---|---|
| GO:0005694 | Chromosome | 8.57E-28 | 1.92E-23 |
| GO:0005634 | Nucleus | 1.05E-26 | 1.17E-22 |
| GO:0044427 | Chromosomal Part | 6.44E-24 | 3.60E-20 |
| GO:0044815 | DNA packaging complex | 2.95E-23 | 9.35E-20 |
| GO:0000786 | Nucleosome | 1.82E-21 | 3.71E-18 |
| GO:0032993 | Protein-DNA complex | 4.64E-21 | 8.66E-18 |
| GO:0044428 | Nuclear part | 7.83E-19 | 1.35E-15 |
| GO:0031981 | Nuclear lumen | 3.36E-18 | 5.37E-15 |
| GO:0005654 | Nucleoplasm | 2.77E-17 | 3.65E-14 |
| GO:0000228 | Nuclear chromosome | 1.48E-15 | 1.58E-12 |

GO, Gene Ontology; DNA, deoxyribonucleic acid.


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**Table S5** Presentation of the 10 most significant downregulated GO terms for molecular function in adult patients with CCHD and RVI

| Category | Term | P value | Adjusted P value |
|---|---|---|---|
| GO:0003677 | DNA binding | 5.29E-22 | 1.18E-18 |
| GO:0003676 | Nucleic acid binding | 1.45E-13 | 9.80E-11 |
| GO:0097159 | Organic Cyclic compound binding | 2.43E-09 | 6.63E-07 |
| GO:1901363 | Heterocyclic compound binding | 3.36E-09 | 8.73E-07 |
| GO:0003700 | DNA binding transcription factor activity | 1.14E-07 | 2.16E-05 |
| GO:0043142 | Single-stranded DNA-dependent ATPase activity | 7.75E-07 | 0.00013 |
| GO:0140110 | Transcription regulator activity | 1.52E-06 | 0.00023 |
| GO:0000981 | RNA Polymerase 2 transcription factor activity, sequence-specific DNA binding | 2.39E-06 | 0.00035 |
| GO:0042393 | Histone binding | 2.43E-06 | 0.00036 |
| GO:0031492 | Nucleosomal DNA binding | 3.18E-06 | 0.00044 |

GO, Gene Ontology; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; RNA, ribonucleic acid.

**Table S6** Presentation of the 10 most significant downregulated GO terms for biological processes in adult patients with CCHD and RVI

| Category | Term | P value | Adjusted P value |
|---|---|---|---|
| GO:0007049 | Cell Cycle | 4.93E-26 | 3.60E-20 |
| GO:1903047 | Mitotic cell cycle process | 1.33E-23 | 5.54E-20 |
| GO:0022402 | Cell cycle process | 1.49E-23 | 5.54E-20 |
| GO:0051276 | Chromosome organization | 2.29E-22 | 6.40E-19 |
| GO:0000278 | Mitotic cell cycle | 2.88E-22 | 7.16E-19 |
| GO:0090304 | Nucleic acid metabolic process | 3.76E-18 | 5.61E-15 |
| GO:0006259 | DNA metabolic process | 1.15E-17 | 1.61E-14 |
| GO:0006261 | DNA-dependent DNA replication | 1.20E-16 | 1.49E-13 |
| GO:0140014 | Mitotic nuclear division | 3.79E-16 | 4.47E-13 |
| GO:0051301 | Cell division | 6.13E-16 | 6.86E-13 |

GO, Gene Ontology; DNA, deoxyribonucleic acid.


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**Table S7** Upregulated genes matching to the selected differentially expressed signaling pathways in adult patients with CCHD and RVI

| | P value | Gene | P value | Adjusted P value | Regulation |
|---|---|---|---|---|---|
| Gene Ontology (PANTHER) | | | | | |
| Extracellular exosome; GO:0070062 | 3.81E-14 | APOB | 3.69E-25 | 2.27E-22 | Up |
| | | SERPINB6 | 6.42E-21 | 2.35E-18 | Up |
| | | ACSL4 | 5,14E-09 | 2.18E-07 | Up |
| | | GPA33 | 4.73E-09 | 2.03E-07 | Up |
| | | DSC1 | 2.80E-11 | 2.00E-09 | Up |
| | | MYL12A | 1.42E-62 | 1.66E-58 | Up |
| | | SERPINA5 | 3.32E-09 | 1.48E-07 | Up |
| | | CAB39 | 2.33E-12 | 2.16E-10 | Up |
| | | CD59 | 6.34E-19 | 1.79E-16 | Up |
| | | DSTN | 8.09E-13 | 8.49E-11 | Up |
| | | SYNC | 5.10E-13 | 5.55E-11 | Up |
| | | PPM1L | 2.96E-19 | 8.65E-17 | Up |
| | | RNF11 | 4.70E-15 | 7.23E-13 | Up |
| | | MME | 3.12E-17 | 7.51E-15 | Up |
| | | CLIC5 | 7.40E-22 | 3.09E-19 | Up |
| | | ANXA7 | 8.92E-18 | 2.27E-15 | Up |
| | | RRAS | 6.83E-12 | 5.63E-10 | Up |
| | | RRAS2 | 2.50E-12 | 2.30E-10 | Up |
| | | RAB5A | 3.32E-09 | 1.48E-07 | Up |
| Extracellular vesicle and cell cell communication; GO:1903561 | 8.00E-14 | APOB | 3.69E-25 | 2.27E-22 | Up |
| | | CD59 | 6.34E-19 | 1.79E-16 | Up |
| | | LGMN | 6.89E-05 | 0.00086 | Up |
| | | LGALS3 | 1,73E-07 | 4.87E-06 | Up |
| | | SERPINB6 | 6,42E-21 | 4.87E-06 | Up |
| | | CD63 | 0.0012 | 0.0092 | Up |
| | | DES | 5.10E-14 | 6.67E-12 | Up |
| | | HSPA4 | 5,37E-15 | 8,21E-13 | Up |
| | | PPM1L | 2.96E-19 | 8.65E-17 | Up |
| | | RNF11 | 4.70E-15 | 7.23E-13 | Up |
| | | MME | 3.12E-17 | 7.51E-15 | Up |
| | | MYL12A | 1.42E-62 | 1.66E-58 | Up |
| | | CLIC5 | 7.40E-22 | 3,09E-19 | Up |
| | | ANXA7 | 8.92E-18 | 2.27E-15 | Up |

**Table S7** (*continued*)


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**Table S7** (*continued*)

| | P value | Gene | P value | Adjusted P value | Regulation |
|---|---|---|---|---|---|
| | | SCN3A | 1.07E-25 | 7.34E-23 | Up |
| | | SYT13 | 5.97E-20 | 2.00E-17 | Up |
| | | GRIK2 | 4.46E-23 | 2.05E-20 | Up |
| | | BDNF | 9.87E-16 | 1.79E-13 | Up |
| | | GRIN2A | 2.40E-21 | 9.19E-19 | Up |
| | | IL6R | 9.89E-13 | 9.98E-11 | Up |
| | | DGKG | 1.61E-27 | 1.21E-24 | Up |
| | | DOCK5 | 4.44E-30 | 4.72E-27 | Up |
| | | PRKG1 | 8.56E-20 | 2.82E-17 | Up |
| | | MYH7 | 9.04E-11 | 5.86E-09 | Up |
| | | RCAN2 | 5.08E-22 | 2.16E-19 | Up |
| | | HOMER2 | 4.05E-23 | 1.90E-20 | Up |
| | | C1orf168 | 1.69E-24 | 8.99E-22 | Up |
| | | ITGBL1 | 6.94E-21 | 2.50E-18 | Up |
| | | SARS | 9.46E-06 | 0.000155 | Up |
| | | CHP1 | 3.64E-08 | 1.24E-06 | Up |
| | | SERPINB1 | 3.50E-08 | 1.20E-06 | Up |
| | | STAMBP | 0,00023 | 0.0024 | Up |
| Response to oxidative stress; GO:0006979 | 8.67E-05 | HBA2 | 3.88E-12 | 3.42E-10 | Up |
| | | TPO | 7.76E-15 | 1.16E-12 | Up |
| | | BECN1 | 2.20E-12 | 2.05E-10 | Up |
| | | HBB | 8.34E-13 | 8.67E-11 | Up |
| | | MAOB | 2.27E-18 | 6.11E-16 | Up |
| | | KPNA4 | 3.76E-29 | 3.39E-26 | Up |
| | | SOD3 | 0.00034 | 0.0033 | Up |
| | | TXN2 | 0.00058 | 0.0052 | Up |
| Contractile Fiber; GO:0043292 | 2.36E-17 | HABP4 | 4.01E-16 | 7.57E-14 | Up |
| | | DUSP27 | 1.55E-27 | 1.21E-24 | Up |
| | | CALM1 | 3.56E-05 | 0.00049 | Up |
| | | MYBPC1 | 6.86E-13 | 7.23E-11 | Up |
| | | CMYA5 | 1.77E-13 | 2.13E-11 | Up |
| | | KLHL41 | 4.98E-42 | 1.46E-38 | Up |
| | | SYNM | 5.47E-16 | 1.02E-13 | Up |
| | | SYNC | 5.10E-13 | 5.55E-11 | Up |

**Table S7** (*continued*)


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**Table S7** (*continued*)

| | P value | Gene | P value | Adjusted P value | Regulation |
|---|---|---|---|---|---|
| | | FHL5 | 2.19E-37 | 3.42E-34 | Up |
| | | FHL1 | 9.46E-35 | 1.30E-31 | Up |
| | | MYH7 | 9.04E-11 | 5.86E-09 | Up |
| | | MYPN | 1.35E-15 | 2.36E-13 | Up |
| | | MYOM3 | 9.39E-15 | 1.38E-12 | Up |
| Signaling pathway (KEGG analysis) | | | | | |
| Retinol metabolism (hsa00830) | 0.0017 | ADH1B | 4.93E-11 | 3.36E-09 | Up |
| | | ADH1C | 0.00037 | 0.0035 | Up |
| | | RDH10 | 0.00076 | 0.0064 | Up |
| | | PNPLA4 | 2.61E-10 | 1.58E-08 | Up |
| | | CYP1A1 | 1.01E-05 | 0.00016 | Up |
| | | CYP4A22 | 0.00098 | 0.0079 | Up |
| | | RETSAT | 1.18E-08 | 4.61E-07 | Up |
| PPAR signaling pathway (hsa03320) | 0.021 | RXRG | 0,0051 | 0,03 | Up |
| | | PPARG | 2.03E-08 | 7.46E-07 | Up |
| | | APOA1 | 0.0019 | 0.014 | Up |
| | | ACSL6 | 1.82E-21 | 7.33E-19 | Up |
| | | ACSL4 | 5.14E-09 | 2.18E-07 | Up |
| | | CYP4A22 | 0.00098 | 0.0079 | Up |
| | | AQP7 | 8.07E-05 | 0.00099 | Up |
| | | ACADM | 5.53E-07 | 1.36E-05 | Up |

KEGG, Kyoto Encyclopedia of Genes and Genomes; has, Homo sapiens (human); GO, Gene Ontology; PANTHER, Protein Analysis Through Evolutionary Relationships.


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**Table S8** Downregulated genes matching to the selected differentially expressed signaling pathways in adult patients with CCHD and RVI

| | P value | Gene | P value | Adjusted P value | Regulation |
|---|---|---|---|---|---|
| Gene Ontology (PANTHER) | | | | | |
| Cell Cycle; GO:0007049 | 4.92E-24 | CDT1 | 5.37E-05 | 0.00069 | Down |
| | | CDC7 | 2.69E-11 | 1.94E-09 | Down |
| | | MCM2 | 1.14E-05 | 0.00018 | Down |
| | | PRIM1 | 3.61E-06 | 6.85E-05 | Down |
| | | ORC6 | 7.67E-08 | 2.39E-06 | Down |
| | | BCAT1 | 1.16E-05 | 0.00018 | Down |
| | | MCM3 | 4.74E-09 | 2.04E-07 | Down |
| | | CDK6 | 2.20E-09 | 1.03E-07 | Down |
| | | MCM5 | 4.20E-15 | 6.55E-13 | Down |
| | | RCC1 | 6.30E-05 | 0.0008 | Down |
| | | E2F7 | 9.65E-08 | 2.92E-06 | Down |
| | | NASP | 8.50E-06 | 0.00014 | Down |
| | | CDK14 | 0.00021 | 0.0022 | Down |
| Cell division; GO:051301 | 6.13E-16 | CDT1 | 5.37E-05 | 0.00069 | Down |
| | | CENPF | 3.34E-08 | 1.15E-06 | Down |
| | | NCAPG | 1.02E-08 | 4.03E-07 | Down |
| | | STAG2 | 8.89E-08 | 2.72E-06 | Down |
| | | CDC7 | 2.69E-11 | 1.94E-09 | Down |
| | | FBXL7 | 6.42E-08 | 2.03E-06 | Down |
| | | PARD6G | 5.22E-05 | 0.00068 | Down |
| | | SPDL1 | 3.99E-06 | 7.46E-05 | Down |
| | | CASC5 | 4.48E-11 | 3.08E-09 | Down |
| | | TPX2 | 5.89E-08 | 1.88E-06 | Down |
| Mitotic cell cycle; GO:0000278 | 2.88E-22 | DNMT3A | 6.29E-09 | 2.60E-07 | Down |
| | | SOX4 | 8.12E-21 | 2.88E-18 | Down |
| | | WDR62 | 7.06E-06 | 0.00012 | Down |
| | | NCAPG | 1.02E-08 | 4.03E-07 | Down |
| | | AJUBA | 6.43E-06 | 0.00011 | Down |
| | | TOP2A | 9.27E-14 | 1.17E-11 | Down |
| | | POLA1 | 3.10E-06 | 5.98E-05 | Down |
| | | SNX30 | 3.65E-13 | 4.11E-11 | Down |
| DNA replication; GO:0006260 | 4.56E-15 | POLD1 | 5.57E-11 | 3.73E-09 | Down |
| | | GINS1 | 1.59E-07 | 4.55E-06 | Down |
| | | ING4 | 1.58E-11 | 1.21E-09 | Down |

**Table S8** (*continued*)


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**Table S8** (*continued*)

| | P value | Gene | P value | Adjusted P value | Regulation |
|---|---|---|---|---|---|
| | | HELB | 3.29E-07 | 8.62E-06 | Down |
| | | DTL | 1.08E-06 | 2.42E-05 | Down |
| | | POLQ | 3.04E-05 | 0.00043 | Down |
| | | RBBP4 | 5.68E-06 | 0.0001 | Down |
| | | FAM111A | 7.36E-06 | 0.00013 | Down |
| Signaling pathway (KEGG) | | | | | |
| Cell cycle (hsa04110) | 0.00098 | CDK6 | 2.20E-09 | 1.03E-07 | Down |
| | | ORC6 | 7.67E-08 | 2.39E-06 | Down |
| | | SKP2 | 6.68E-08 | 2.11E-06 | Down |
| | | CDC7 | 2.69E-11 | 1.94E-09 | Down |
| | | MCM5 | 4.20E-15 | 6.55E-13 | Down |
| DNA replication (hsa03030) | 0.0019 | LIG1 | 5.68E-14 | 7.38E-12 | Down |
| | | POLA1 | 3.10E-06 | 5.98E-05 | Down |
| | | PRIM1 | 3.61E-06 | 6.85E-05 | Down |
| | | POLE | 2.85E-10 | 1.67E-08 | Down |
| | | RNASEH2C | 1.10E-06 | 2.45E-05 | Down |
| Ribosome (hsa03010) | 0.012 | RPL18 | 5.85E-05 | 0.00075 | Down |
| | | MRPS6 | 0.0086 | 0.045 | Down |
| | | RPS8 | 0.0004 | 0.0038 | Down |

KEGG, Kyoto Encyclopedia of Genes and Genomes; has, Homo sapiens (human); GO, Gene Ontology; PANTHER, Protein Analysis Through Evolutionary Relationships; DNA, deoxyribonucleic acid.


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**Table S9** Results of a separate study representing the top 50 genes identified as potential markers for cardiac development and heart maturation (15). The 50 developmental markers listed below were used for identification of genes with the same expression in our study, symbolizing developmentally regulated genes. The detected overlapping genes were not included for further analysis (Figure S1)

| Cluster of the top 50 genes | Developmental markers | Expression |
|---|---|---|
| Cluster 1 | Myh6 | Increased |
| Cluster 1 | Atp2a2 | Increased |
| Cluster 1 | Pln | Increased |
| Cluster 1 | Cox6a2 | Increased |
| Cluster 1 | Cox7b | Increased |
| Cluster 1 | Ndufa1 | Increased |
| Cluster 1 | Uqcrq | Increased |
| Cluster 1 | Atp5e | Increased |
| Cluster 1 | Cox7a1 | Increased |
| Cluster 1 | Cox6c | Increased |
| Cluster 1 | Tnni3 | Increased |
| Cluster 1 | Fabp3 | Increased |
| Cluster 1 | Myl2 * | Increased |
| Cluster 2 | Pgam1 | Decreased |
| Cluster 2 | Tubb5 | Decreased |
| Cluster 2 | Nme1 | Decreased |
| Cluster 2 | Gm5506 | Decreased |
| Cluster 2 | Eif5a | Decreased |
| Cluster 2 | Ngfrap1 | Decreased |
| Cluster 2 | Cks1b | Decreased |
| Cluster 2 | Cdkn1c | Decreased |
| Cluster 2 | Mest * | Decreased |
| Cluster 2 | Gpc3 | Decreased |
| Cluster 2 | H2afz | Decreased |
| Cluster 2 | Tnni1 * | Decreased |
| Cluster 2 | Mif | Decreased |
| Cluster 2 | Hmgn2 | Decreased |
| Cluster 2 | Gyg | Decreased |
| Cluster 2 | Myl7 * | Decreased |
| Cluster 2 | Myl4 * | Decreased |
| Cluster 3 | Hadha | Early low expression, then increasing |
| Cluster 3 | Ryr2 | Early low expression, then increasing |
| Cluster 3 | Srl | Early low expression, then increasing |

**Table S9** (*continued*)


http://dx.doi.org/10.21037/cdt-20-894

**Table S9** (*continued*)

| Cluster of the top 50 genes | Developmental markers | Expression |
|---|---|---|
| Cluster 3 | Nfib | Early low expression, then increasing |
| Cluster 3 | Nfia | Early low expression, then increasing |
| Cluster 3 | Klf6 | Early low expression, then increasing |
| Cluster 3 | Itm2b * | Early low expression, then increasing |
| Cluster 3 | Ech1 * | Early low expression, then increasing |
| Cluster 3 | Phyh | Early low expression, then increasing |
| Cluster 3 | Oxct1 * | Early low expression, then increasing |
| Cluster 3 | Gpc1 | Early low expression, then increasing |
| Cluster 3 | Fhl2 * | Early low expression, then increasing |
| Cluster 3 | Mt1 | Early low expression, then increasing |
| Cluster 3 | Mgst3 | Early low expression, then increasing |
| Cluster 3 | Acadl | Early low expression, then increasing |
| Cluster 3 | Lpl | Early low expression, then increasing |
| Cluster 3 | Brp44l | Early low expression, then increasing |
| Cluster 3 | D830015G02Rik | Early low expression, then increasing |
| Cluster 3 | Lars2 | Early low expression, then increasing |
| Cluster 3 | S100a1 * | Early low expression, then increasing |

*, detected overlapping genes excluded for further analysis.

**Table S10** Presentation of overexpressed fetal genes in adult patients with CCHD and RVI

| Fetal gene | P value | Adjusted P value | Expression |
|---|---|---|---|
| MEF2C | 0.0081 | 0.043 | Increased |
| NPPA | 0.00072 | 0.0062 | Increased |
| ACTA1 | 4.55E-09 | 1.96E-07 | Increased |
| DES | 5.10E-14 | 6.67E-12 | Increased |
| DMD | 0.0062 | 0.035 | Increased |


http://dx.doi.org/10.21037/cdt-20-894

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## 8.3 Appendix 2

### 8.3.1 Supplementary of the original article “Compound mutation in cardiac sarcomere proteins is associated with increased risk for major arrhythmic events in pediatric onset hypertrophic cardiomyopathy”

**Supplementary Material**

**Methods S1: Molecular genetic testing.**

Genetic testing strategy included amplification with polymerase chain reaction followed by sanger sequencing in 17 patients (34.7%), next generation sequencing as sequencing by synthesis by illumina in 14 patients (28.6%) and whole exome analysis in 5 patients (10.2%). The type of molecular genetic testing was not available in the case of 13 patient (26.5%). Multiple gene diagnostic was performed in 24 patients. Gene panels included at least sequencing of the *MYH7* and *MYBPC3* genes or the combination of MYH7 and TNNT2. The biggest panel consisted of 16 known causative genes like ACTC1, ACTN2, ANKRD1, CSRP3, JPH2, MYBPC3, MYH7, MYL2, MYL3, PLN, PRKAG2, TCAP, TNNC1, TNNI3, TNNT2 and TPM1. Single gene analysis was conducted in a total of 7 patients, with amplification and sequencing of the MYH7 gene in 4 patients and the TNNT2 gene in 3 patients. Based on the classification system and guidelines of the American College of Medical Genetics and Genomics (ACMG) and The Association for Molecular Pathology (AMP) mutations were interpreted [1,2]. Genotype-positive was defined as the result of molecular genetic testing, revealing a pathogenic or likely pathogenic variant as per ACMG criteria or re-analysis in ClinVar. Patients with negative result of molecular genetic testing and patients with variants of uncertain significance were defined as genotype negative. Genotype-positive/phenotype-positive, genotype-positive/phenotype-negative as well as genotype-negative/phenotype-positive individuals were included for comparison. Patients identified as genotype positive and still phenotype negative at the time of the study were also included, as this cohort has a genetic predisposition to develop a phenotype typical of hypertrophic cardiomyopathy and may still become clinically conspicuous during childhood. In addition, there are patients who have detected a disease-causing mutation and yet do not develop a phenotype, presumably due to less harmful mutational variants within the affected gene or influence of other nongenetic or epigenetic factors.

**Table S1.** Mutation description of molecular genetic testing of genotype-positive patients with hypertrophic cardiomyopathy.

| Gene | Reference Sequence Transcript | Nucleotid change | Protein modification | Mutation type | ClinVar Classification | Number of patients carrying mutation | Mutation origin |
|---|---|---|---|---|---|---|---|
| MYH7 [1] | NM_000257.3 | c.1988G>A | p.Arg663His | missense | pathogenic | 1 | inherited [8] |
| | NM_000257.2 | c.5135G>A | p.Arg1712Gln | missense | likely pathogenic | 1 | inherited |
| | NM_000257.2 | c.1357C>T | p.Arg453Cys | missense | likely pathogenic to pathogenic | 1 | inherited |
| | NM_000257.3 | c.1208G>A | p.Arg403Gln | missense | pathogenic | 2 | de novo [9], inherited |
| | NM_000257.3 | c.2155C>T | p.Arg719Trp | missense | pathogenic | 4 | inherited |
| | NM_000257.4 | c.1331A>G | p.Asn444Ser | missense | likely pathogenic | 1 | inherited |
| | NM_000257.3 | c.1816G>A | p.Val606Met | missense | likely pathogenic to pathogenic | 2 | inherited |
| | NM_000257.3 | c.3613G>A | p.Glu1205Lys | missense | likely pathogenic | 1 | inherited |
| | NM_000257.3 | c.3346G>A | p.Glu1116Lys | missense | likely pathogenic | 1 | inherited |
| | NM_000257.4 | c.2207T>C | p.Ile736Thr | missense | likely pathogenic to pathogenic | 1 | inherited |
| | NM_000257.3 | c.2678C>A | p.Ala893Glu | missense | likely pathogenic | 2 | inherited |
| | NM_000257.4 | c.4472C>G | p.Ser1491Cys | missense | likely pathogenic | 1 | inherited |
| | NM_000257.4 | c.1207C>T | p.Arg403Trp | missense | pathogenic | 1 | inherited |
| MYBPC3 [2] | NM_000256.3 | c.[821+1G>A] | N/A [7] | N/A | pathogenic | 1 | inherited |
| | NM_000256.3 | c.1484G>A | p.Arg495Gln | missense | likely pathogenic to pathogenic | 1 | inherited |
| | NM_000256.3 | c.1468G>A | p.Gly490Arg | missense | likely pathogenic to pathogenic | 2 | de novo, inherited |
| | NM_000256.3 | c.26-2A>G | N/A | splice mutation | pathogenic | 1 | inherited |
| | NM_000256.3 | c.2864_2865delCT | p.Pro955ArgfsX95 | frameshift | pathogenic | 1 | inherited |
| | NM_000256.3 | c.3599T>C | p.Leu1200Pro | missense | likely pathogenic to pathogenic | 1 | inherited |
| | NM_000256.3 | c.3767_3769delCCA | p.Thr1256del | deletion | likely pathogenic | 1 | inherited |
| TPM1 [3] | NM_001018005.1 | c.287A>G | p.Glu96Gly | N/A | likely pathogenic | 1 | inherited |
| TNNT2 [4] | NM_001001430.2 | c.274C>T | p.Arg92Trp | missense | pathogenic | 3 | inherited |
| | NM_001001430.2 | c.311C>T | p.Ala104Val | missense | likely pathogenic | 3 | inherited |
| | NM_001001430.2 | c.291G>T | p.Lys97Asn | N/A | likely pathogenic | 1 | inherited |
| MYL2 [5] | NM_000432.2 | c.173G>A | p.Arg58Gln | missense | pathogenic | 1 | de novo |
| TNNI3 [6] | NM_000363.4 | c.557G>A | p.Arg186Gln | missense | pathogenic | 1 | inherited |

| | | | | | | |
|---|---|---|---|---|---|---|
| NM_000363.4 | c.587A>G | p.D196G | N/A | likely pathogenic | 1 | inherited |

Detailed list of affected genes in pediatric onset hypertrophic cardiomyopathy patients, including NCBI (The National Center for Biotechnology Information) Reference Sequence Transcript, nucleotid change, their effect on protein modification and mutation type. [1], ß-myosin heavy chain; [2], myosin binding protein C; [3], cardiac troponin T2; [4], cardiac troponin I3; [5], α tropomyosin; [6], myosin light chain 2; [7], not available; [8], considering the presence of a positive molecular genetic test of the parents and / or positive family history for HCM phenotype in parents; [9], considering the presence of a negative molecular genetic test of the parents and / or negative family history for HCM phenotype in parents.

**Table S2.** Clinical characteristics and imaging parameter of pediatric hypertrophic cardiomyopathy patients with compound mutations.

| **Patients characteristics** | **Patient 1** | **Patient 2** | **Patient 3** | **Patient 4** |
|---|---|---|---|---|
| Sex (Male/Female) | Male | Female | Male | Male |
| Age at first diagnosis (years) | 6.0 | 7.0 | 1.0 | 0.3 |
| Age at last follow up (years) | 39.6 | 22.6 | 14.6 | 26.6 |
| Genotype / phenotype expression | Genotype positive / phenotype positive | Genotype positive / phenotype positive | Genotype positive / phenotype positive | Genotype positive / phenotype positive |
| Identified mutation | MYH7 [21] + TNNT2 [22] | MYH7 + TNNI3 [23] | MYH7 + TNNT2 | MYH7 + TNNT2 |
| Mutation origin | Inherited [24] | Inherited | Inherited | Inherited |
| **Family history** | | | | |
| Positive for HCM [1] | Yes | Yes | Yes | Yes |
| Positive for SCD [2] | Yes | No | Yes | Yes |
| **Mortality** | | | | |
| Death | No | No | Yes | No |
| **Arrhythmia** [3] | | | | |
| None, Mild [4], Severe [5] | Severe | Severe | None | Severe |
| MAEs [6] | No | Appropriate ICD discharge | SCD | Appropriate ICD discharge |
| **Morbidity** | | | | |
| Hospitalization | Yes | Yes | No | Yes |
| Age at first hospitalization (years) | 37.8 | 7.0 | - | 10.9 |
| ICD [7] implantation | Yes | Yes | No | Yes |
| Age at ICD implantation (years) | 37.8 | 14.3 | - | 10.9 |
| Primary or Secondary prevention | Primary | Secondary | - | Secondary |
| Appropriate discharge | No | Yes | - | Yes |
| Number of cardiac medications | 1 | 2 | 0 | 1 |
| NYHA [8] class I, II, III, IV | II | II | I | II |
| **Morphology** | | | | |
| CMR [9] EDVI [10] (mL/m$^2$) | N/A [25] | 64.0 | 63.0 | N/A |
| CMR ESVI [11] (mL/m$^2$) | N/A | 16.0 | 16.0 | N/A |
| LVOTO [12] | No | No | No | No |
| **Hypertrophy** | | | | |
| CMR myocardial mass (g/m$^2$) | N/A | 117.0 | 43.0 | N/A |
| TTE [13] IVSd [14] z-score | 4.1 | 3.3 | 3.1 | 4.9 |
| TTE LVPWd [15] z-score | 2.5 | 3.3 | 3.2 | 1.7 |
| **Fibrosis** | | | | |
| CMR LGE [16] | N/A | LGE | LGE | N/A |
| CMR LGE localization | N/A | Entire myocardium | Uncertain detection | N/A |

| | | | | |
|---|---|---|---|---|
| **Systolic function** | | | | |
| TTE EF[17] (%) | 61.0 | 57.0 | 78.0 | 41.0 |
| GLS [18] average | N/A | N/A | N/A | -8.2 |
| GLS dispersion | -18.0 | N/A | -4.0 | -2.0 |
| GLS minimum | -13.0 | N/A | -17.0 | -9.0 |
| GLS maximum | 5.0 | N/A | -13.0 | -7.0 |
| **Diastolic function** | | | | |
| TTE MV [19] E/A Ratio | 2.0 | N/A | 1.1 | 2.0 |
| TTE MV E Deceleration (m/s) | 195.0 | N/A | 217.0 | 255.0 |
| TTE E maximum (m/s) | 0.8 | N/A | 0.6 | 0.5 |
| LA [20] Diameter (cm) | 4.6 | N/A | 4.3 | 5.1 |

[1], hypertrophic cardiomyopathy; [2], sudden cardiac death; [3], arrhythmia on Holter or cardiopulmonary exercise test; [4], premature ventricular or supraventricular beats; [5] non-sustained or sustained ventricular or supraventricular tachycardia; [6], resuscitation and/or appropriate implantable cardioverter defibrillator discharge and/or sudden cardiac death; [7], implantable cardioverter defibrillator; [8], New York Heart Association or Modified Ross classification according to age; [9], cardiac magnetic resonance imaging; [10], end-diastolic volume index; [11], end-systolic volume index; [12], left ventricular out-flow tract obstruction; [13], transthoracic echocardiography; [14], end-diastolic inter-ventricular septal; [15], end-diastolic left ventricular posterior wall thickness; [16], late gadolinium enhancement; [17], ejection fraction; [18], global longitudinal strain; [19], mitral valve; [20], left atrium; [21], β myosin heavy chain; [22], cardiac troponin T2; [23], cardiac troponin I3; [24], presence of a positive molecular genetic test of the parents and / or positive family history for HCM with clinically conspicuous parents; [25], not available.

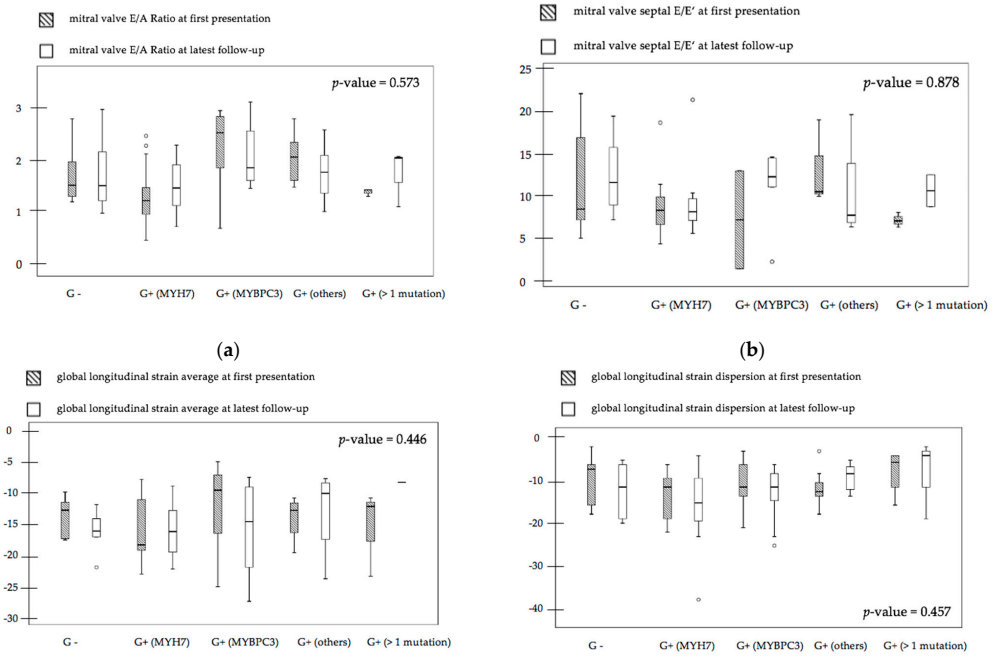


**Figure S1.** Disease progression Part II. Shown are distinct disease features at the time of first and last presentation grouped according to underlying genotype. Left ventricular diastolic function is presented by echocardiographic measurements of the mitral valve E/A ratio (**a**), and septal E/E´ ratio (**b**) on tissue Doppler imaging. Global longitudinal strain average (**c**) and global longitudinal strain dispersion (**d**) present echocardiographic signs of left ventricular systolic function. P-values were calculated with non-parametric Kruskal-Wallis test. The delta of respective parameter between first presentation and last follow up did not differ between consecutive groups. G−: genotype-negative patients, G+ MYH7: genotype-positive patients with β-myosin heavy chain single-mutation, G+ MYBPC3: genotype-positive patients with myosin binding protein C single-mutation, G+ others: genotype-positive patients with cardiac troponin T2, cardiac troponin I3, $\alpha$ tropomyosin and myosin light chain 2 single-mutations, G+ multiple mutation: genotype-positive patients with compound mutations, °: outliers.

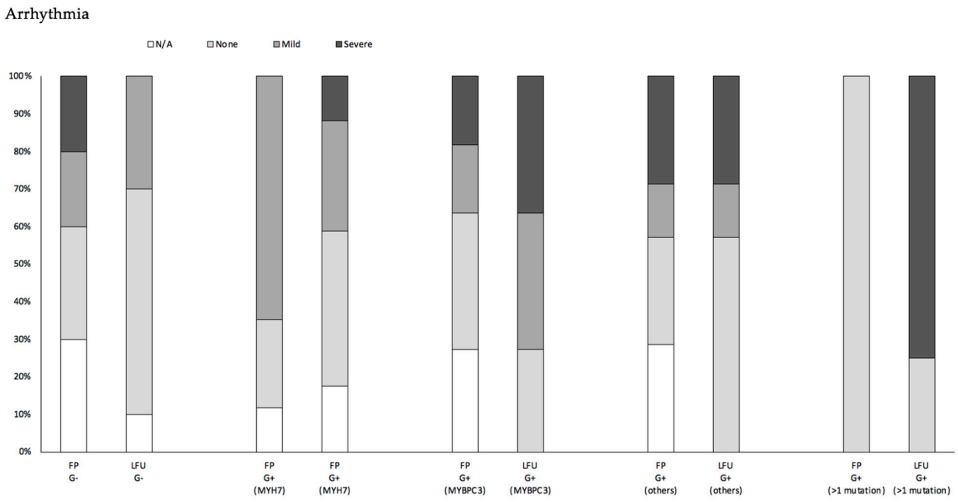


**Figure S2.** Progress of arrhythmia in patients with pediatric onset hypertrophic cardiomyopathy. Shown are defined arrhythmia classes on first and last presentation grouped according to underlying genotype. Arrhythmia were detected on Holter or cardiopulmonary exercise test. N/A, not available; mild, premature ventricular or supraventricular beats; severe, non-sustained or sustained ventricular or supraventricular tachycardia. P-values were calculated with non-parametric Kruskal-Wallis test. The delta of respective parameter between first presentation and last follow up did not differ between consecutive groups ($p$-value 0.179). G–: genotype-negative patients, G+ MYH7: genotype-positive patients with β-myosin heavy chain single-mutation, G+ MYBPC3: genotype-positive patients with myosin binding protein C single-mutation, G+ others: genotype-positive patients with cardiac troponin T2, cardiac troponin I3, α tropomyosin and myosin light chain 2 single-mutations, G+ multiple mutation: genotype-positive patients with compound mutations.

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## 8.4 Appendix 3

### 8.4.1 Publisher permissions

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> Best regards and have a nice day,
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> Kathrin Pollmann

### 8.4.2 Presentation of interim results

May 2019: Oral Presentation “Molecular signaling pathways in right ventricular impairment in adult patients after tetralogy of Fallot repair“ at the annual meeting of the Association for European Pediatric and Congenital Cardiology (AEPC) in Sevilla, Spain.