Tampere University

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Cardiac Diseases with Molecular and Clinical Aspect

JPH2-RyR2-SCN5A gene mutations

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To all those who contribute to genetic cardiac diseases

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Tampere, October 2019

ABSTRACT

The incidence of sudden cardiac death (SCD) in all age groups is one per thousand life-years. In recent decades, with the development of genetic research, molecular genetics has been able to clarify the diagnosis of serious life-threatening heart disease and to optimize treatments, and to assess the risk of disease and necessity of heart monitoring for the affected relatives. Additionally, the development of stem cell-based technologies has further helped to study genetic disease at cellular level.

In the outpatient clinic for genetic cardiac diseases, we found interesting families whose diseases we further studied in more detail clinically and under laboratory conditions. Monogenic, dominantly inherited cardiac diseases were the focus. All these diseases are associated with an increased risk of sudden death at a young age. Early diagnosis and well-targeted treatments can significantly improve patients' prognosis.

A large family carrying a sodium channel *SCN5A* gene mutation D1275N was found in the Pirkanmaa area in Finland. A clinical phenotype of family members included severe conduction defect and atrial arrhythmias. In this thesis, we analyzed 12-lead electrocardiogram (ECG) in that family and discovered ECG changes that predict conduction defect progression and cardiogenic thromboembolic complication. With these results, we will be able to predict the need for anticoagulation and pacemaker treatments of gene mutation carriers before complications appear.

Hypertrophic cardiomyopathy (HCM) is a common monogenic heart disease that is estimated to appear in one in 500 individuals. So far, hypertrophic cardiomyopathy-causing gene defects have been most commonly found in sarcomere proteins corresponding to cardiac contraction. In the second part of the work, we were able to demonstrate for the first time the calcium handling protein junctophilin-2 (*JPH2*) gene to cause hypertrophic cardiomyopathy. This protein is a non-sarcomeric calcium regulator. This *JPH2* p.(Thr161Lys) variant is a new Finnish mutation causing atypical HCM in many unrelated families.

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inheritable rare cardiac disorder associated with exercise- and emotional stress-induced ventricular extrasystole and tachycardia in the absence of structural heart disease. The efficacy of medications in these patients is poor. CPVT is caused by mutations in ryanodine receptor 2 (*RyR2*) gene. Combining a clinical trial with stem cell study,

we investigate the effect of a certain medication (dantrolene) on patients and their stem cell-derived cardiomyocytes. First of all, we observed a clear mutation-specific response – N-terminal mutations responded well to the treatment while the drug had no effect in patients having mutations in the C-terminal part of the gene. Secondly, we did a major discovery the patients and their stem cell-derived cardiomyocytes responded similarly on the medication – if the patient responded, also his stem cell-derived cardiomyocytes responded, and if the patient did not, neither the cells did. The results demonstrated that the location of the mutation has a significant role in drug-responsiveness, and that stem cell-derived cell can be used for treatment optimization in patients with genetic cardiac diseases without testing the drugs in patients.

Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are generally called pluripotent stem cells (hPSCs). These cells can be differentiated into any cell of human body. In this thesis, we analyze stem cell derived cardiomyocytes. Some electrophysiological aspects of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) can be studied using microelectrode arrays (MEAs), where the cells are cultured on electrodes and ECG-type of field potential (FP) recordings can be obtained. The QT interval in 12-lead ECG is beating-rate-dependent and correction formulas have been created to abolish the effect of the rate. We investigated whether the same formulas could be used when studying stem cell-derived cardiomyocytes. We compared the FP duration (FPD) to the QT time of electrocardiogram (ECG) from healthy individuals with low basic heart rate and performed stress exercise test to increase heart rate. We found that the repolarization parameter FPD in hPSC-cardiomyocytes (hPSC-CMs) behaved similarly as QT time in ECG recordings, and thus the same rate correction formula can be applied to electrical recordings in cell culture situations.

In conclusion, the identification of the underlying genetic detect aims at identifying individuals at increased risk, and clinical parameters can be found to predict the need of further treatments. Additionally, stem cell technologies will be powerful in the future to optimize treatments in patient- and mutation-specific manner.

TIIVISTELMÄ

Sydänperäisen äkkikuoleman ilmaantuvuus kaikissa ikäryhmissä on yksi tuhatta henkilövuotta kohden. Viime vuosikymmeninä geenitutkimusten kehittymisen myötä on molekyyligenetiikan avulla kyetty selventämään vakavien nuorellakin iällä henkeä uhkaavien sydänsairauksien diagnostiikkaa, optimoimaan hoitoja sekä arvioimaan sairastuneen lähisukulaisten sairastumisriskiä ja sydänseurantatarvetta. Lisäksi kantasolutekniikoiden kehittyminen helpottaa geneettisten sairauksien tutkimista solutasolla.

Perinnöllisiä sydänsairauksia hoitaessa löysimme mielenkiintoisia sukuja, joiden sairauksia tutkimme kliinisesti ja laboratorio-oloissa. Tämä väitöskirja käsittelee monogeenisia vallitsevasti periytyviä sydänsairauksia, joihin erityisesti diagnosoimattomana liittyy äkkikuoleman riski nuorena. Varhaisella diagnostiikalla ja oikein kohdennetuilla hoidoilla potilaiden sairauden ennustetta voidaan merkittävästi parantaa.

Totesimme laajassa pirkanmaalaisessa suvussa esiintyvän natriumkanava *SCN5A* D1275N geenimutaation, joka aiheuttaa sydämessä vakavia johtumishäiriöitä ja eteisperäisiä rytmihäiriöitä johtaen fataaleihin sydänperäisiin tromboembolisiin komplikaatioihin. Selvitimme 12-kytkentäisen sydänsähkökäyrän (EKG) löydöksien liittymistä *SCN5A* D1275N geenivirheeseen ja geenivirheen tromboembolisille komplikaatioille altistavien EKG löydösten ja tahdistin hoitoa vaativien johtumishäiriöiden ennakoitavuutta. Löysimme geenivirheeseen liittyviä sydänsairauden etenemistä ennakoivia EKG-muutoksia, joiden perusteella voimme jatkossa suunnitella geenivirheen kantajien hoitoja mahdollisimman oikea-aikaisesti ennen komplikaatioiden ilmaantumista.

Hypertrofinen eli paksuseinäinen sydänlihassairaus on yleinen vallitsevasti periytyvä sydänsairaus esiintyen yhdellä henkilöllä 500:sta. Toistaiseksi hypertrofista kardiomyopatiaa aiheuttavia geenivirheitä on eniten löytynyt sydämen supistumisesta vastaavista sarkomeeriproteiineista. Toisessa osatyössä pystyimme osoittamaan ensimmäistä kertaa ei-sarkomeerisen kalsiumin säätelyyn vaikuttavan junktofiliiniproteiinia koodaavan *JPH2* – geenivirheen vaikuttavan perinnöllisen hypertrofisen kardiomyopatian syntyyn. Analysoimalla yhdeksän perheen *JPH2* (Thr161Lys) geenivirheen kantajien sydänmanifestaatioita totesimme *JPH2* geenivirheen yksinään aiheuttavan hypertrofista kardiomyopatiaa.

Katekoliamiiniherkkä polymorfinen kammiotakykardia (CPVT) on harvinainen perinnöllinen rytmihäiriösairaus, joka ilmaantuu fyysisen tai henkisen rasituksen yhteydessä katekolamiinien provosoidessa kammiolisälyöntisyyttä ja monimuotoista kammiotakykardiaa terverakenteisessa sydämessä. Rytmihäiriöiden hoidossa käytettävien lääkitysten teho on huono ja vaihteleva tautia aiheuttavan ryanodiinireseptori 2 *(RyR2)* mutaation kantajilla. Yhdistimme kliinisen ja kantasolututkimuksen tutkimalla dantroleeni-lääkkeen vaikutusta *RyR2* mutaatiopotilailla ja heidän kantasoluistaan johdetuilla sydänsoluilla. Erityisesti, tutkimme mutaatiospesifistä lääkevastetta – N-terminaalimutaatioiden vaste hoitoon oli hyvä, kun taas C-terminaaliosan geenivirheen kantajilla ei ollut vastetta tutkitulle lääkkeelle. Merkittävin havaintomme oli huomata potilailla ja heidän kantasoluistaan johdetuilla sydänsoluilla (hiPSC-CM) olevan samanlainen lääkevaste. Jos potilas respondoi, myös hänen hiPSC-CM respondoivat lääkeaineelle, ja jos lääke ei tehonnut potilaaseen, se ei toiminut myöskään soluviljelmillä. Tutkimustulos kuvastaa mutaation sijainnilla olevan merkittävän vaikutuksen lääkevasteeseen. Siten voimme hiPSC-sydänsolumalleja käyttäen optimoida geneettisesti sydänsairaan potilaan hoitoja testaamatta lääkettä potilaalla.

Ihmisen alkion kantasoluja (hESC) ja indusoituja kantasoluja (hiPSC) kutsutaan yleisesti pluripotenteiksi kantasoluiksi (hPSC). Näitä soluja voidaan muuntaa erilaisiksi ihmisen soluiksi. Soluviljelytekniikoilla tuotettujen ihmisen sydänsolujen (hPSC-CM) sähköisiä ominaisuuksia voidaan mitata käyttäen mikroelektrodeja (MEA). Näiden avulla rekisteröidään solun sähköistä potentiaalia (FP), jonka ajatellaan vastaavan ihmiseltä otettavaa EKG-rekisteröintiä. QT-aika 12-kytkentäisessä EKG:ssa on sykeriippuvainen ja korjauskaavoilla pyritään poistamaan sykkeen muutoksen vaikutusta QT-aikaan. Tutkimme Bazettin kaavan soveltuvuutta hPSC-CM-solumalleissa. Vertasimme FP-aikaa (FPD) EKG:n QT-aikaan eri syketaajuuksilla terveillä henkilöillä syketaajuutta polkupyörärasituksella nopeuttaen. Totesimme hPSC-CM solujen repolarisaatioparametrien käyttäytyvän samoin kuin QT-aika EKG:ssa, siten sama korjauskaava sopii soluviljelmien sähköiseen rekisteröintiin.

Molekyyligeneettinen diagnoosi helpottaa potilaiden ja heidän terveiden lähisukulaisten sydänsairauden sairastumisriskin arviota, sairauden diagnostiikkaa ja hoitoa. Lisäksi kantasolutekniikoilla voimme tulevaisuudessa optimoida potilas- ja mutaatiospesifisiä hoitotapoja.

CONTENTS

ABBREVIATIONS

AA	amino acid
AF	atrial fibrillation
ACMG-AMP	American College of Medical Genetics and Genomics- Association for Molecular Pathology
AP	action potential
APD	action potential duration
ARVC	arrhythmogenic right ventricular cardiomyopathy
AS	atrial standstill
AV	atrioventricular
AVN	atrioventricular node
AVNRT	atrioventricular nodal re-entry tachycardia
BBB	bundle branch block
bpm	beats per minute
BR	beating rate
BrS	Brugada syndrome
Ca^{2+}	calcium ion
Ca_v	voltage-gated calcium channel
cAMP	cyclic adenosine monophosphate
CASQ2	calsequesterin 2
cFPD	corrected field potential duration
CHD	coronary heart disease
CM	cardiomyocyte
CPVT	catecholaminergic polymorphic ventricular tachycardia
Cx40	connexin 40
DCM	dilated cardiomyopathy
DNA	deoxyribonucleic acid

ECG	electrocardiogram
ExAC	Exome Aggregation Consortium
FAF	familial atrial fibrillation
FP	field potential
FPD	field potential duration
fQRS	fragmented QRS
gnomAD	the Genome Aggregation database
HCM	hypertrophic cardiomyopathy
hESC	human embryonic stem cell
HF	heart failure
hiPSC	human induced pluripotent stem cell
hiPSC-CM	human induced pluripotent stem cell-derived cardiomyocyte
hPSC	human pluripotent stem cell
hPSC-CM	human pluripotent stem cell-derived cardiomyocyte
I_{Ca}	inward calcium (Ca^{2+}) channel current
ICD	implantable cardioverter-defibrillator
I_{Na}	inward sodium (Na^{+}) channel current
JMC	junctional membrane complexes
JPH2	junctophilin 2
K^{+}	potassium ion
K_v	voltage-gated potassium channel
LAFB	left anterior fascicular block
LBBB	left bundle branch block
LQTS	long QT syndrome
LTCC	L-type calcium channel
LV	left ventricle
LVH	left ventricular hypertrophy

LVOT	left ventricular outflow tract
LVOTO	left ventricular outflow tract obstruction
MYBPC3	myosin binding protein C
MEA	microelectrode arrays
Na^+	sodium ion
$[Na^+]$	sodium concentration
Na_v	voltage-gated sodium channel
NCX	Na^+/Ca^{2+}-exchanger
NGS	next generation sequencing
NSVT	non-sustained ventricular tachycardia
PAMA	pacemaker
PCCD	progressive cardiac conduction disease
PKA	protein kinase A
PPI	peak-to-peak interval
proBNP	brain (B-type) natriuretic propeptide
PVC	premature ventricular contractions
QTc	corrected QT time
RBBB	right bundle branch block
RCM	restrictive cardiomyopathy
RyR2	ryanodine receptor 2
SA	sinoatrial
SAM	septal anterior motion
SAN	sinoatrial node
SCD	sudden cardiac death
SCN5A	sodium channel 5A
SERCA2a	sarcoplasmic reticulum Ca^{2+}-ATPase
SR	sarcoplasmic reticulum
SSS	sick sinus syndrome

T_a	atrial repolarization
TPM1	alpha tropomyosin
TTE	transthoracic echocardiography
T-tubules	Transverse tubules
VF	ventricular fibrillation
VT	ventricular tachycardia
VUS	variant of uncertain significance
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following four original publications listed below. These publications are referred to in the text by using their Roman numerals (I-IV).

Publication I **Sari U. M. Vanninen**, Kjell Nikus & Katriina Aalto-Setälä. Electrocardiogram changes and atrial arrhythmias in individuals carrying sodium channel *SCN5A* D1275N mutation. *Annals of Medicine*, 2017, 49(6), 496-503. doi: 10.1080/07853890.2017.1307515. PMID: 28294644

Publication II **Sari U. M. Vanninen**, Krista Leivo, Eija H. Seppälä, Katriina Aalto-Setälä, Olli Pitkänen, Piia Suursalmi, Antti-Pekka Annala, Ismo Anttila, Tero-Pekka Alastalo, Samuel Myllykangas, Tiina M. Heliö*, Juha W. Koskenvuo* Heterozygous junctophilin-2 (*JPH2*) p. (Thr161Lys) is a monogenic cause for HCM with heart failure. *PLoS One*, 2018, 13(9):e0203422. doi: 10.1371/journal.pone.0203422. PMID: 30235249

Publication III Penttinen K*, Swan H*, **Vanninen S**, Paavola J, Lahtinen AM, Kontula K, Aalto-Setälä K. Antiarrhythmic effects of dantrolene in patients with catecholaminergic polymorphic ventricular tachycardia and replication of the responses using iPSC models. *PLoS One*, 2015, 10(5):e0125366. doi: 10.1371/journal.pone.0125366. PMID: 25955245#

Publication IV **Sari U. M. Vanninen***, Ville J. Kujala*, Ilkka Pörsti, Katriina Aalto-Setälä. A quantitative comparison of 12-lead electrocardiograms and *in vitro* cardiac field potentials with different beating frequencies. *International Journal of Stem Cell Research & Therapeutics*, 2019, 1(1), 1-8. https://symbiosisonlinepublishing.com/stem-cell-research-therapeutics/stem-cell-research-therapeutics04.pdf

Publication was previously included in the doctoral dissertation "Induced pluripotent stem cell-derived disease model for catecholaminergic polymorphic ventricular tachycardia" by Kirsi Penttinen at the Tampere University of Technology 2016.

*Authors contributed equally

1 INTRODUCTION

Two groups of familial diseases are responsible for sudden cardiac death especially in young: cardiomyopathies and channelopathies. Congenital cardiac channelopathies are primary electrical disorders related to mutations in genes encoding cardiac ion channels and/or their regulatory proteins leading to channelopathies and life-threatening arrhythmias. On the other hand, cardiomyopathies are related to mutations in genes encoding several categories of structural proteins. (Magi et al., 2017; Garcia-Elias & Benito, 2018.) Therefore, genotype studies have become an essential part of clinical diagnosis (Magi et al., 2017). Molecular genetic diagnosis improves the ability to predict risk for diseases and enable understanding of the pathophysiology, which improve prevention and therapy (Zipes et al., 2019).

Mutations in the *SCN5A* gene cause cardiac sodium (Na^+) channel dysfunction associating with variable conduction diseases, arrhythmias, atrial standstill, even fatal ventricular arrhythmias and dilative cardiomyopathy (Remme et al., 2008). The carriers of a *SCN5A* mutation need a clinical and electrocardiogram (ECG) follow-up because of the risk associated with severe conduction defects (Probst et al., 2006). Mutations in the cardiac Na^+ channel encoded by the gene *SCN5A* can result in many different phenotypes (Remme et al., 2008). Some of the Finnish *SCN5A* D1275N mutation carriers had progressive conduction disease and atrial arrhythmias before they died of a cardioembolic stroke at a young age (Laitinen-Forsblom et al., 2006).

Hundreds of different disease-causing mutations have been identified in genes that encode proteins of the sarcomere, which is the basic contractile unit of cardiac myocyte (Roma-Rodrigues & Fernandes, 2014; Zipes et al., 2019). The critical role of calcium (Ca^{2+}) in excitation-contraction coupling and linking alterations in Ca^{2+} handling with hypertrophic remodeling is known. Consequently, investigators have suggested that mutations in genes encoding Ca^{2+}-handling proteins, such as junctophilin 2 (*JPH2*), might be associated with hypertrophic cardiomyopathy (HCM). (Landstrom & Ackerman, 2012.) HCM affects 0.2% of the global population (Frey et al., 2012). In HCM, typically the left ventricular wall is asymmetric thickened

(Elliot et al., 2008). Phenotype is varied, and HCM can be asymptomatic or cause fatal arrhythmias and heart failure (Roma-Rodrigues & Fernandes, 2014).

Ryanodine receptor 2 (RyR2*)* is responsible for calcium regulation in the cardiomyocyte (Priori & Chen, 2011). Catecholaminergic polymorphic ventricular tachycardia (CPVT) caused by *RyR2* gene mutation is a malignant stress-induced arrhythmogenic disease that may lead to sudden death in children and young adults (Priori et al., 2002; Zipes et al., 2019). CPVT belongs to a group of inheritable disorders referred to as channelopathies without structural heart disease, caused by mutations in genes coding for channel-proteins that regulate cardiac electrical function (Imberti et al., 2016).

The first stable human embryonic stem cell (hESC) lines were derived in the 1990s (Thomson et al., 1998). Human induced pluripotent stem cells (hiPSCs) by derived programming of differentiated human somatic cells into a pluripotent state allow creation of patient- and disease-specific stem cells (Takahashi et al., 2007). It is possible to record the electrical activity of cardiomyocytes with field potentials (FPs) using microelectrode arrays (MEAs) (Stett et al., 2003; Reppel, 2004). In MEA, peak-to-peak interval (PPI) is determined as time between two depolarizing sodium [Na^+] peaks, PPI is analogues with RR in the ECG. The field potential duration (FPD) has been claimed to be analogous with the QT interval in the ECG. (Stett et al., 2003.)

In the first publication, the aim was to investigate the significance of the gene mutation in ECG changes. In the second publication, the aim was to find out the pathogenicity of the likely HCM causing gene by exploring the heart expressions of the gene mutation carriers. In next two publications, the aim was to find out the suitability of the human pluripotent stem cell-derived cardiomyocyte (hPSC-CM) cell model to study changes in the human heart rate. The optimal management of heart disease requires the understanding of the association between the causative mutations and the clinical phenotype.

2 LITERATURE REVIEW

2.1 Heart and cardiomyocytes

The heart consists of four chambers: the right and left atria and ventricle. Cardiomyocytes within the heart are required to pump in order to provide effective contraction–relaxation cycle of myocytes action that can ensure adequate blood perfusion of the various organs and tissues in the body through a network of blood vessels. (Woodcock & Matkovich, 2005.) Cardiomyocytes (atrial, ventricular and nodal cells) form approximately 75% of the total ventricular volume and weight, but only one third of the total number of cells (Zipes et al., 2019). The heart consists different of cell types, each with their own contribution to structural, biochemical, mechanical and electrical properties of the functional heart (Xin et al., 2013) (Figure 1). The sarcomere is the basic contractile unit of cardiac myocyte, composed of thick myosin and thin actin filaments (Knollmann & Roden, 2008; Zipes et al., 2019) (Figure 2).

Specialized cardiomyocytes form is the cardiac conduction system, which is responsible for the control of rhythmic beating of the heart (Woodcock & Matkovich, 2005).

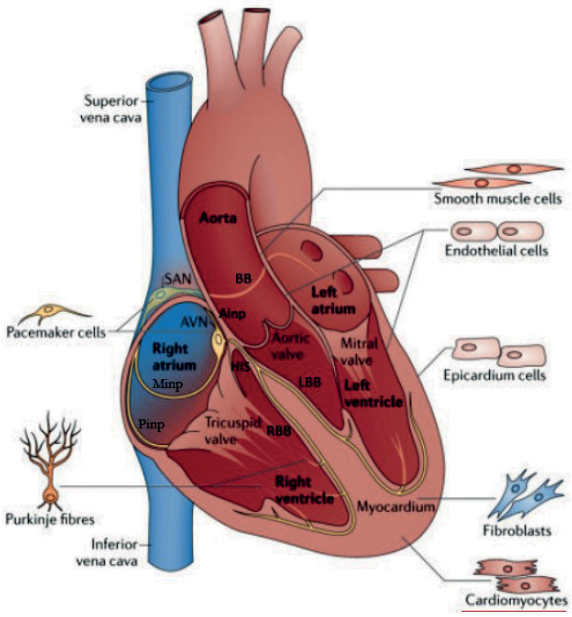


Figure 1. Heart cell types and conduction pathways.
SAN, sinoatrial node; AVN, atrioventricular node; BB, Bachmann bundle; Ainp, Minp, Pinp, anterior, middle, and posterior internodal pathways; HIS, his bundle; RBB, right bundle branch; LBB, left bundle branch.
Modified from Xin et al., 2013.

2.2 Electrophysiology of the heart

2.2.1 Cardiac excitation

The mechanical activity of the heart is controlled by electrochemical impulses. These impulses are intrinsic to the heart but are also modulated by neuronal activity through the autonomic nervous system. (Keating & Sanguinetti, 2001.) Atrial activation begins in the sinoatrial node (SAN) or the neighboring atrial pacemakers. SAN is primary pacemaker. It spreads in radial fashion to depolarize the right atrium, the interatrial septum, and then the left atrium. (Nerbonne & Kass, 2005; Surawicz & Knilans, 2008.) This impulse is transmitted to all atrial myocytes, leading to coordinated depolarization and the contraction of the atria (Keating & Sanguinetti, 2001).

Three specialized pathways (the anterior, middle, and posterior internodal pathways) link the SAN to the atrioventricular node (AVN). An interatrial pathway, the Bachmann bundle, connects the right and left atria. (Sedmera & Gourdie, 2014.) The main function of the AVN is the generation of a delay between the activation of the atria and the ventricles. A bundle of His is the continuation of the penetrating bundle on the ventricular side of the AVN before the left and right bundles (Zipes et al., 2019). Purkinje fibers connect with the ends of the bundle branches. The Purkinje system is a complex network of cardiac cells located at the endocardium that is specialized in the rapid conduction of electrical signals in the ventricles. It is necessary to coordinating the myocardial activation and coordination of AV valves. (Guiraudon & Jones, 2014; Sedmera & Gourdie, 2014.) (Figure 1). The parasympathetic nerves, which slow heart rate, are distributed mainly to the SAN and AVN, to a lesser extent to the muscle of atria, and very little directly to the ventricular muscle. The sympathetic nerves, which increase heart rate, are distributed to all parts of heart. (Hall, 2016.)

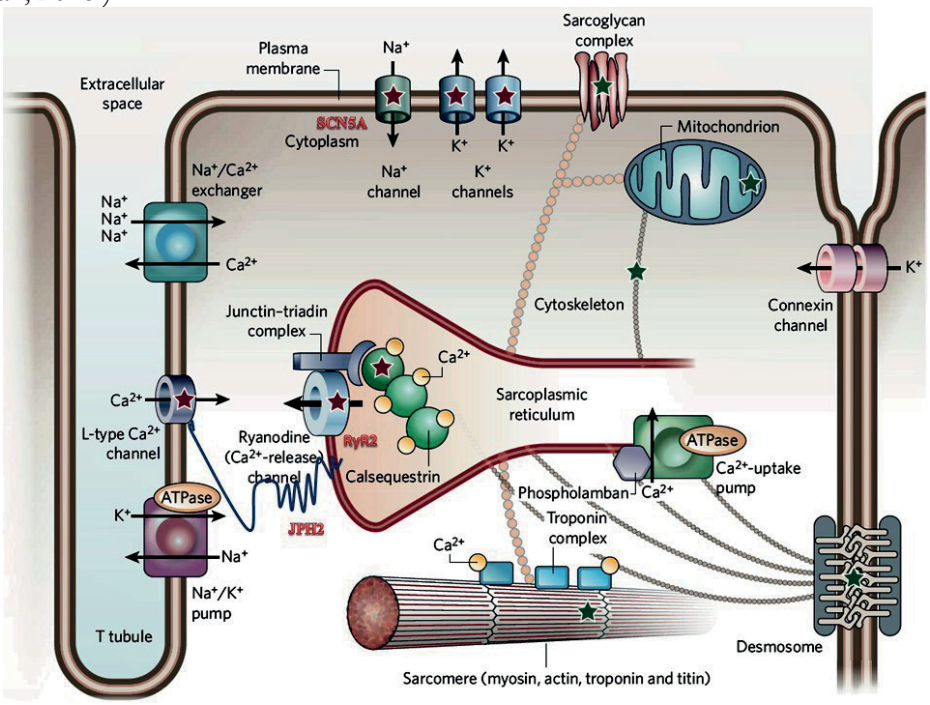


Figure 2. A ventricular cardiomyocyte. Illustrated are the protein complexes (especially **SCN5A, RyR2** and **JPH2**), cardiomyocyte architecture and intracellular organelles involved in cardiac excitation–contractile coupling, which is explained in the text. Red stars indicate proteins encoded by genes that are mutated in primary arrhythmia syndromes, two of them are *RyR2* and *SCN5A*. Green stars indicate protein complexes in which mutations in multiple genes cause cardiomyopathies often associated with arrhythmias; these complexes include the sarcomere. Modified from Knollmann & Roden, 2008.

2.2.2 Cardiac action potential

Electrical signaling in the heart involves the passage of ions through ionic channels: sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}). The movements of these ions across the cell membrane through channel pores generate excitation and signals in cardiac myocytes that are required for normal electrophysiological activity. (Nerbonne & Kass, 2005; Zipes et al., 2019.) The regulation of the contractility of the individual cardiomyocytes is tightly mediated by multiple ion channels and regulatory proteins and exchangers that accurately control Ca^{2+} entry into and out of the cell and the sarcoplasmic reticulum (SR) (Woodcock & Matkovich, 2005) (Figure 2).

Potassium currents determine the resting membrane potential and govern repolarization in cardiac myocytes. The transfer rate of potassium ions is dependent of the beating rate. (Aziz et al., 2018.) The starting event in the cardiac cycle is membrane depolarization, which occurs with ion entry through connexin channels from a neighbouring cardiomyocyte followed by opening of voltage-gated Na^+ channels and Na^+ entry (Knollmann & Roden, 2008). Sodium channels play an essential role in the initiation, propagation, and maintenance of cardiac excitation throughout the heart (Terrenoire et al., 2007). The cardiac action potential (AP) is initiated by the depolarization of the sarcolemma (Woodcock & Matkovich, 2005). The resultant rapid depolarization of the membrane inactivates Na^+ channels and opens both K^+ channels and Ca^{2+} channels. The entry of Ca^{2+} into the cell triggers the release of Ca^{2+} from the sarcoplasmic reticulum through the ryanodine channel 2 (RyR2). Ca^{2+} binds to the troponin complex and activates the contractile apparatus. Cellular relaxation occurs on the removal of Ca^{2+} from the cytosol by the Ca^{2+}-uptake pumps of the SR and by Na^+/Ca^{2+}- exchange (NCX) with the extracellular fluid. Intracellular Na^+ homeostasis is achieved by the Na^+/K^+ pump. (Knollmann & Roden, 2008.) (Figure 3).

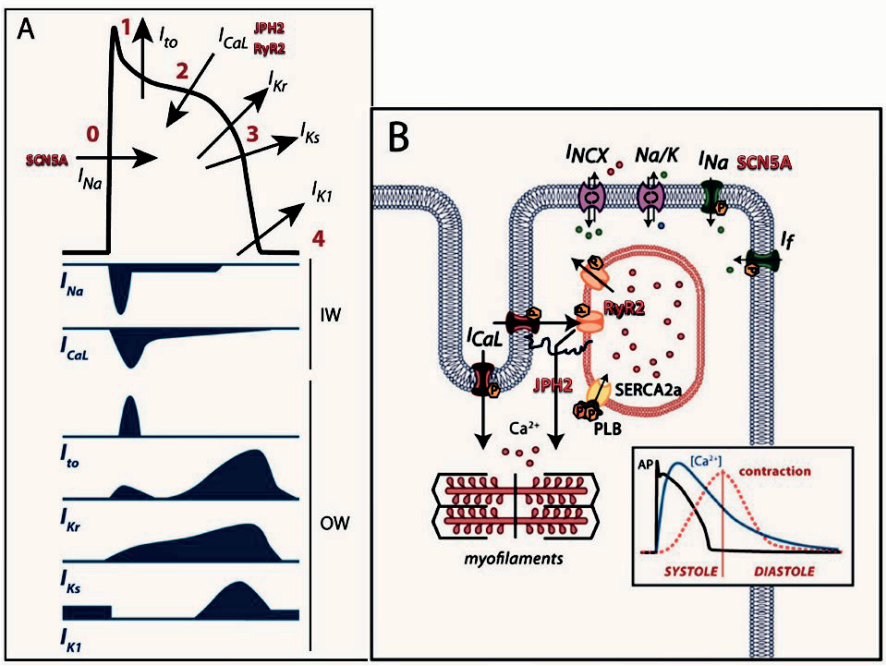


Figure 3. (A) Cardiac action potential and transmembrane ionic currents that participate in each phase. Phase 0: rapid depolarization due to entrance of Na^+ currents into the cell. *SCN5A* encodes the rapid sodium channel. Phase 1: early repolarization initiated by outward K^+ currents. Phase 2: a plateau phase marked by the Ca^{2+} entry into the cell against K^+ outward repolarizing currents. Phase 3: end of repolarization produced by K^+ currents upon Ca^{2+} channel-inactivation. Phase 4: resting membrane potential (about -90 mV) determined by inward-rectifier K^+ currents. OW, outward currents; IW, inward currents. I_{Ks}, delayed-rectifier; I_{Kr}, rapid delayed-rectifier; I_{CaL}, inward Ca^{2+} currents through the L-type voltage-dependent calcium channels

(B) Excitation-contraction coupling: during action potential, Ca^{2+} entry in phase 2 induces a large release of Ca^{2+} from the sarcoplasmic reticulum through the RyR2 receptor that allows cell contraction. After repolarization, Ca^{2+} is extruded from the cell through the Na^+/Ca^{2+} exchanger or taken back into the SR through SERCA2a to allow cell relaxation. JPH2 has an important role in the maintenance of effective Ca^{2+} handling. Figure modified from Garcia-Elias & Benito, 2018.

2.2.3 The cardiac sodium channel

Voltage-gated sodium channels (Na_v) are broadly expressed in the human body. The Na_v 1.5 is the main Na_v expressed in the heart (Moreau & Chahine, 2018). The $Na_v1.5$ channels are transmembrane proteins of cardiomyocyte for the rapid upstroke of the cardiac action potential and for rapid impulse conduction through cardiac tissue (Balser, 1999). $Na_v1.5$ channels mediate the inward sodium current (I_{Na}) and induce fast depolarization, thereby initiating the excitation–contraction coupling cascades in the cells. The upstroke speed of the AP and conduction is determined by the numbers of $Na_v1.5$ channels that are available for opening. The inactivation process is usually rapid and stable for most ion channels. (Han et al., 2018.) The number of sodium channels and their electrophysiological function differ between various parts of the heart (Remme et al., 2008; Remme et al., 2013). The voltage-gated sodium channel is encoded by the *SCN5A* gene (Remme & Bezzina, 2010).

2.2.4 The cardiac potassium channel

Voltage-gated potassium channels (K_v) are the most various family of voltage-dependent channels in the heart (Zipes et al., 2019). K^+ channels play a pivotal role in the AP repolarization process. Some of the K-channels are primarily responsible for early repolarization, whereas others drive late repolarization and others are open throughout the cardiac cycle. (Schmitt et al., 2014.) Reduced potassium channel protein levels or function causes prolonged repolarization and long QT, for example the genetic syndromes long-QT 1 and 2, even drug-induced long-QT. Reduced potassium channel expression or function may lead to other disease states, as in heart failure and hypertrophy. (Huang et al., 2013.)

2.2.5 The cardiac calcium channel

The regulation of intracellular calcium (Ca^{2+}) is very important in all cell types (Kushnir et al., 2018). The main function of cardiac muscle cells is to enforce cardiac excitation-contraction-relaxation, which depends on the electrical Ca^{2+} transport and contractile properties (Zipes et al., 2019). Therefore, calcium is an important second messenger in cardiac function (Bers, 2002). Ca^{2+} release through RyR2 is triggered by Ca^{2+} entering via $Ca_V1.2$ during depolarization. In the heart, RyR2 are organized on the SR in Ca^{2+} release units associated with $Ca_V1.2$ on the transverse tubules

(T-tubules) forming SR/T-tubule junctions. (Franzini-Armstrong et al., 2005.) The depolarization of the cell membrane causes Ca^{2+} to enter the cardiomyocyte through $Ca_V1.2$ during the prolonged plateau phase of the cardiac action potential. This Ca^{2+}-induced Ca^{2+}-release is required for myocardial contraction. (Wong et al., 1992.) Ca^{2+} is also important for the activation of signal transduction pathways responsible for hypertrophic cardiac remodeling and heart failure by controlling gene transcription via Ca^{2+}-dependent signaling as well as for cardiac development, cardiac energy homeostasis, and eventually for cell death (Freichel et al., 2017).

2.2.6 Cardiac ryanodine receptors

Ryanodine receptors (RyR) are intracellular Ca^{2+}-permeable channels that provide the sarcoplasmic reticulum Ca^{2+} release required contractions for skeletal underlying RyR1 and RyR2 for cardiac muscle (Betzenhauser & Marks, 2010). RyRs are massive structures comprising the largest known ion channel-bearing macromolecular complex in the human genome (Santulli et al., 2018). RyR2 encodes a calcium ion transporter, which is responsible for the calcium induced calcium release that results in ventricular contraction. RyR2 is responsible for calcium regulation in the cardiomyocyte. (Priori & Chen, 2011.)

2.2.7 Junctophilin 2

Junctophilin 2 (JPH2) is an important membrane-binding structural protein in cardiomyocytes for the coupling of transverse (T) tubule-associated L-type Ca^{2+} channels (LTCC) and type-2 ryanodine receptors on the sarcoplasmic reticulum within junctional membrane complexes (JMC) (Nishi et al., 2000; Takeshima et al., 2000). JPH2 provides a structural bridge between the plasmalemma and sarcoplasmic reticulum and is essential for precise Ca^{2+}-induced Ca^{2+} release during excitation-contraction coupling in cardiomyocytes (Guo et al., 2015). Signaling between these two Ca^{2+} channels is required for normal cardiac contractility (Matsushita et al., 2007) (Figure 2 and 3).

2.3 Electrocardiogram

The primary clinical tool for evaluating cardiac electrical events is an electrocardiogram (ECG). The APs are responsible for the atrial and ventricular activations, and this electrical activation is recorded by ECG. (Surawicz & Knilans, 2008.) The ECG is characterized by different waves that represent specific phases of the cardiac activation sequence: the P wave reflects atrial depolarization, the QRS complex represents ventricular depolarization, and the T wave indicates ventricular repolarization (Terrenoire et al., 2007) (Figure 4).

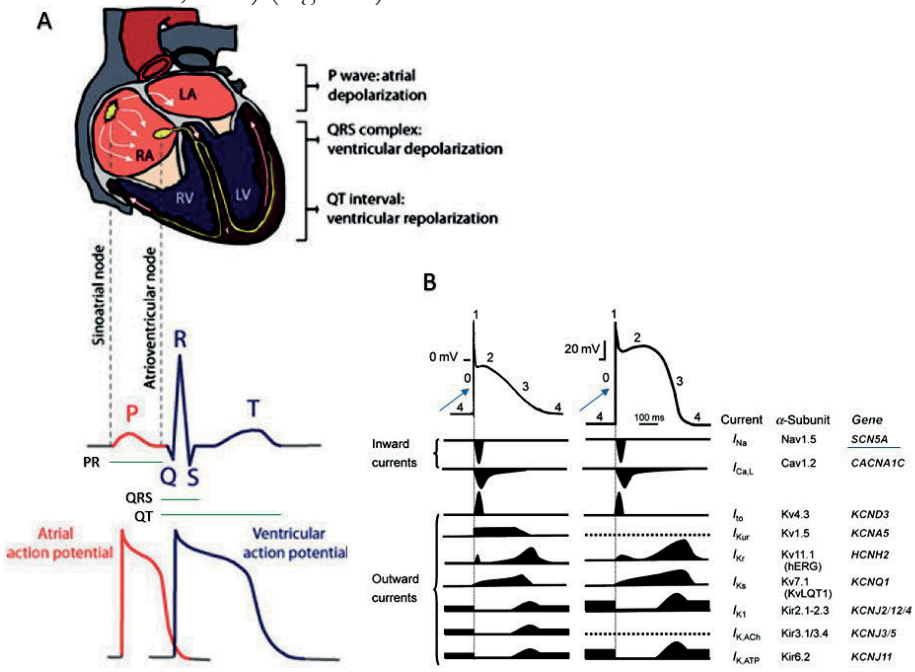


Figure 4. Cardiac electrical activity: (A) ECG and action potential. Green lines indicate PR, QRS and QT intervals. (B) Action potential and transmembrane ionic currents in each phase, especially sodium current. Modified from Amin et al., 2010 and Ravens & Cerbai, 2008.

2.3.1 P wave and PR interval

The P wave corresponding to atrial depolarization, the early part of the P wave may be considered to represent the electrical potential generated by the upper part of the right atrium, and the late part as that by the left atrium and the inferior right atrial wall (Surawicz & Knilans, 2008).

The PR interval includes atrial depolarization and atrial repolarization (T_a) (Surawicz & Knilans, 2008; Childers, 2011).

2.3.2 QRS complex and QT interval

The QRS complex corresponding to ventricular depolarization from which the axis, duration and configuration are determined and from these results we can evaluate the normality of ventricular activation (Surawicz & Knilans, 2008).

The QT interval stands for the duration of ventricular electrical systole (Surawicz & Knilans, 2008). The QT interval also includes ventricular depolarization. ST segment and T wave are ¾ of the QT interval which is used to represent ventricular repolarization. (Airaksinen et al., 2016.) The QT interval is determined by the cardiac action potential, which is balanced between inward and outward currents of myocardial cells (Visken, 2009).

2.3.3 QTc

Normally the QT interval decreases with increasing heart rate (Karjalainen et al., 1994) due to an increase in potassium flux across the cell membrane (Aziz et al., 2018). There are many formulas describing this relation. QTc formula attempts to separate the dependence of the length of the QT interval from the length of the RR interval (Surawicz & Knilans, 2008).

The duration of the mechanical ventricular systole is determined by the original Bazett's formula: Systole = K√cycle. The normal value for K is 0.37 for men and 0.4 for women. (Bazett, 1920.) The most widely used is the formula derived by Bazett: QTc = QT/√RR (Funck-Brentano & Jaillon, 1993). Rate correction of the QT interval should not be efforted when RR interval variability is large (Rautaharju et al., 2009). QT interval and QTc are expressed in milliseconds and RR interval in seconds. Nevertheless, the upper limit of the normal QTc value is less than 0.441 seconds. (Surawicz & Knilans, 2008.)

QT/RR hysteresis value means how quickly; and QT/RR adaptation how much QT interval changes when heart rate changes (Malik et al., 2018). QT/RR hysteresis is independent of the static QT/RR relationship (Malik et al., 2008).

2.3.4 Pathologies of conduction time

Cardiac conduction, as assessed by the PR interval and QRS duration, is an important electrophysiological character and a determinant of arrhythmic risk (Smith J.G. et al., 2009). A wide, notched or low-amplitude Ta can predict an interatrial

conduction disturbance with or without atrial enlargement. The Ta wave represents atrial repolarization and the interval from the beginning of the P wave to the end of Ta is the atrial equivalent of the ventricular QT interval. (Childers, 2011.) PR prolongation is also associated with an increased risk of atrial fibrillation (Shulman et al., 2015), a need for pacemaker implantation, and excess mortality (Cheng et al., 2009). Progressively increasing conduction defects predict atrial paralysis and atrial arrhythmias (Probst et al., 2003; Sajeev et al., 2006). Many investigators considered fragmented QRS (fQRS) as an independent predictor of cardiac events (Morita et al., 2008; Das & Zipes, 2009; Chatterjee & Changawala, 2010). The abnormal duration of the QT interval has been found in epidemiologic studies to identify individuals at high risk of sudden cardiac death (Zhang et al., 2011).

The definition of normal QT time is difficult because abnormality QT interval can be either "too long" or "too short". Large population studies suggest that, for the adult population, normal QTc values for males are 350 to 450 ms and for females 360 to 460 ms. (Visken, 2009.) However, the upper limit for the normal QTc for both genders is 440 milliseconds (Surawicz & Knilans, 2008). Nevertheless, the Bazett's correction overestimates the number of patients with a prolonged QT (Patel et al., 2016). Nonetheless, marked abnormalities of the QT interval may be caused by many different reasons and situations: genetic disorders (e.g., long/short QT syndrome), pharmacologic agents (e.g., antiarrhythmics, antipsychotics, antibiotics), electrolyte abnormalities (e.g., hypokalemia and hypomagnesemia), and their interactions (Zhang et al., 2011). Especially when pharmacologic agents cause QT changes, it is possible to choose individual treatments for patients by combining evidence from *in vivo* and *in vitro* studies using human pluripotent stem cell-derived cardiomyocyte (hPSC-CM) techniques (Yamazaki et al., 2018).

2.3.5 Electrophysiological studies on human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs)

With the microelectrode arrays (MEAs) technology, it is possible to record field potentials (FPs) generated by cells with electrical activity, including cardiomyocytes (Stett et al., 2003; Reppel et al., 2004). The MEA biosensor is *in vitro* system to monitor both acute and chronic effects of drugs and toxins and to perform functional studies under physiological or induced pathophysiological conditions that mimic *in vivo* damages (Stett et al., 2003).

In the heart, FP represents spread of excitation and the conduction velocity in MEA, and FP corresponds intrinsic AP, and AP duration is the same than FP duration (FPD) (Halbach et al., 2003). Peak-to-peak interval (PPI) is determined as time between two depolarizing sodium [Na^+] peaks, PPI is analogues with RR interval. The FPD has been claimed to be analogous with the QT interval on the ECG. (Stett et al., 2003.) (Figure 5)

The Bazett's formula for correcting FPD (cFPD) for rate-depended modulation is commonly used with MEA recordings: $cFPD = FPD/\sqrt{PPI}$ (Caspi et al., 2009; Itzhaki et al., 2011).

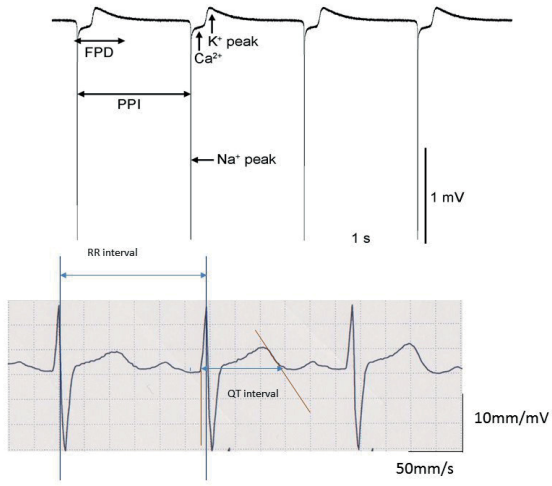


Figure 5. Characteristics of field potential (FP) (upper panel) and ECG (lower panel) recordings. A FP trace from one of the recording electrodes on the microelectrode array generated by the spontaneously beating human embryonic stem cell–derived cardiomyocytes. The field potential duration (FPD) and peak-to-peak interval (PPI) is also depicted in the first cardiac FP cycle along with the sodium (Na^+), calcium (Ca^{2+}), and potassium (K^+) currents in the cardiac FP components.

2.4 Methods to study genetics of cardiac diseases

2.4.1 Genetic testing in clinical practice

Genes are the fundamental units of hereditary (Krebs et al., 2018). Over the past several decades, the causative genes have been identified for a range of cardiovascular diseases. This knowledge has provided explanations to patients, improved the ability to predict risk for diseases, and enabled understanding of the pathophysiology; therefore, we can improve prevention and therapy. (Zipes et al., 2019.)

Genes are encoded by deoxyribonucleic acid (DNA), which comprises four different bases: adenine (A), cytosine (C), guanine (G) and thymine (T). DNA has two opposing strands that form A-T and G-C base pairs, and coil into a double helix structure. (Watson & Crick, 1953.) The human genome contains about 6 billion base pairs across the 46 chromosomes. About 1% of the genomic DNA encodes the estimated 20 000 genes in humans. (Zipes et al., 2019.) The DNA sequence of a gene that codes protein is known as the exon. Mutations are heritable changes in the base sequence of DNA. (Krebs et al., 2018.)

2.4.1.1 Modes of inheritance

Disease can be a result from a defect in a single gene (monogenic) or from many genes (polygenic) (Zipes et al., 2019). For monogenic diseases, modes of inheritance include autosomal dominant, autosomal recessive and X-linked heritability. Mitochondrial genetic disorders are maternally inherited. In autosomal dominant disorders, a single defective allele of a gene from the mother or father suffices to the cause phenotype, whereas in autosomal recessive disorders both copies from mother and father need to be defective to lead to the phenotype. (Zipes et al., 2019.) Genetic variants are inherited (familial) or represent spontaneously derived (sporadic) *de novo* variants occurring for the first time in an individual (Tester & Ackerman, 2011).

Most common cardiovascular diseases are polygenic disorders requiring variants in more than one gene to cause a disease (Zipes et al., 2019). Many cardiomyopathies and channelopathies are the consequences of a single gene defect and are thus inherited according to Mendelian law (Corrado et al., 2005), in which case the change is necessary and sufficient to cause disease (Zipes et al., 2019). Studies on genotype–phenotype correlations led to the identification of affected asymptomatic individuals and healthy mutation carriers in every condition (Corrado et al., 2005).

Cardiac disease mutations are remarkable for substantial variation in clinical expression. Both genetic heterogeneity (e.g. several genes that cause the same disease) and allelic variation (different mutations in the same gene) contribute to variable morphological phenotypes and disease severity. Genetic variation, lifestyle, and other contributory factors that account for differences have an influence on clinical manifestations in family members with identical mutations. (Richards et al., 2015; Burke et al., 2016.)

2.4.1.2 Pathogenicity classification of genomic variants

Genetic diagnostics has proven as an effective strategy to differentiate potential underlying causes and to rule out phenocopies. Accurate molecular genetic diagnosis helps to detect those phenocopies that might require different treatments. (Ackerman et al., 2011; Vago et al., 2016.)

When classifying such variants as pathogenic, we know that a mechanism of pathogenicity is consistent with the established inheritance pattern for the disease (Richards et al., 2015; Jarvik & Browning, 2016). Variants with very low population frequency and with strong evidence for cosegregation with disease phenotype, conservation across species, or functional evidence are classified as pathogenic. Variants are likely pathogenic, when they have lower strength for consegregation or limited functional evidence. Otherwise likely pathogenic variants are like pathogenic ones. Designation "variant of uncertain significance (VUS)" is defined as a variant with restricted evidence for consegregation, limited or contradictory functional evidence, and variable conservation across species. (Richards et al., 2015.)

The genotype–phenotype correlation is complicated when some phenotypes do not manifest in all individuals carrying the same gene mutation (incomplete penetrance), and when the type and severity of the phenotypes varies between genotype-positive individuals (variable expressivity) (Giudicessi & Ackerman, 2013).

2.4.1.3 Next-generation DNA sequencing technology

In the last decade, sequencing technology (next-generation sequencing [NGS]) has facilitated the search for such genetic components spread over the genome. NGS is a sequencing technology that enables sequencing at a massive scale across the

genome, and it can be used to identify additional genetic loci involved in a disease. NGS can be used in gene panels to sequence multiple genes simultaneously (e.g. 20–109 comprehensive arrhythmia or cardiomyopathy-associated genes). (Myllykangas et al., 2011; Mizusawa, 2016.)

Disease-causing genes for inherited arrhythmias have been successfully identified in the last three decades, resulting in a large impact on patient care (Mizusawa, 2016). Mutations in any gene encoding an ion channel protein can cause ion channel dysfunction, a so-called channelopathy. This channelopathy may lead to cardiac arrhythmias and sudden cardiac death as the most severe clinical manifestation. (Brugada J. et al., 2007.) In the absence of structural heart disease, these arrhythmias, especially in the younger population, are often an outcome of genetic defects in specialized membrane proteins (Knollmann & Roden, 2008).

During the last two decades, mutations especially in sarcomere genes have been found to comprise the most common cause for hypertrophic cardiomyopathy (HCM), but still a meaningful number of patients with dominant HCM are left without molecular genetic diagnosis. NGS does not only enable evaluation of known HCM genes, but also candidate genes for cardiomyopathy are frequently tested, which may lead to a situation where conclusive interpretation of the variant requires extensive family studies. Diagnostic yield of molecular genetic testing in daily practice is 25–40% in HCM. (Walsh et al., 2017.)

2.4.2 Stem cells

Stem cells signify natural units of embryonic development and tissue regeneration. Pluripotent stem cells (PSC) have a nearly unlimited self-renewal capacity and developmental potential to differentiate into virtually any cell type of an organism. (Wobus & Boheler, 2005.) An embryonic stem cell line is derived from the inner cell mass of a blastocyst. The resulting human embryonic stem cell line can then be differentiated into various cell types of the different embryonic lineages as well as into male or female germ cells. (Wobus & Boheler, 2005; Mountford, 2008.)

The first stable human embryonic stem cell (hESC) lines were derived in 1998 (Thomson et al., 1998) and first human induced pluripotent stem cell (hiPSC) lines in 2007. These cells are collectively called human pluripotent stem cells (hPSCs). (Takahashi et al., 2007.) Naturally found cells, pluripotency is associated with the cells of early embryos, which can generate all of the tissues in the organism. iPSCs are similar pluripotent stem cells, but they are produced from fully differentiated

cells by inducing the expression of genes necessary for pluripotency (Park et al., 2008).

2.4.2.1 Induced pluripotent stem cells

The ability to generate induced pluripotent stem cells (iPSCs) by reprogramming somatic tissues is one of the greatest breakthroughs in biomedical science in the last decade. Notable advances have been made in reprogramming technologies beyond the stable integration of four core genes (octamer-binding transcription factor 4 (Oct4), sex determining region Y-box 2 (Sox2), Kruppel-like factor 4 (Klf4) and myelosytomatosis viral oncogene homolog (c-Myc)) originally described by Yamanaka and colleagues in 2006 for mouse cells (Takahashi et al., 2006; Yamanaka, 2008) and soon reproduced from human cells. (Takahashi et al., 2007; Park et al., 2008.) Although the integrated viral transgenes become transcriptionally silenced after reprogramming, insertional mutations and residual transgene or virally induced gene expression may interfere with differentiation or alter sub-sequent cell phenotypes, leading to questions of whether iPSCs and their derivatives really reflect a patient's own cells (Saha & Jaenisch, 2009).

The iPSCs approach, pioneered by Takahashi and Yamanaka, allows the reprogramming of adult somatic cells into pluripotent stem cells by ectopic expression of a set of transcription factors. Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) can be using for cardiac research. (Takahashi et al., 2007.) HiPSC-CMs have been used in many studies to assess molecular mechanism of different heart diseases. (Saha & Jaenisch, 2009; Smith A.S.T. et al., 2017.) (Figure 6) These cardiomyocytes have been extensively used for *in vitro* electrophysiological recordings (Kehat et al., 2001; Reppel et al., 2005; Liang et al ., 2010). The role of patient- and disease-specific iPSC-CMs is a new platform with significant scientific and clinical potential in studying disorders related to sudden cardiac death (SCD) (Sallam et al., 2015).

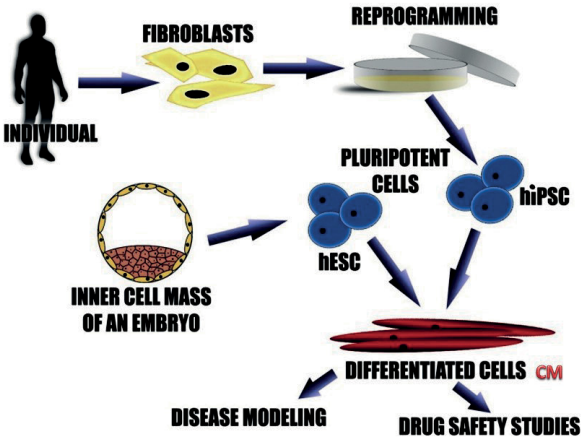


Figure 6. Pluripotent hESCs and hiPSCs can be differentiated into various cell types using specific differentiation protocols. CM, cardiomyocyte. Modified from Laurila et al., 2016.

2.5 Genetic cardiac diseases pathophysiology and gene mutations

2.5.1 Sudden cardiac death

Sudden cardiac death (SCD) depicts a natural and unexpected death from cardiac causes occurring within a short period of time (generally within 1 h of symptom onset) in the absence of any other potentially lethal condition (Magi et al., 2017). In adult populations, cardiomyopathies and cardiac channelopathies, along with coronary heart disease (CHD), are the most common conditions that predispose patients to SCD. According to recent epidemiological studies of SCD in Western countries, CHD underlies about 75%, cardiomyopathies 10–15%, and ion channelopathies 1–2% are diagnosed of the SCD cases. (Hayashi et al., 2015; Mizusawa, 2016.) The limits between inherited primary electrical heart diseases (channel-pathy) and structural heart muscle disease (cardiomyopathy) are still undefined (Corrado et al., 2005). A susceptible substrate can be present even in the absence of any structural heart disease detectable by conventional techniques, such as echocardiography or magnetic resonance imaging (Knollmann & Roden, 2008; Ackerman et al., 2011).

2.5.2 Cardiac channelopathies

Cardiac channelopathies, primary electrical disorders result from mutations in genes encoding cardiac ion channels and/or their regulatory proteins, which result in modifications in the cardiac action potential or in the intracellular calcium handling that lead to electrical instability (Garcia-Elias & Benito, 2018). The electrical substrate of cardiac channelopathies can develop depending on the affected gene and the effect of a particular mutation (loss- or gain-of-function) on the channels, receptors and regulators that participate in this process. A mutation may make a channel non-functional, underactive, overactive or leaky. (Garcia-Elias & Benito, 2018; Skinner et al., 2019.) It is said that each cardiac channelopathy has its own ECG signature and typical mode of presentation (Skinner et al., 2019). Typical channelopathies are long QT syndrome, short QT syndrome, Brugada syndrome and catecholaminergic polymorphic ventricular tachycardia (CPVT) (Fernández-Falgueras et al., 2017).

2.5.2.1 Catecholaminergic polymorphic ventricular tachycardia

Catecholaminergic polymorphic ventricular tachycardia is an inheritable cardiac disorder associated with adrenergic exercise- and emotional stress-induced ventricular tachycardia (VT) in the absence of structural heart disease and high incidence of sudden death with life-threatening polymorphic or bidirectional VT or ventricular fibrillation (VF) (Watanabe & Knollmann, 2011; Priori et al., 2015; Imberti et al., 2016). CPVT is rare, estimated prevalence at 1:10 000 (Priori et al., 2002). Approximately 30% of patients with CPVT develop symptoms before 10 years of age, and 60% before 40 years (Ackerman et al., 2011). A natural history of CPVT is showing up to 30% SCD before age 40 in the absence of antiadrenergic therapy (Napolitano et al., 2012).

Pathophysiology of CPVT

During stress and exercise, catecholamines are released by the sympathetic nervous system and bind to beta-adrenergic receptors resulting in generation of cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA) which has both direct functional as well as developmental effects (Kushnir et al., 2018). During sympathetic nervous system mediated stress, activation of RyR2 plays a key role in

increases in Ca^{2+} transient resulting in sustained enhancement of cardiac contractility (Bers, 2002; Kushnir et al., 2018). The slightest modification in the functioning of one of these players may modify the AP and/or the intracellular Ca^{2+} dynamics, potentially favouring an arrhythmogenic substrate (Garcia-Elias & Benito, 2018).

CPVT is caused by mutations in genes involved in the intracellular calcium homeostasis of cardiac cells (Obeyesekere et al., 2015). Arrhythmias in CPVT occur from diastolic Ca^{2+} leak (Kushnir et al., 2018). Two causative genes of CPVT have been identified: *RyR2*, encoding the cardiac ryanodine receptor Ca^{2+}- release channel, and calsequestrin 2 (*CASQ2)*, encoding cardiac calsequestrin (Watanabe & Knollmann, 2011). CPVT is inherited as an autosomal dominant or autosomal recessive trait, usually with high penetrance (Laitinen et al., 2004). Approximately 60% of patients with CPVT have a mutation in the *RyR2* (CPVT1), and CPVT2 cases are autosomal recessive mutations caused by mutations in *CASQ2* gene, which encodes the calcium binding protein (calsequestrin). These are much rarer and cause about 3–5% of CPVT cases (Skinner et al., 2019).

Diagnosis of CPVT

Diagnosis is based on (family) history of adrenergically induced syncope, response to stress testing and genetic analysis (Siegers et al., 2014). Bidirectional VT is considered the diagnostic marker of CPVT. During an exercise stress test, CPVT patients display isolated premature beats at the beginning of exercise with a progressive worsening of the complexity of ventricular arrhythmias in response to an increased workload. Typically, when the heart rate reaches 90 to 110 bpm, runs of non-sustained or sustained VT appear and they may degenerate into sustained VT and VF unless exercise is promptly terminated. However, not all patients with CPVT manifest this form of arrhythmia. Therefore, molecular screening of the *RYR2* and *CASQ2* genes are important to diagnose in patients with less typical phenotypic manifestations. (Leenhardt et al., 1995; Priori & Chen, 2011.) Delayed diagnosis leads to suboptimal treatment and unnecessary risk of SCD (Siegers et al., 2014).

2.5.2.1.1 Cardiac ryanodine receptors *RyR2* mutation

Sympathetic activation during exercise or emotional stress induces ventricular arrhythmias above a threshold heart rate in *RyR2* mutation carriers (Priori et al., 2002; Laitinen et al., 2004; Kontula et al., 2005). Gain-of-function mutations of the *RyR2* receptor cause increased calcium (Ca^{2+}) sensitivity, which leads to a premature and increased release of calcium from the sarcoplasmic reticulum during diastole, and adrenaline stimulate further calcium release, resulting in delay after depolarizations and triggered activity (Priori & Chen, 2011; Leenhardt et al., 2012; van der Werf & Wilde, 2013). *RyR2* receptors are divided into four regions (i.e. N-terminal: 1–600; central: 2000–2500; C-terminal: 3700–4200 and 4500–5000) (Ono et al., 2010) (Figure 7).

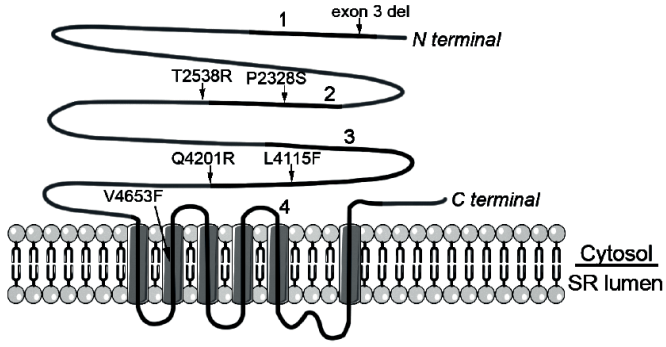


Figure 7. RyR2 protein, mutations studied in the study III and mutation clusters 1–4. Mutations in the study III (arrows) are located in different parts of the RyR2 protein and mutation clusters. Clusters are represented as black lines numbered from 1 to 4. Cluster 1 consists of amino acids (AA) 44–466, cluster 2 AA 2246–2534 and cluster 3 AA 3778–4201, and these three clusters are located in the N-terminal and central regions of the protein and form the cytoplasmic domain. Cluster 4 comprises AA 4497–4959 and forms the transmembrane domain, which is located in the C-terminal region. Modified from Priori & Chen, 2011.

2.5.2.2 Cardiac conduction defect and atrial arrhythmias and sodium channel *SCN5A* gene mutations

Abnormalities in cardiac conduction can occur due to a variety of factors, including developmental and congenital defects, acquired injury or ischemia of portions of the conduction system, or less frequently due to inherited diseases that alter cardiac conduction system function (Wolf & Berul, 2006).

The gene *SCN5A* is located on chromosome 3p21 and encodes the alpha-subunit of the voltage-gated cardiac sodium channel Na_V 1.5 (Remme et al., 2008; Veerman et al., 2015). It is expressed in heart tissue and plays a critical role in the excitability of cardiomyocytes (Amin et al., 2010). Mutant $Na_v1.5$ causes alterations in the peak and late sodium current and is associated with a wide range of congenital arrhythmias. More than 400 *SCN5A* mutations have been found in the *SCN5A* gene. (Han et al., 2018.) The importance of I_{Na} for normal cardiac electrical activity is the reason for the high incidence of arrhythmias in cardiac sodium channel-pathies (Amin et al., 2010).

SCN5A encodes the rapid sodium channel. Regulation of the expression and proper function of voltage-gated ion channels at the plasma membrane of excitable cells is essential in maintaining cellular excitability and electrical impulse propagation. In the heart, voltage-gated sodium (Na^+) channels determine the amplitude and slope of the action potential upstroke, which are especially important in the control of impulse conduction velocity, and in the maintenance of appropriate waves of excitation through the working myocardium. (Nerbonne & Kass, 2005; Wilde & Amin, 2018.)

The dysfunction of these channels can lead to life-threatening cardiac arrhythmias (Herfst et al., 2003). Mutations in the *SCN5A* gene are involved in numerous inherited cardiac arrhythmias: long QT syndrome (LQTS), Brugada syndrome (BrS) (Brugada, J. et al., 2007; Makita et al., 2008), and sick sinus syndrome (SSS) (Gui et al., 2010), progressive cardiac conduction disease (PCCD) (Herfst et al., 2003), familiar atrial fibrillation (FAF) (Darbar et al., 2008; Remme & Bezzina, 2010) and atrial standstill (AS) (Groenewegen et al., 2003), which all potentially can lead to fatal arrhythmias at a relatively young age (Miles & Behr, 2016) (Figure 8). Very rare AS is associated with an increased risk of sudden death due to the occurrence of very low escape rhythm and an increased risk of thromboembolic stroke due to the high incidence of atrial fibrillation/flutter (AF) (Castro et al., 2009).

Familiar atrial standstill has been associated with the *SCN5A* D1275N mutation combined with rare polymorphisms in an atrial-specific connexin40 (*Cx40*) gene (Groenewegen et al., 2003; Makita et al. 2005; Makita, 2009). In a large Finnish

family, cardiac conduction defects and atrial arrhythmias were also associated with the same *SCN5A* D1275N mutation, although not linked to the polymorphism in the *Cx40* gene (Laitinen-Forsblom et al., 2006). For all that, no sudden cardiac death was documented in the Finnish family, at least four affected members suffered from cardioembolic stroke by the age of 31 and two of them died of stroke (Laitinen-Forsblom et al., 2006).

Makita et al. described progressive atrial dysfunction leading to the AS associated with *SCN5A* mutation L212P (Makita et al., 2005). The missense mutation, D1275N has been associated with a variety of unusual phenotypes, including dilated cardiomyopathy (McNair et al., 2004; Hayano et al., 2017). *SCN5A* mutations with loss-of-function properties have been identified in patients with cardiac conduction defects, SSS (Makita et al., 2005, Gui et al., 2010). Probst et al. described progressively increased QRS duration in patients with *SCN5A* linked hereditary Lev–Lenegre disease affecting the His bundle and its branches (Probst et al., 2003).

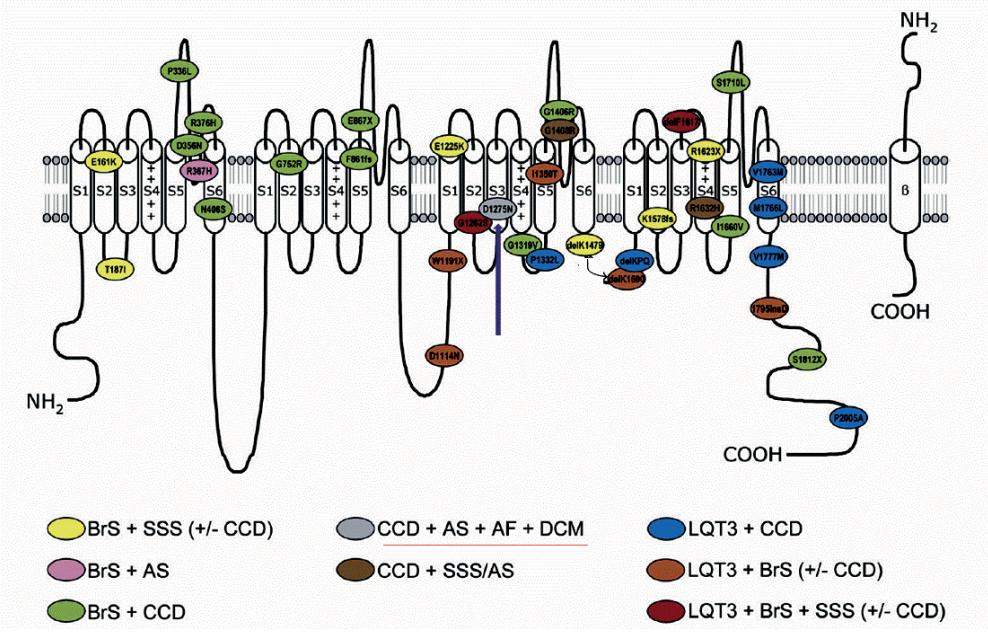


Figure 8. Schematic representation of the primary structure of the cardiac sodium channel with location of *SCN5A* mutations associated with sodium channel overlap syndromes. Arrow shows where D1275N mutation is located in *SCN5A* gene. BrS indicates Brugada syndrome; LQT3, long-QT syndrome type 3; SSS, sick sinus syndrome; CCD, cardiac conduction defect; AS, atrial standstill; AF, atrial fibrillation; DCM, dilated cardiomyopathy. Modified from Remme et al., 2008.

2.5.2.2.1 *SCN5A* D1275N and loss of I_{Na} function

SCN5A mutations cause arrhythmias through both gain- and loss-of-function mechanisms of the sodium channel (Tan, 2006; Han et al., 2018; Moreau & Chahine, 2018). Gain-of-function mutations in *SCN5A* lead to more sodium influx into cardiomyocytes through aberrant channel gating and cause for example long QT syndrome, whereas loss-of-function mutations in *SCN5A* lead to lower expression levels of the SCN5A or production of defective $Na_v1.5$ proteins. Some of the *SCN5A* mutations are loss- or gain-of mutations, and in overlap syndromes mutations have both loss-of-function and gain-of-function effects. (Wilde & Amin, 2018.) (Figure 9). *SCN5A* mutations causing early-onset AF has been demonstrated to be gain-of-function mutations (Olesen et al., 2012).

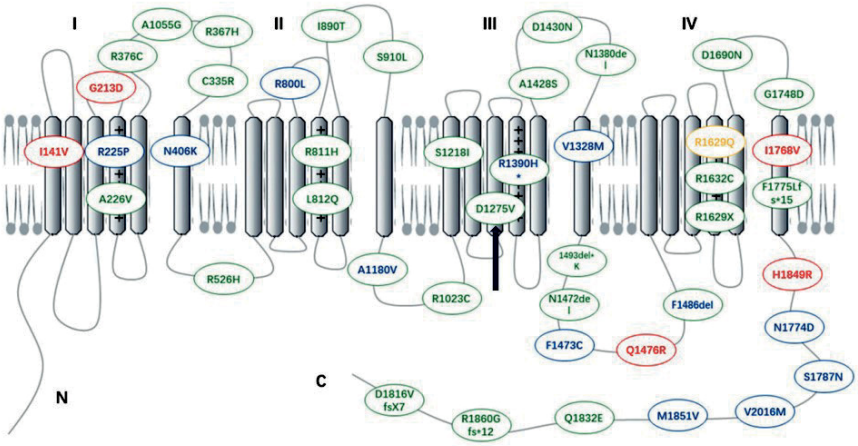


Figure 9. *SCN5A* mutations. Arrow shows where D1275N mutation is located in *SCN5A* gene. The red represents gain of $I_{Na\text{-}P}$, blue represents gain of $I_{Na\text{-}L}$, green represents loss of $I_{Na\text{-}P}$, yellow represents loss of $I_{Na\text{-}L}$, the mixed colors (text and ellipsoid) represent coexistence of gain- and loss-of-function mutations. The sodium current mediated by Nav1.5 consists of peak and late components ($I_{Na\text{-}P}$ and $I_{Na\text{-}L}$) Domains I-IV. Modified from Han et al., 2018.

The mutation present in the Finnish family *SCN5A* D1275N causing conduction defects and atrial arrhythmias (Laitinen-Forsblom et al., 2006) manifests a loss-of-function when analyzed in transfected cells (Gui et al., 2010). Reduced sodium channel availability leads to conduction defects (Remme & Bezzina, 2010) leading to prolongation of atrial action potential duration (APD) (Roberts & Gallop, 2010). P-wave broadening and a tendency to PR-prolongation are manifested in the ECG of individuals with the *SCN5A*/5280delG mutation, presumably due to a reduction in Na^+ current (Herfst et al., 2003). Reduced inward Na^+ current causes also QRS-widening, slow heart rate including abnormal pauses (>2 second) and slow-fast alternation of heart rhythm (Detta et al., 2015). *In vitro* electrophysiological studies showed reduced peak cardiac Na^+ current as a key defect and this is consistent with the observed reduced conduction velocity (Watanabe et al., 2011) (Figure 10).

Wilde and Brugada have described how loss of I_{Na} function could induce atrial fibrosis, which may cause atrial fibrillation in individuals with associated structural abnormalities including increased diastolic ventricular pressure in the setting of dilated cardiomyopathy with subsequent enlargement of the atria (Wilde A.A. & Brugada R., 2011). Fibrosis is a complex process of abnormal tissue healing which inevitably leads to loss of physiological organ structure and function (Schaefer, 2018).

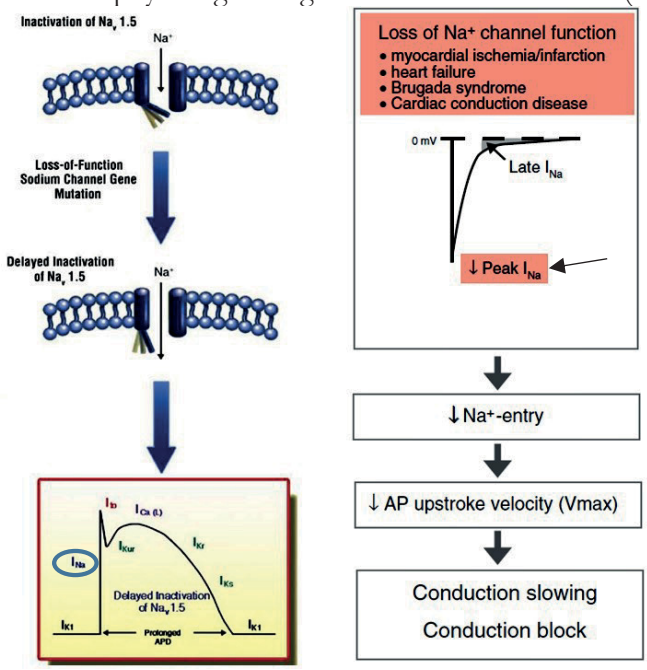


Figure 10. Schematic representation of *SCN5A* D1275N and loss of I_{Na} function. Modified from Roberts & Gallop, 2010; Remme & Wilde, 2014.

2.5.3 Cardiomyopathies

Cardiomyopathy is a myocardial disorder in which the heart muscle is structurally and functionally abnormal. Cardiomyopathies are grouped into specific morphological and functional phenotypes: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), arrhythmogenic right ventricular cardiomyopathy (ARVC), restrictive cardiomyopathy (RCM). (Elliot et al., 2008.)

DCM is defined as left ventricular dilatation and systolic dysfunction without coronary artery or valve disease. It may be caused by genes encoding sarcomeric proteins, cytoskeletal and even ion channel and gap junction proteins. (McKenna et al., 2017.) Many different conditions, such as tachycardia, infections, autoimmune disease et cetera, can also cause DCM (Japp et al., 2016).

RCM is a myocardial disease characterized by impaired ventricular filling and reduced diastolic volume in the presence of normal systolic function and normal or near normal myocardial thickness (Elliot et al., 2008; McKenna et al., 2017).

ARVC is a genetically determined cardiomyopathy characterized by fibrofatty replacement of the myocardium (Zipes et al., 2019). Mutations of genes encoding for various cardiac proteins are an alternative approach to reclassify cardiomyopathies according to the causative genetic defect (Elliot et al., 2008).

The diagnosis of heart failure (HF) can be combined with clinically assisted examinations, such as ECG or echocardiography. A brain natriuretic peptide (BNP) is a laboratory test, which is also widely used as significant indicators for cardiac dysfunction. (Elliot et al., 2014.)

2.5.3.1 Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is a primary disorder of the heart without pathology in another organ (Roma-Rodrigues & Fernandes, 2014). HCM is considered the most common form of inherited cardiomyopathies, estimated to affect one in 500 in general population (Maron B.J. et al., 1995; Semsarian et al., 2015) and the most common identifiable cause of sudden death in young athletes (Maron B.J., 2002).

Diagnosis of HCM

Diagnosis of HCM is made by two-dimensional echocardiography showing hypertrophied end-diastolic left ventricular wall $\geq$ 15 mm in the absence of other cardiac or systemic causes of hypertrophy such as aortic valve stenosis or hypertension. In a subject with a definitive HCM-causing mutation or in first-degree relatives with definite HCM, left ventricular maximal wall thickness $\geq$ 13 mm is sufficient for hypertrophic cardiomyopathy diagnosis. (Elliott et al., 2008, Elliott et al., 2014.) Cardiac hypertrophy is usually asymmetric with greatest involvement most commonly of the basal interventricular septum subjacent to the aortic valve. Other myocardial regions can also be affected: the apex, the mid-portion as well as the posterior wall of the left ventricle. (Marian & Braunwald, 2017.) Intraventricular obstruction usually occurs at the left ventricular outflow tract (LVOT) but can be midventricular caused by hypertrophied papillary muscles and midventricular hypertrophy (Veselka et al., 2017).

Primary abnormalities of the mitral apparatus are common such as leaflet elongation, papillary muscle hypertrophy or bifidity, and abnormal origins or insertions of the papillary muscles (Veselka et al., 2017). Approximately one-third of patients have resting septal anterior motion (SAM) of the mitral valve leaflets that results in obstruction to the LVOT, while another third has latent obstruction only during maneuvers that change loading conditions and LV contractility (Elliott et al., 2014). Sometimes severe septal-leaflet contact is even a sole finding of HCM (Sen-Chowdhry et al., 2016), but it is not yet enough for HCM diagnosis (Elliott et al., 2014) (Figure 11). At the cellular level, cardiac myocytes are hypertrophied, disorganized, and separated by areas of interstitial fibrosis (Marian & Braunwald, 2017).

The disease is often sub-clinical and goes unrecognized mainly because many HCM patients are asymptomatic, but up to 25% will develop significant symptoms: chest pain, shortness of breath, exercise intolerance, palpitations, presyncope and syncope. However, sudden cardiac death may be the first manifestation of the disease. (Marziliano et al., 2012; Roma-Rodrigues & Fernandes, 2014; Veselka et al., 2017.) Atrial and ventricular arrhythmias occur in approximately 20% of HCM patients, leading to a poorer prognosis because these patients have a higher probability of suffering stroke and HF (Roma-Rodrigues & Fernandes, 2014).

More than 1400 mutations in 20 or more genes, mainly encoding proteins of cardiac sarcomere, have been identified to underlie hereditary HCM (Maron B.J. & Maron M.S., 2013; Pinto & Reckman, 2018). HCM is a genotypically and phenotypically heterogeneous disease (Maron B.J. et al., 1995; Landstrom et al., 2011). The variability of the phenotype is caused, at least in part, by the causal mutation acting in concert with a number of other genetic and of non-genetic influences. Approximately 60% of patients with HCM have a familial disease typically inherited in an autosomal dominant pattern. (Walsh et al., 2017.) Autosomal recessive and X-linked modes of inheritance are rare (Marian & Braunwald, 2017).

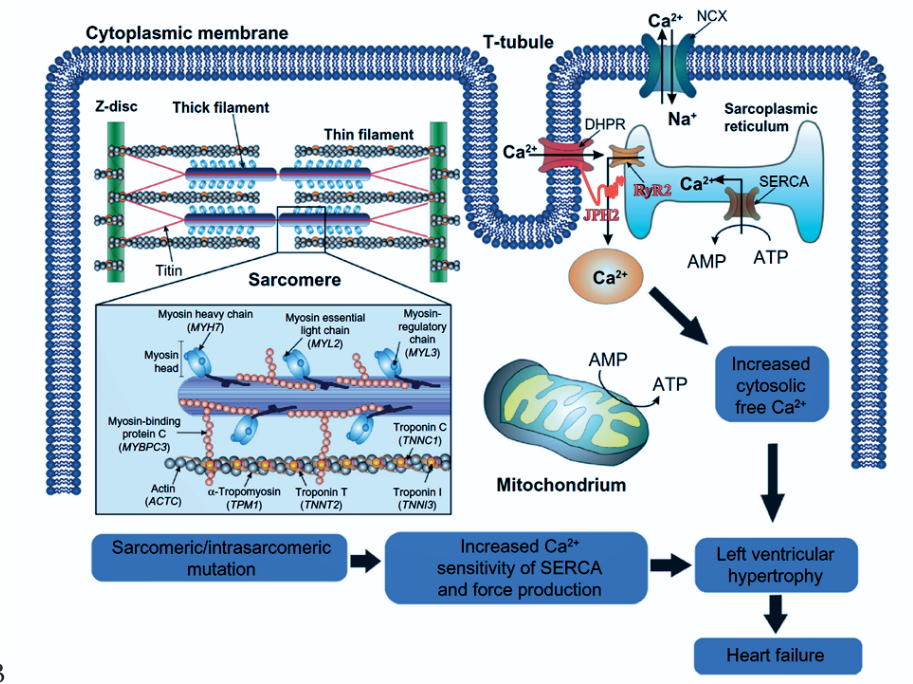
Rarer causes of HCM include metabolic and mitochondrial disorders, congenital malformations and endocrinopathies (Elliott et al., 2014); cardiomyopathies of this kind are called phenocopies of HCM. Phenocopies of HCM are for example Anderson-Fabry disease (mutations in galactosidase alpha, *GLA*), Danon disease (mutations in lysosomal-associated membrane protein 2, *LAMP2)*, *PRKAG2* related glycogen storage disease (mutations in protein kinase AMP-activated non-catalytic subunit gamma 2, *PRKAG*2), cardiac amyloidosis, neuromuscular diseases and malformation syndromes (Noonan spectrum syndromes) (Vago et al., 2016).

The most established genes associating with HCM are myosin binding protein C *(MYBPC3)*, myosin heavy chain 7 *(MYH7)*, troponin I3, cardiac type *(TNNI3)*, troponin T2, cardiac type *(TNNT2)*, alpha tropomyosin 1 *(TPM1)*, myosin light chain 2 *(MYL2)*, myosin light chain 3 *(MYL3)* and actin, alpha, cardiac muscle 1 *(ACTC1)* (Walsh et al., 2017). The role of candidate genes such as troponin C1, slow skeletal and cardiac type (*TNNC1)* (Schmidtmann et al., 2005) and actinin alpha 2 *(ACTN2)* (Chiu et al., 2010) has remained obscure.

2.5.3.1.1 The junctophilin-2 mutation

Human genetic mutations in junctophilin subtypes are linked to HCM (Takeshima et al., 2015), but the molecular mechanisms underlying JPH2 downregulation remain not fully understood (Guo et al., 2015). The *JPH2* gene encodes the main junctophilin isoform in the heart (Garbino & Wehrens, 2010). JPH2 has a critical role in the maintenance of effective Ca^{2+} handling suggesting involvement in excitation–contraction process linking alterations in Ca^{2+} handling with hypertrophic remodeling (Landstrom et al., 2007).

A



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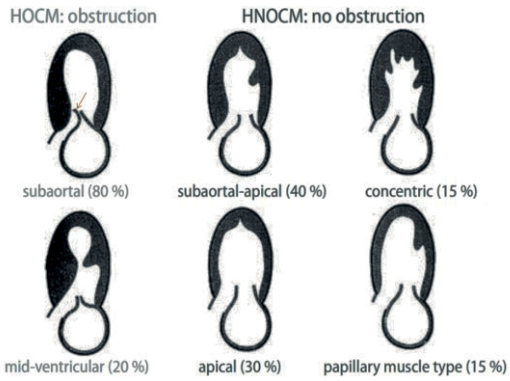

Figure 11. (A) Schematic representation of HCM and heart failure due to sarcomeric and *JPH2* mutations. Mutations in the proteins responsible for the maintenance of cardiomyocyte structure and function result in inefficient or excessive use of ATP and a consequent energy deficiency. The energy deficiencies then compromise calcium homeostasis in the cell, resulting in, among other events, an increase in calcium sensitivity of the ATPase SERCA and increased cytosolic free calcium. The contractibility of the myocyte is compromised, resulting in cell death and consequent myocardial fibrosis. This myocardial fibrosis may result in left ventricular hypertrophy, increasing the risk of heart failure. *Abbreviations:* NCX, Na^+/Ca^{2+}- exchanger; DHPR, dihydropyridine receptor; RyR2, ryanodine receptor; JPH2, junctophilin 2; SERCA, sarco-/endoplasmic reticulum Ca^{2+}-ATPase; AMP, adenosine monophosphate; ATP, adenosine triphosphate.
(B) Types of HCM. Arrow shows anterior mitral valve leaflet. Modified from Roma-Rodrigues & Fernandes, 2014 and Lauschke & Maisch, 2009.

The JPH2 decreases the time required for calcium activation to induce the type II ryanodine receptors to release calcium into the calcium-induced calcium release receptors (Roberts R., 2017). Additionally, maintaining the ultrastructure of the cardiac dyad, JPH2 negatively regulates RyR2 activity and helps retain Na^+/Ca^{2+}-exchanger (NCX) within the dyadic cleft to regulate local Ca^{2+} signaling. Loss of JPH2 promotes RyR2-mediated SR Ca^{2+} leak and concurrently impairs NCX function due to subcellular redistribution of NCX, which further drives Ca^{2+} leakage. (Wang at al., 2014.)

Disruption of the junctional membrane complexes (JMC) is a common finding in failing hearts. It is assumed that *JPH2* mutations affect JMC, which might have a role in SR Ca^{2+} release (Landstrom et al., 2007). Wang et al. also demonstrated a novel cellular mechanism underlying an enhanced SR Ca^{2+} leakage, following a JPH2 knockdown associated with HF (Wang et al., 2014). The downregulation of *JPH2* gene has been associated with HF, and it has been suggested that mutations in this gene are associated with HCM (Takeshima et al., 2015) (Figure 11).

2.5.3.1.2 Cardiomyopathy manifestations of *JPH2* mutations

JPH2 was initially published as a candidate gene for HCM in 2007 when Matsushita et al. found p.(Gly505Ser) in four probands among 148 Japanese HCM patients (Matsushita et al., 2007), but this variant was later found to be a common polymorphism present in up to 4–6% in Asian/African populations (Manrai et al., 2016). Before our *JPH2* study, no convincing evidence of segregation within large pedigrees except for the p.(Glu85Lys) (Sabater-Molina et al., 2016), and no *de novo JPH2* mutations have been reported in patients with HCM. In 2007, Landstrom et al. found two rare missense and one frameshift variant in *JPH2* in three probands with HCM, but their families were not studied or genotyped (Landstrom et al., 2007). Role of the JPH2 in cardiomyopathies has been obscure, as only one rare variant segregating with any type of cardiomyopathy causing DCM has been published. In 2016, Sabater-Molina showed segregation of the *JPH2* p.(Glu85Lys) with dilated cardiomyopathy with or without left ventricular non-compaction cardiomyopathy (LVNC) features in a large family. (Sabater-Molina et al., 2016.) About 10% of the HCM cases evolve into DCM with unknown causes (Nanni et al., 2003).

2.6 Treatments

Channelopathy and cardiomyopathy mutations can cause severe brady- or tachyarrhytmias and/or diastolic or systolic heart failure, but so far there is no cure, and we can only treat symptoms. Patients with CPVT with structural healthy heart and patients with hypertrophic heart, ventricular arrhythmias of both different patient groups are treated by betablockers and implantable cardioverter-defibrillator (ICD). (Zipes et al., 2019.) The asymptomatic nature of channelopathies and cardiomyopathies is cause for search in family members who may be carrying genetic risk factors, making the identification of these disease manifestations of significant clinical importance (Fernández-Falgueras et al., 2017).

2.6.1 SCN5A

The term atrial cardiomyopathy is used, when we can show atrial electrical and/or mechanical dysfunction, and/or fibrosis. These changes increase the risk of atrial fibrillation and stroke. (Guichard & Nattel, 2017.) Progressively increased conduction defects predict atrial paralysis and atrial arrhythmias, which are a risk for cardioembolic stroke without anticoagulation (Probst et al., 2003; Sajeev et al., 2006). The antiarrhythmic drug is determined on a patient-by-patient basis depending on the conduction disorders.

2.6.2 Hypertrophic cardiomyopathy

Left ventricular hypertrophy predispose to both ventricular and atrial tachyarrhythmias and different conduction defects. HCM is the leading cause of sudden death in young individuals and an important cause of heart failure at any age. (Cooper et al., 2017.) Fibrosis progression predicts an increased risk of coming clinical events in HCM. Impaired energetics and perfusion abnormalities may cause fibrotic process. (Raman et al., 2019.)

The prevalence of atrial fibrillation within HCM is estimated to be 18%–28% (Cooper et al., 2017). The risk of thromboembolism is exaggerated in HCM and aggressive anticoagulation is recommended in European as well as American guidelines (Gersh et al., 2011; Elliott et al., 2014). Catheter ablation for AF should be

considered in patients without severe left atrial enlargement, who have drug refractory symptoms or are unable to take antiarrhythmic drugs (Elliott et al., 2014).

Asymptomatic patients without LVOT obstruction (LVOTO) do not currently warrant medical therapy (Elliott et al., 2014; Cooper et al., 2017). Diastolic dysfunction is common, when the left ventricular end diastolic pressure is elevated. Beta-blockers, regardless of whether there is coexisting LVOTO, is a good choice for treatment. Nondihydropyridine calcium-channel blockers (verapamil or diltiazem) must be used with caution in the presence of significant LVOT gradient. (Elliott et al., 2014; Marian & Braunwald, 2017.) β-blockers with vasodilatory effect nebivolol and carvedilol are contraindicated (Makavos et al., 2019). LVOT obstruction in HCM is associated with greater morbidity and mortality (Cooper et al., 2017). ß-blockers are often sufficient to reduce the incidence and severity of exercise-induced LV outflow tract gradients. Disopyramide, used in combination with ß-blockers, also has negative ino-tropic effects and reduces basal LVOT gradients without proarrhythmic effects. If medical therapy is not enough, LVOT can be treated by surgical myectomy or alcohol septal ablation (surgical or percutaneous septum reduction). (Elliott et al., 2014.)

The treatment of diastolic dysfunction can be needed also by diuretics. Spironolactone is being tested for relief and/or regression of the fibrotic process in HCM. (Makavos et al., 2019.) Patients with HCM with symptoms of HF and impaired LVEF < 50% without LVOT obstruction should be treated with conventional therapy for systolic heart failure including angiotensin-converting-enzyme inhibitors, angiotensin-receptor blockers, β-blockers, and diuretics if necessary (Elliott et al., 2014; Marian & Braunwald, 2017). If a HCM patient has severe heart systolic or diastolic failure with maximal drug therapy, heart transplantation must be considered (Elliott et al., 2014; Zipes et al., 2019).

2.6.3 CVPT

Current therapeutic options are beta-antiadrenergic drugs, flecainide, implantable cardioverter-defibrillators (ICD) (Leenhardt et al., 2012; van der Werf & Wilde, 2013; Priori et al., 2013) and left cardiac sympathetic denervation (Wilde et al., 2008; Hayashi et al., 2009).

Beta-antiadrenergic medication is the cornerstone of antiarrhythmic treatment for all CPVT patients (Leenhardt et al., 2012; van der Werf & Wilde, 2013, Priori et al., 2013). The dual Na^{2+} and cardiac Ca^{2+}-release channel inhibitor flecainide has

also shown beneficial effects (Watanabe et al., 2009). Flecainide blocks RyR2 channels, which is reduced by Ca^{2+} overload *in vitro* or by high dose catecholamine challenge *in vivo* (Hwang et al., 2019).

ICDs are used if severe arrhythmic events occur despite optimal β-blocking treatment. However, the use of ICDs is not without risk since ICD-shocks may further aggravate catecholamine release and initiate an uncontrolled electric storm.

Left cardiac sympatectomy may be highly effective in patients with refractory to medical therapy. The left cardiac sympathetic denervation is an antifibrillatory intervention that largely prevents norepinephrine release in the heart, reducing these adrenergically mediated life-threatening arrhythmias. (Wilde et al., 2008; Cho, 2016.)

2.6.3.1 Dantrolene

The ryanodine receptor type-1 (RyR1) is the skeletal muscle counterpart. Mutations in RyR1 result in malignant hyperthermia, a rare but life-threatening complication of general anesthesia occurring upon administration of volatile anesthetics or depolarizing muscle relaxants. Dantrolene is a specific and an effective treatment for malignant hyperthermia. (Krause et al., 2004.)

In animal models of CPVT1, dantrolene has been shown to have antiarrhythmic effects restoring interdomain interactions critical for the closed state of the RyR2 Ca^{2+} channel (Kobayashi et al., 2005; S. Kobayashi, 2009). Dantrolene has also been demonstrated to have beneficial effects in iPSC derived CMs from a CPVT1 patient with an N-terminal S406L mutation (Jung et al., 2012), but no studies in patients have so far been reported.

3 AIMS OF THE STUDY

The main aims of the present thesis were as follows:

I To determine the progressive 12-lead ECG changes caused by the genetic defect *SCN5A* D1275N and to investigate the ECG findings which may lead to thromboembolic complications and conduction disorders requiring pacemaker treatment.

II To determine the pathogenicity of *JPH2* c.482C>A, p. (Thr161Lys) variant.

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III To determine the response of dantrolene medication in patients with different *RyR2* mutations and whether the *in vivo* responses of patients correspond to the *in vitro* data obtained from hiPSC-CMs derived from the same patients.

IV To compare the dynamic electrophysiological characteristics of ECG to corresponding MEA recordings of hPSC-CMs.

4 MATERIALS AND METHODS

4.1 Ethical consideration

The local Ethics Committee gave their approval for the study I (R01128) and the study IV (R07110M and R08070).

Study II has been approved by the Ethical Review Committee of the Department of Medicine, University of Helsinki (Dnro 307/13/03/01/11) and by the Ethical Review Committee of the Department of Medicine, University of Tampere (R08070) and conforms to the ethical principles outlined in the Declaration of Helsinki.

The third study was approved by the Ethical Review Committee of the Helsinki University Hospital (HUS 396/13/03/01/12) and was performed in accordance with the institutional guidelines and the Declaration of Helsinki. Clinical trial was registered with EudraCT (2012-005292-14).

Oral information and written informed consent were obtained from all patients (I-IV).

4.2 Patients and molecular genetic studies

All participants are of Finnish ethnicity.

4.2.1 Study I

A Finnish family with 45 members was evaluated at the Heart Center, Tampere University Hospital. This study includes eleven *SCN5A* D1275N mutation carriers and two deceased obligate carriers. Their ages were 12–73 years, when ECGs were recorded. The *SCN5A* D1275N genotype analysis of the family members has been previously described in detail by Laitinen-Forsblom et al. Four (31%) affected family members had a stroke before the age of 31 years and two experienced a premature death. (Laitinen-Forsblom et al., 2006.)

4.2.2 Study II

Nine index patients with the *JPH2* variant c.482C>A, p.(Thr161Lys) and their relatives from four Finnish hospitals were included. We described the heart manifestations of 26 patients with *JPH2* c.482C>A, p.(Thr161Lys) mutation, one of them was deceased obligate carrier (17 males and nine females, range 8–80 years old). The adult participants were assessed clinically at Heart and Lung Center, Helsinki University Hospital, at the Heart Hospital, Tampere University Hospital or at the Seinäjoki Central Hospital by physical examination, resting 12-lead ECG, appropriate laboratory tests and transthoracic echocardiography (TTE). The children were evaluated clinically at Children's Hospital, Helsinki University Hospital or at Department of Pediatrics, Tampere University Hospital.

4.2.3 Study III

The patients consisted of six individuals (mean age 50±10 years, range 37–59 years, five females), who were molecularly defined as heterozygous carriers of different gain-of-function *RyR2* mutations causing CPVT1. They carried the following mutations*:* c.168-301_c.273+ 722del1128 mutation (later called as exon 3 deletion) or point mutations p.P2328S (c.6982C>T), p.T2538R (c.7613C>G), p.L4115F (c.12343C>T), p.Q4201R (c.12602A>G) or p. V4653F (c.13957G>T) (Figure 7). They all used a beta-adrenergic blocking agent, two of the patients with ICD showed atrial pacing. Basic laboratory parameters and cardiac echo were normal in all patients.

4.2.4 Study IV

Five healthy volunteers (mean age 38.4±20 years, range 18–52 years, three males and two females), who have bradycardic heart rate (< 50 bpm).

4.3 Methods

4.3.1 ECG/ Studies I, II, IV

Heart rate, P-wave duration and amplitude, PR interval and deviation from the baseline, QRS duration, S-wave amplitude and upstroke, QT, and corrected QT (QTc; Bazett's formula) intervals were measured manually according to established methodology. Normal values were internationally accepted references values (Surawicz & Knilans, 2008).

For QRS-complex fragmentation (fQRS), we used the definition by Das et al.: additional R wave (R' prime), notching in nadir of the S wave, notching of R wave, or the presence of more than one R prime (fragmentation). Typical bundle branch block (BBB) pattern (QRS $\geq$ 120 ms) – right or left – and incomplete RBBB were excluded from the definition of fQRS. (Das & Zipes, 2009; Basaran et al., 2011.) In the left anterior fascicular block (LAFB), QRS duration may be normal or slightly prolonged. The upper limits of the S-wave amplitude in leads V1–V3 were 1.8, 2.6, and 2.1mV. (Surawicz & Knilans, 2008.) The QT interval was measured according to established methodology (Postema & Wilde, 2014). In Figure 5, it is shown how heart rate is determined by RR cycle lengths (ms) before measured QT interval. QT interval is measured from the beginning of the QRS complex to the tangent to the end of the T wave.

The diagnosis of junctional escape rhythm needed at least three successive complexes. Both the typical finding with a narrow QRS and possible cases with broad QRS due to pre-existing intraventricular conduction defect were included. (Surawicz & Knilans, 2008.)

Atrial standstill is characterized by bradycardia (HR<50 bpm), the absence of P waves, and a junctional narrow or slightly wide QRS complex and regular escape rhythm (Groenewegen et al., 2003; Surawicz & Knilans, 2008).

4.3.1.1 Study I

Systematic 12-lead ECG analysis was performed in 13 family members carrying an *SCN5A* D1275N mutation. Conduction defects and supraventricular arrhythmias, including atrial fibrillation/flutter, atrioventricular nodal re-entry tachycardia (AVNRT) and junctional rhythm were searched for.

4.3.1.2 Study IV

We compared sinus rhythm 12-lead ECG from healthy individuals to cardiac FP recordings of hESC-CMs recoded with MEAs to investigate how well the intricate components of the ECG and cardiac FP recordings correspond to each other.

RR and QT interval were measured on a single selected lead V2 or V3 because of the clearest T wave during exercise. Heart rate was determined by RR cycle lengths (ms) before measured QT interval. Measurements were rounded to the nearest 5 ms. The QT interval and heart rate measured by the analysis program was used in the heart rate adjustment formulae from the Framingham Study, adjusting the measured QT intervals for heart rate using Bazett's formula (Bazett, 1920; Sagie et al., 1992) and QT/RR values (Malik et al., 2008).

4.3.2 Classification of genetic findings

4.3.2.1 Study II

Genetic testing was carried out from genomic DNA using the OS-Seq™ (oligo-nucleotide-selective sequencing) NGS method (Myllykangas et al., 2011; Akinrinade et al., 2015). The genetic evaluation of the index patients with hypertrophic cardiomyopathy was performed using the Blueprint Genetics Core Cardiomyopathy or Pan Cardiomyopathy Panels covering 69 and 103 genes, respectively, associated with cardiomyopathies and their genetic phenocopies including *JPH2* c.482C>A, p.(Thr161Lys) (NM_020433.4) variant.

The ACMG-AMP (American College of Medical Genetics and Genomics-Association for Molecular Pathology) pathogenicity classification guidelines offer a set of categories that for classification of a variant as pathogenic and criterion for the evidence for pathogenicity (Richards et al., 2015). N (= probability of observed cosegregation if not pathogenic) is required to be smaller if all the segregation evidence comes from a single family, rather than two or more families, solely due to the concern that evidence from a single family can be due to physical linkage between the observed variant and an unobserved causal variant. For example, under a dominant model, this probability is $N = (½)^m$, where m is the number of meioses of the variant of interest that are informative for cosegregation. (Jarvik & Browning, 2016.)

4.3.3 Echocardiography and cardiac magnetic resonance imaging

4.3.3.1 Study II

HCM was clinically diagnosed according to ESC Guidelines (Elliott et al., 2008). In adults, the diagnosis of HCM requires LV wall thickness ≥15 mm as measured by any imaging technique. Correspondingly, the clinical diagnosis of HCM in first-degree relatives of patients with left ventricle hypertrophy (LVH) ≥15 mm is based on the presence of otherwise unexplained increased LV wall thickness ≥13 mm. In children, the diagnosis of HCM requires LV wall thickness more than two standard deviations greater than the predicted mean. (Elliott et al., 2008.) Cardiac magnetic resonance imaging (cardiac MRI = CMR) was performed in some cases especially in patients with borderline diagnostic findings at echocardiography. The presence of the *JPH2* mutation c.482C>A, p.(Thr161Lys) mutation in the family history was obtained and pedigrees were drawn.

4.3.4 Exercise stress test

4.3.4.1 Study III

The antiarrhythmic potential of dantrolene in the treatment of CPVT was studied with both patients and their iPSC-CMs generated from them. The clinical tests and the iPSC studies were performed separately and blinded. The follow up time of the patients was three days after the dantrolene infusion.

All *RyR2* patients underwent the exercise stress test three times: a baseline, the first day after intravenous infusion of dantrolene sodium (Dantrium, 1.5 mg per kg of body weight) and on the second day to assess the effects after dantrolene sodium washout and to demonstrate the reproducibility of the basic exercise test. The exercise test was performed with a bicycle ergometer. The initial load was 30 W, followed by increments of the load by 15 W each minute.

Numbers of premature ventricular contractions (PVCs) during exercise and at recovery phase as well as the maximum number of consecutive PVCs were counted.

4.3.4.2 Study IV

12-lead ECG was recorded at rest until heart rate decreased slower than 50 bpm. Starting at 20 W and the work rate was then increased 5 W manually by degrees. Exercise stress test was interrupted when the target heart rate (130 bpm) was reached.

4.3.5 hPSC lines and studies

4.3.5.1 Study III

Studied iPSC lines were UTA.05605.CPVT generated from the patient with *RyR2* exon 3 deletion, UTA.05208.CPVT from the patient with mutation P2328S, UTA.07001.CPVT from the patient with mutation T2538R, UTA.03701.CPVT from the patient with mutation L4115F, UTA.05503.CPVT from the patient with mutation Q4201R, UTA.05404.CPVT from the patient with mutation V4653F and UTA.04602.WT from a healthy control individual. All the CPVT-iPSC lines were characterized for their karyotypes, mutations, pluripotency, immunocytochemistry, embryoid body and teratoma formation.

iPSCs were co-cultured with murine visceral endoderm-like (END-2) cells (Humbrecht Institute, Utrecht, The Netherlands) to differentiate them into spontaneously beating CMs.

The percentage of abnormal Ca^{2+} transients, such as multiple peaks comprising of two peaks, irregular phases, oscillations, and varying amplitude manifested as low peaks, were calculated from each studied cell line. Beating frequency and diastolic Ca^{2+} levels of CMs were analyzed during spontaneous baseline beating, and during adrenaline perfusion. These parameters were compared between mutated and control cell lines and between each mutated cell line.

For dantrolene studies, the changes in Ca^{2+} were recorded during spontaneous baseline beating, spontaneous beating during 1 μM adrenaline perfusion and spontaneous beating during 1 μM adrenaline together with 10 μM dantrolene (Sigma) perfusion. Diastolic Ca^{2+} levels and beating frequency were compared between adrenaline and dantrolene of CMs.

In "responder" group dantrolene abolished virtually all the Ca^{2+} handling abnormalities, in "semi-responder" group dantrolene reduced them by more than 50%, in "non-responder" group dantrolene reduced them by less than 50%.

4.3.5.2 Study IV

We used commercial cell culture H7 hESCs (WiCell), and the hPSCs were differentiated into cardiomyocyte clusters by co-culturing them with mouse endoderm cell line. FPs were measured by MEA. The same formula was used for FP recordings as well, only then QTc became cFPD, QT was FPD and RR was PPI (determined as time between two depolarizing sodium [Na^+] peaks). FPD was determined as the time between the onset of initial deflection and return to baseline as described before. (Caspi et al., 2009.)

We compared FPD to QT time and PPI to RR (Figure 5).

4.3.6 Statistical Analysis

4.3.6.1 Study III

Statistical analysis of *in vivo* studies was made with SPSS 21.0 statistical software package (SPSS, Chicago, IL). Data are presented as average + 1 standard deviation (SD). Comparisons between phases were performed by the non-parametric Wilcoxon test. The significance of *in vitro* differences between two groups was evaluated with the unpaired Student's t-test. The significance of changes within a group was evaluated with the paired Student's t-test. Data are expressed as average ± standard error of the mean (S.E.M.) and n refers to the number of cells. $P < 0.05$ was considered statistically significant in both *in vivo* and *in vitro*.

4.3.6.2 Study IV

Data are presented as mean ± SD. Statistical analyses were performed between the groups with two-tailed t-test using SPSS software (IBM).

5 RESULTS

5.1 Study I: Pathognomonic ECG findings in patients with *SCN5A* D1275N mutation

Notching of the R and S waves – including initial QRS fragmentation – and prolonged S-wave upstroke were present in all the affected family members (Table 1, Figures 12 and 13).

Five (38%) mutation carriers had fascicular or bundle branch block, nine (69%) had atrial arrhythmias; no ventricular arrhythmias were found. Five mutation carriers had a VVI-type of pacemaker (PAMA) (Figure 14). Two of them were upgraded to a physiologic PAMA. One mutation carrier underwent an electrophysiology study proving to have fibrosis of the atrial lateral wall.

Table 1. Distribution of 12-leads ECG parameters of the *SCN5A* D1275N patients.

ECG parameters	n = 13 (%)	Individual
Junctional rhythm	3 (23%)	(II:2, III:6, III:7)
Atrial fibrillation or flutter	9 (69%)	(I:1, II:1, II:3, II:4, III:1, III:3,III:4, III:5, III:7)
Atrial tachycardia	1 (8%)	(III:5)
Flat P wave in lead II	4 (31%)	(II:1, II:2, III:2, III:7)
P wave > 140 ms	2 (15%)	(I:1, III:2)
PR interval > 200 ms	4 (31%)	(II:1, III:1, III:2, III:5)
Fragmentation of initial QRS	13 (100%)	
S-wave upstroke ≥ 55 ms in leads V2-3	13 (100%)	
Notching in nadir of the S wave in leads V1, V2, V3	13 (100%)	
QRS ≥ 120 ms	10 (77%)	(all except II:3, III:1, III:3)
RBBB	4 (31%)	(I:1, III:6, III:7, IV:1)
LAFB	4 (31%)	(I:1, II:3, III:6, III:7)
Pacemaker	5 (38%)	(I:1, II:4, III:5, III:6, III:7)

RBBB, presence of right/left bundle branch block; LAFB, left anterior fascicular block
Generations I-IV, from oldest to youngest. Age order in generation 1-7 from oldest to youngest

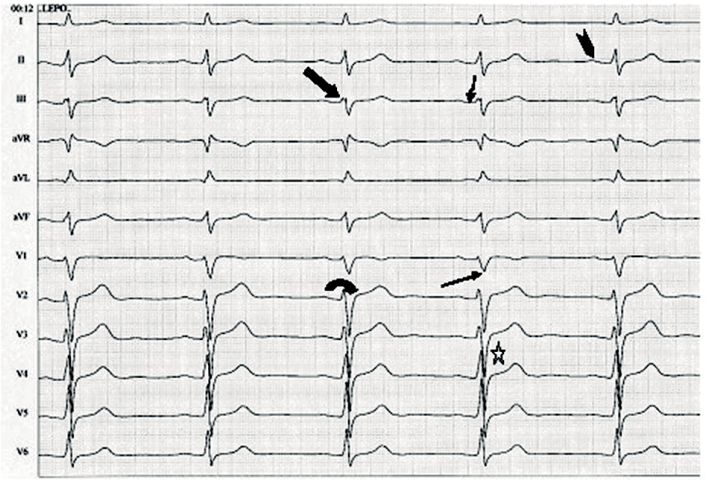

Figure 12. A typical 12-lead ECG in an asymptomatic patient with the *SCN5A* D1275N mutation.
Wide arrow: low amplitude signal at the beginning QRS complex.
Narrow arrow: notch in S wave
: flat P wave
: PR depression
: deep S wave in chest leads
: prolonged S-wave upstroke ≥ 55 ms

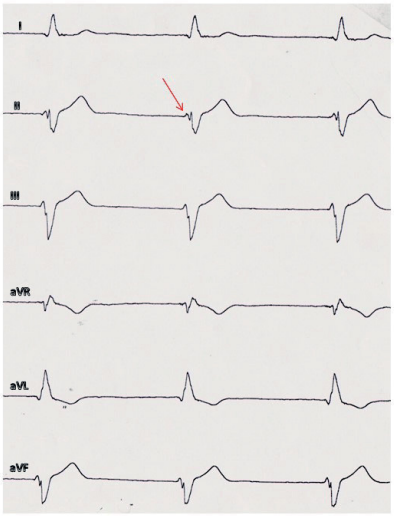

Figure 13. The initial fragmentation of the QRS complex. Arrow shows a changing point with increasing amplitude in *SCN5A* D1275N patients, who progressed conduction defect from sinus rhythm to atrial standstill.

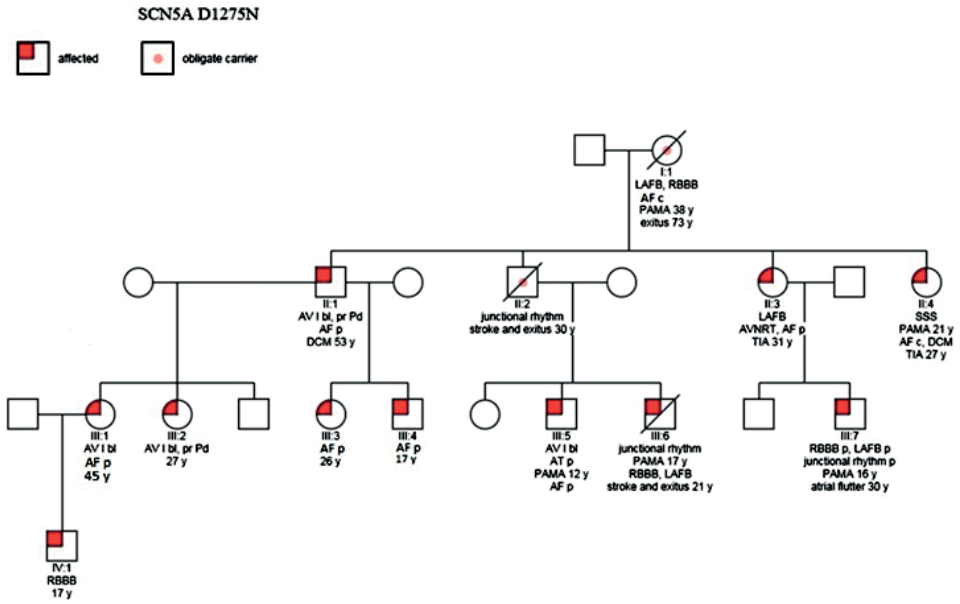

Figure 14. *SCN5A* D1275N pedigree indicating the ECG findings. Four of nine children of the oldest obligate mutation carrier (patient I:1) had the *SCN5A* mutation, only these individuals were significant to genetic analysis and they together with their children are included. AF, atrial fibrillation or flutter; AT, atrial tachycardia; AV I, grade I atrioventricular block; AVNRT, atrioventricular nodal re-entry tachycardia; c, chronic; DCM, dilated cardiomyopathy; LAFB, left anterior fascicular block; p, paroxysmal; PAMA, implanted pacemaker; pr Pd, prolonged P duration; RBBB, right bundle branch block; SSS, sick sinus syndrome; TIA, transient ischemic attack; y, years. Generations I-IV, from oldest to youngest. Age order in generation 1-7 from oldest to youngest.

5.2 Study II: *JPH2* c.482C>A, p.(Thr161Lys) clinical characteristics of the probands and their family members

A heterozygous *JPH2* c.482C>A, p.(Thr161Lys) (NM_020433.4) variant was observed in nine unrelated Finnish probands with cardiomyopathy. Altogether the p.(Thr161Lys) was detected in 20 affected individuals. The variant cosegregated with HCM in six families (Families 2–4, 6–8) and in three families the mutation was found only in the probands (Family 9), in probands and young family member without HCM (Family 1) or in probands and in another family member without HCM (Family 5). Proband (III.1) is a girl who developed HCM by the age of 7 years. She has also a well-established pathogenic nonsense mutation in *MYBPC3* (c.3181C>T, p.(Gln1061*)). She has inherited both of the variants from her mildly affected grandfather through her mother who is obligate carrier of both variants. Systolic heart failure or conduction abnormalities were observed in every family. (Table 2 and Figure 15). Average at diagnosis was 26.9±20.6 years in the nine probands and their maximum LV wall thickness was 20.4±5.2 mm. PAMA or ICD was implanted in 6/20 (30%) and reduced LVEF (<47%) or elevated brain natriuretic peptide (proBNP) concentration (>300 ng/l) was observed in 9/20 (45%) of the patients. Penetrance of HCM was 48%, 71% and 100% by age of 40, 60 and 80, respectively.

Table 2. Clinical characteristics of the *JPH2* probands and their family members.

Family	Age M/F	Geno-type	Conduction defect	Arrhyth-mias	PM, ICD	LV-WT	LVEDD/ EF (%)	pro-BNP (ng/l)	Age at dg	Pheno-type
Family 1										
I.1	52M	+/-	no	AF	ICD	20	46/56%	400	47	HCM
Family 2										
I.1	44M	+/-	no	no	no	16	46/72%	76	44	HCM
II.1	12M	+/-	no	VT	no	23	48/77%	315	12	HCM
II.2	16M	+/-	pRBBB	no	no	6	51/63%	NA	-	normal
Family 3										
I.1	48M	+/-	RBBB	no	no	15	49/72%	44	41	HCM
II.1	24M	+/-	LBBB	VT	ICD	29	44/75%	145	13	HOCM
Family 4										
I.2	45M	n.a.	AVB3, LAFB, RBBB	AF, VT	PM	28	55/20%	8425	17	HOCM, SHF
I.3	57F	-/-	no	no	no	14	43/60%	33	-	LVH/ Hyper-tens
II.1	25M	+/-	no	SVT	no	22	45/67%	104	9	HCM
II.2	17M	+/-	LAFB	no	no	20	49/55%	NA	1	HCM
III.3	8M	+/-	no	no	no	11	35/>50%	NA	1	HCM
Family 5										
I.1	54M	+/-	no	SVT	no	10	50/70%	NA	-	normal
I.2	53F	-/-	no	no	no	11	44/83%	164	-	normal
II.1	25F	+/-	RBBB, LAFB	VES, VT, SVT	no	22	43/60%	5700	12	HCM, SHF
Family 6										
I.1	62M	+/-,*	LAFB	AF	no	15	40/52%	998	61	HCM
I.2	60F	-/-	NA	NA	NA	NA	NA	NA	-	NA
II.1	F	(+/-,*)	NA	NA	NA	NA	NA	NA	-	HCM
III.1	8F	+/-,*	NA	no	no	11	33/>60%	3928	7	HCM
Family 7										
I.2	76F	+/-	LAFB	AF	no	27	49/56%	NA	?	HCM
I.3	72F	+/-	no	AF	no	17	45/60%	NA	?	HCM
I.4	80F	(+/-)	no	AF	no	NA	NA	NA	?	?
II.1	52M	+/-	AVB1	VES, SVT	no	23	49/63%	NA	43	HCM
II.2	49M	+/-	AVB1	no	no	21	60/50%	NA	?	HCM
II.3	48M	+/-	no	no	no	11	NA	NA	-	normal
III.1	20F	+/-	no	SVT	no	12	44/66%	NA	19	normal

Family 8										
I.1	43F	(+/-)	AVB3, LBBB	AF, VT	PM	18	70/30%	6637	25	HCM, SHF
I.2	60F	-/-	no	no	no	13	40/71%	160	-	LVH/Hypertens
I.3	76F	+/-	AVB3, LAFB, RBBB	no	PM	12	46/40%	5267	67	HCM, SHF
II.1	47M	+/-	AVB1, LBBB	AF, VT	ICD	15	51/37%	4426	36	HCM, SHF
II.2	49F	-/-	no	no	no	10	49/66%	31	-	normal
III.1	15M	+/-	no	no	no	7	46/66%	NA	-	normal
Family 9										
I.1	63F	+/-	no	AF	PM	19	45/60%	4082	63	HCM

Index patients are marked in bold. Symbols and abbreviations: Age (M/F), age and gender (M, male; F, female); Genotype – +/- is heterozygous and (+/-) obligatory heterozygous for p.(Thr161Lys) in *JPH2* and -/- is wild type, * heterozygous for *MYBPC3* Gln1061*; R/LBBB, right/left bundle branch block; LAFB, left anterior fascicular block; AVB1-3, atrioventricular block types 1-3; Arrhythmias – AF, atrial fibrillation/flutter; SVT, supraventricular tachycardia (>10 short episodes per day or SVT requiring cardioversion); VT, ventricular tachycardia ≥ 3 beats with frequency >100/min; VES, ventricular extrasystoles >1000 per day; SVES, supraventricular extrasystoles >5000 per day; PM, ICD, CRT-P/D – pacemaker, implantable cardioverter-defibrillator, cardiac resynchronization therapy device; LV-WT, maximal left ventricular wall thickness; LVEDD & EF, left ventricular end-diastolic diameter (mm) and ejection fraction (%); Age at dg-age at diagnosis of cardiomyopathy; Phenotype – phenotype at diagnosis; HCM, hypertrophic cardiomyopathy; LVH, left ventricular hypertrophy; SHF, systolic heart failure

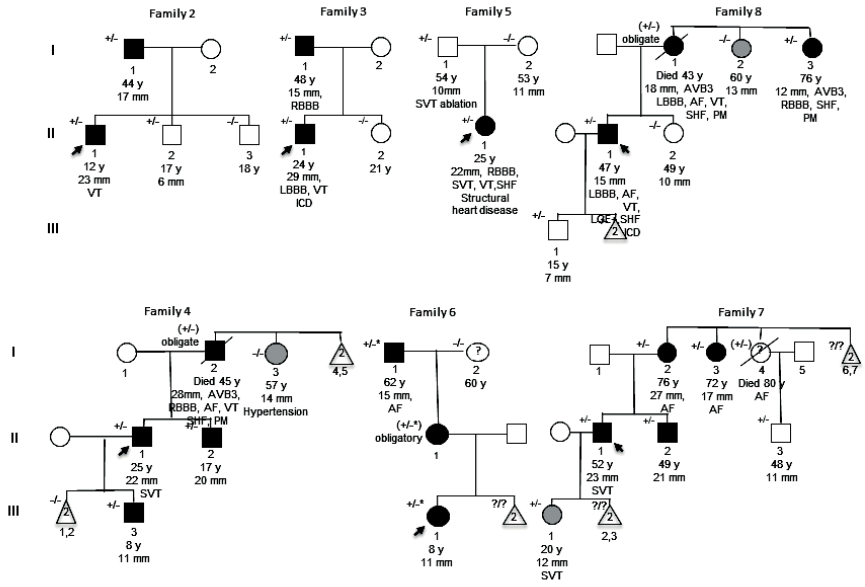

Figure 15. Pedigrees of three families affected with the *JPH2* c.482C>A, p.Thr161Lys*, (rs587782951, NM_020433.4*) variant. Circles represent women, squares men and triangles gender blinded. Black-filled symbols represent individuals who fulfill ESC 2008 diagnostic criteria for HCM (Elliot et al., 2008). One family member (Family 8:I.3) who had imminent cardiomyopathy with borderline LVH (12 mm), 3-degree AV block (AVB3), RBBB and severe systolic heart failure (SHF) was also considered as affected with HCM. Genotypes: +/- heterozygous for the *JPH2* p.Thr161Lys, -/- wild type allele, * *MYBPC3* p. Gln1061*. Age of the family members at last follow-up, maximum LV wall thickness and some other key signs of clinical disease are listed below the symbols. Symbols and abbreviations; arrows indicate index patients; RBBB, right bundle branch block; AF, atrial fibrillation; VT, ventricular tachycardia; SVT, supraventricular tachycardia; PM, pacemaker. Generations I-III, from oldest to youngest. Age order in generation 1-7 from oldest to youngest.

5.3 Study III: Antiarrhythmic effects of dantrolene in CPVT1 patients and their iPSC derived CPVT1 CMs

5.3.1 Antiarrhythmic effects of dantrolene in CPVT1 patients

In the baseline study, patients exercised on an average 8±2 minutes reaching a maximum heart rate of 134±17 bpm. Exercise bicycle testing induced polymorphic premature ventricular contractions (PVCs) in all six patients and non-sustained ventricular tachycardia (NSVT, episodes of 3 to 4 consecutive PVCs) in three of them. The average threshold sinus rate for the appearance of PVCs was 105±9 bpm. The total count of PVCs during the workload was 172±119 (range 43–391).

Figure 16 illustrates an example of the PVCs and NSVT episodes during the baseline study and after dantrolene. Dantrolene significantly increased the threshold at which the arrhythmias appeared from 105±9 to 120±17 bpm. The duration of the exercise phase and the maximal heart rate achieved during the exercise were similar to those in the baseline study. On day 2, after wash-out of dantrolene, the prevalence of PVCs was approaching that in the first baseline test.

Dantrolene decreased the prevalence of PVCs in four patients, whereas in two patients the number of PVCs remained virtually the same (Figure 17). Dantrolene reduced arrhythmias in patients with the mutation in the N-terminal or central region of the RyR2 protein. Thus, dantrolene abolished 97% of PVCs in the patient with exon 3 deletion (cluster 1), 88% of PVCs in the patient with P2328S mutation (cluster 2), 33% of PVCs in the patient with T2538R mutation (right after cluster 2) and 77% of PVCs in the patient with L4115F mutation (cluster 3). In contrast, dantrolene abolished only 1 to 2% of PVCs in patients carrying mutation closer to (Q4201R, end of cluster 3) or within the transmembrane region (V4653F, cluster 4) (Figures 17 and 18).

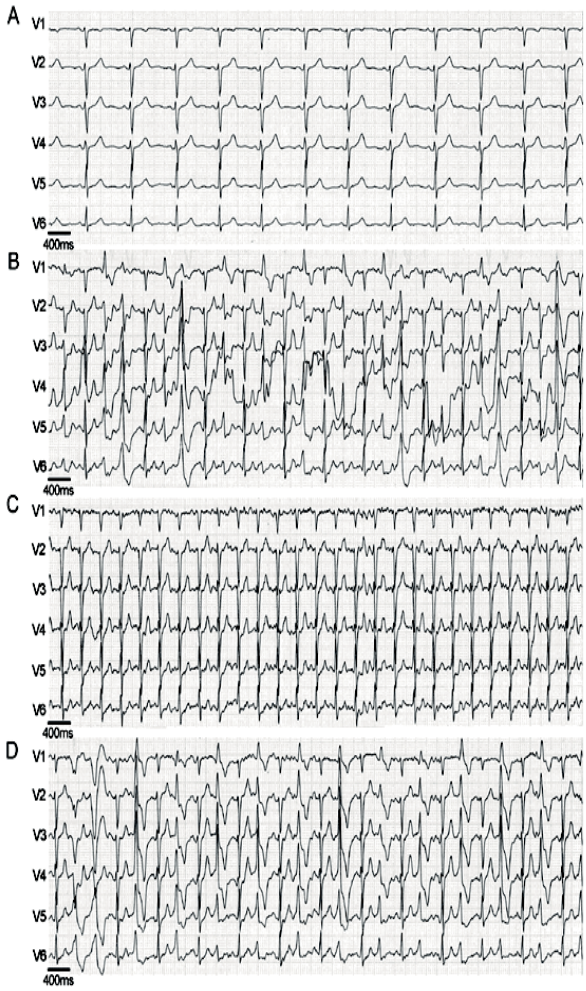

Figure 16. ECG examples of a 38-year-old patient carrying the *RyR2* P2328S mutation. (A) Resting ECG showing sinus rhythm and normal QRS morphology. (B) Exercise ECG at the highest workload of 105Win the baseline study before dantrolene. PVCs include couplets and polymorphic NSVTs. (C) Disappearance of ventricular arrhythmias after administration of dantrolene (workload 105 W). (D) Exercise test on day two after 20-hours wash-out of dantrolene showing return of PVCs (workload 105 W).

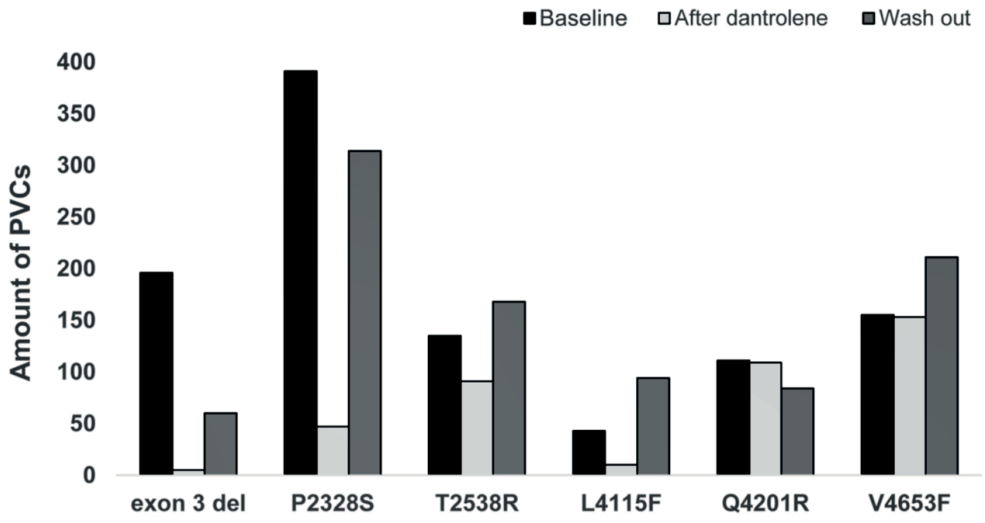

Figure 17. Features of the PVCs. Number of PVCs in exercise stress test before (baseline) and after administration of intravenous dantrolene and 24 hours after dantrolene wash out.

5.3.2 iPSC derived CPVT1 cardiomyocytes reproduced the clinical antiarrhythmic responses to dantrolene

The effects of dantrolene were divided into three groups based on their Ca^{2+} responses. In the "responder" group dantrolene abolished all the Ca^{2+} handling abnormalities, in the "semi-responder" group dantrolene reduced them by more than 50% and in the "non-responder" group dantrolene reduced them by less than 50%. iPSC-CMs were found to clearly reproduce the varying individual clinical responses of dantrolene. In cell lines with the mutation in the N terminal or central region of the *RyR2* protein, dantrolene abolished or reduced the majority of Ca^{2+} transient abnormalities. These mutations were within or in close proximity of clusters 1, 2 or 3. A detailed analysis indicated that in cardiomyocytes (CMs) with exon 3 deletion, P2328S, T2538R or L4115F, dantrolene abolished or reduced by more than 50% of Ca^{2+} abnormalities in 65–97% of cells.

In contrast, the effect of dantrolene was only minimal in CMs carrying a mutation at the end of cluster 3 (Q4201R) or in the transmembrane region (cluster 4, mutation V4653F), in accordance with the *in vivo* dantrolene infusion data (Figure 18). Dantrolene had no effect on the Ca^{2+} transients in control CMs. Dantrolene did not

significantly affect the diastolic Ca^{2+} levels of CMs in which Ca^{2+} transient abnormalities were abolished. Dantrolene increased significantly the diastolic Ca^{2+} levels of control and Q4201R CMs where Ca^{2+} transients were unaltered by the drug. There was no correlation between the antiarrhythmic effect of dantrolene and its effect on beating frequency.

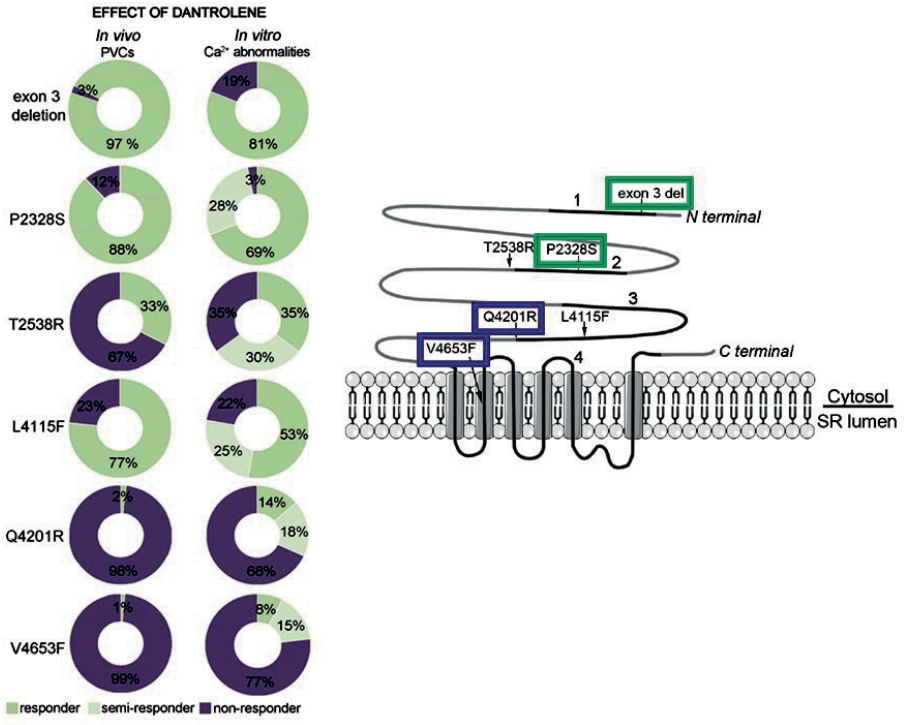

Figure 18. iPSC derived CMs reproduced the clinical responses of dantrolene. *In vivo* and *in vitro* effects of dantrolene correspond within each *RyR2* mutation. *In vitro* drug effects were categorized into three groups (responders, semi-responders and non-responders) depending on how dantrolene affected to the amount of Ca^{2+} abnormalities when compared to adrenaline response. *In vivo* responder group show the percentage of the abolished PVCs when compared to the baseline. Numbers of cells analyzed in exon 3 del n = 16, P2328S n = 32, T2538R n = 17, L4115F n = 36, Q4201R n = 22, V4653F n = 13. Modified from Priori & Chen, 2011.

5.4 Study IV: Findings of repolarization parameters *in Vivo* 12-lead ECG and *in Vitro* FP of hPSC-CM

The heart rate in ECG ranged from 39 to 120 bpm and the beating hPSC-CMs clusters were selected to match this range. The uncorrected mean FPD value was 375 ms with a mean beating rate of 73 bpm, while the uncorrected mean QT was 397 ms with a mean heart rate 76 bpm (Figure 19 and Table 3). When FPD was corrected with Bazett's formula, cFPD was 400 ms with SD 64 ms. Volunteers had a mean QTc 432 ms with SD 39 ms.

QT/RR mean value of our volunteers was 0.433, ranging 0.40–0.46. The mean FPD/PPI value was 0.44.

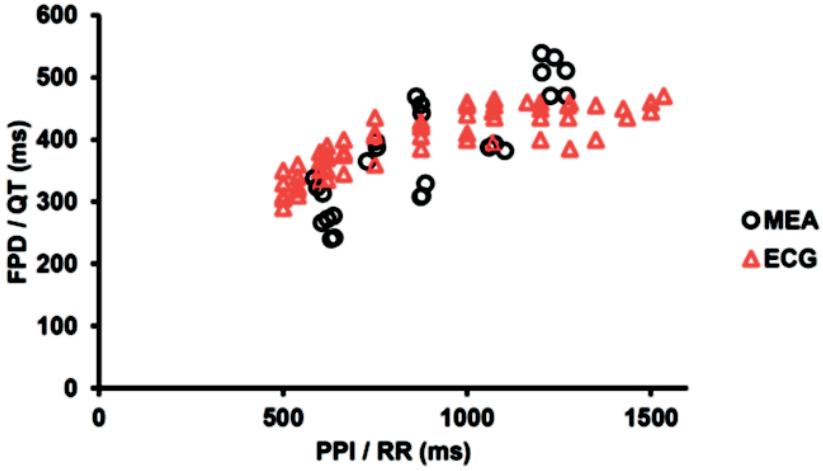

Figure 19. Correlation of beating rate and QT and FPD duration. RR-interval (ms) of electrocardiograms is plotted against QT-intervals (ms) and peak-to-peak interval (PPI, ms) of pluripotent stem cell–derived cardiomyocyte (hPSC-CM) recordings against field potential durations (FPD, ms). For hPSC-CMs, linear fitting shows a value of approximately 0.71 for the coefficient of determination and for ECGs about 0.68.

Table 3. The cardiac parameters measured from the electrocardiogram (ECG) and microelectrode array (MEA) recordings. Parameters were QT and RR interval (ms) in ECG measurements and field potential duration (FPD) and peak-to-peak interval (PPI) (ms) in MEA measurements. The first measurement is heart rate 40 beats per minute (bpm), second measurement 80 and third measurement 120 bpm in the ECG.

		ECG	
Parameter (Volunteer/hPSC-CM Cluster #)	first	second	third
QT (Volunteer #1)	405	365	290
RR (Volunteer #1)	1350	750	500
QT (Volunteer #2)	450	410	330
RR (Volunteer #2)	1500	750	500
QT (Volunteer #3)	455	410	350
RR (Volunteer #3)	1500	750	500
QT (Volunteer #4)	435	385	310
RR (Volunteer #4)	1435	750	500
QT (Volunteer #5)	460	390	305
RR (Volunteer #5)	1425	750	500
		MEA	
FPD (Cluster #1)	382	393	387
PPI (Cluster #1)	1103	1076	1060
FPD (Cluster #2)	240	240	242
PPI (Cluster #2)	632	632	640
FPD (Cluster #3)	308	309	329
PPI (Cluster #3)	875	879	888
FPD (Cluster #4)	442	469	456
PPI (Cluster #4)	877	862	875
FPD (Cluster #5)	365	387	397
PPI (Cluster #5)	729	756	753
FPD (Cluster #6)	313	323	338
PPI (Cluster #6)	608	593	585
FPD (Cluster #7)	277	272	266
PPI (Cluster #7)	637	621	606
FPD (Cluster #8)	470	470	539
PPI (Cluster #8)	1271	1228	1203
FPD (Cluster #9)	511	532	508
PPI (Cluster #9)	1269	1238	1204

6 DISCUSSION

In this thesis, we investigated mutations in three different genes causing inherited cardiac rhythm disorders or structural abnormalities. Mutation-specific ECG changes are caused by the D1275N mutation in *SCN5A* gene. Additionally through extensive genealogy research, we demonstrated the mutation p.Thr161Lys in the *JPH2* gene causing HCM in several, unrelated families. We further investigated drug responsiveness of patients and hiPSC-CMs carrying different mutations in *RyR2* gene. Finally, the ECG parameters were compared to MEA parameters. It is important that we have methods to examine cardiomyocyte function that correlates with *in vitro* models of the native human myocardium, especially for basic research, but also for pharmaceutical industry.

A family history is an important risk factor in many cardiovascular diseases. Studies of rare familial syndromes can identify molecules whose dysfunction leads to cardiomyopathies and arrhythmias with channelopathies, but more generally they also teach us about normal heart physiology and lead to an era of mechanism-based therapeutics. (Zipes et al., 2019.)

Channelopathy and cardiomyopathy mutations have remarkable variation in their clinical manifestations, including variable morphological phenotypes and disease severities (Burke et al., 2016). Other co-morbidities and other (non)-genetic factors can cause changes in myocardial as well as ion channel remodeling (Verkerk et al., 2018).

6.1 ECG findings caused by the D1275N mutation in *SCN5A* gene

The *SCN5A* D1275N mutation combined with rare polymorphisms in an atrial-specific connexin40 (*Cx40*) gene was first identified by Groenewegen's group in 2003 (Groenewegen et al., 2003). The *SCN5A* D1275N mutation is unique because it is associated with both arrhythmias and the DCM phenotype (Hayano et al., 2017). McNairs's research team proposed that D1275N alteration in the ion channel gene *SCN5A* is associated with DCM and dysfunction in electrical excitability, caused by disruption of sodium channel function, also leads to dilation remodeling (McNair et

al., 2004; Gui et al., 2010). A study using a human *SCN5A* D1275N knock-in mouse (Watanabe et al., 2011) and later hiPSC-based model for *SCN5A* D1275N-related sodium channelopathy demonstrated reduced maximum sodium conductance resulting from reduced $Na_V1.5$ protein expression, which is dependent on proteasomal degradation (Hayano et al., 2017).

Earlier, Aalto-Setälä's group described a large family with a mutation in the *SCN5A* gene D1275N without *Cx40* mutation with variable clinical phenotypes, including supraventricular arrhythmias, atrial standstill, and sudden death due to thromboembolic stroke. Atrial fibrillation or flutter and AS were probable etiologic factors for thromboembolic stroke, and the deaths occurred at the age of 31 or younger. (Laitinen-Forsblom et al., 2006.)

A 12-lead ECG is an easy and inexpensive tool for finding risk patients. In our *SCN5A* D1275N study, we justified ECG changes in cardiac molecular events due to a possible reduced sodium flow causing progressive conduction disorders. Typical changes of the 12-lead ECG predict conduction defects and atrial arrhythmias in the patients with the *SCN5A* D1275N. Additionally, the 12-lead ECG appeared to be abnormal in all affected family members. Many rare and common *SCN5A* variants are associated with cardiac conduction defects (Magnani et al., 2014) and atrial fibrillation with or without underlying heart disease (Darbar et al., 2008; Savio-Galimberti & Darbar, 2014). The mutation present in the Finnish family *SCN5A* D1275N causing conduction defects and atrial arrhythmias is a loss-of-function mutation when analyzed using the hiPSC-based model (Hayano et al., 2017). The final outcome *SCN5A* mutation on the phenotype depends on, which gating property is affected and how it is affected (Wilde & Amin, 2018).

6.1.1 PR-segment changes

Three of our *SCN5A* gene mutation D1275N carriers in sinus rhythm had a remarkable PR depression in many leads just before junctional rhythm episodes. Therefore, we propose that abnormal PR depression in many inferolateral leads may predict atrial conduction defects associated with P-wave prolongation. We suggest that PR depression may be a precursor of atrial arrhythmias including conduction disorders, atrial fibrillation, and atrial standstill. Later also other investigators have documented PR depression in patients with *SCN5A* D1275N mutation (Moreau et al., 2018). Theoretically, the analysis of atrial repolarization may reveal important information regarding arrhythmic propensity, as does QT-interval analysis in case of ventricular

arrhythmias. PR depression may be considered as a marker of atrial repolarization abnormality with similar arrhythmogenic potential as the ST elevations for ventricular arrhythmias. We propose that changes of PR interval are similar risk to atrial arrhythmias than changes of QT interval to ventricular arrhythmias. Conditions that significantly reduce Na^+ current (i.e. Brugada syndrome mutations) may selectively shorten the duration of the epicardial action potential. This condition creates a temporal imbalance between endocardial and epicardial repolarization, and such electrical heterogeneity may underlie the ST-segment elevation and proarrhythmic manifestations of the Brugada syndrome. (Balser, 2001.)

SCN5A mutations with loss-of-function properties have been identified in patients with cardiac conduction defects, SSS (Makita et al., 2005), and AS (Groenewegen et al., 2003; Makita et al., 2005). PR prolongation was also associated with increased risk of atrial fibrillation (Shulman et al., 2015), need for pacemaker implantation, and excess mortality (Cheng et al., 2009). We also found these changes in 31% of our *SCN5A* family members even though the mutation was different. One of our patients, who underwent an electrophysiology study, proved to have fibrosis of the right atrial lateral wall. Loss of I_{Na} function could induce atrial fibrosis, which may cause atrial fibrillation in individuals with associated structural abnormalities including increased diastolic ventricular pressure in the setting of dilated cardiomyopathy with subsequent enlargement of the atria (Wilde A.A & Brugada R., 2011).

Watanabe et al. studied the same *SCN5A* mutation as we did, and they observed slowed and disordered cardiac conduction and decreased contractile function in mice bearing the *SCN5A* mutation; mice with two D1275N alleles displayed worse phenotypes than those with one variant allele (Watanabe et al., 2011). Two of our patients needed upgrading from a VVI-type to a physiologic pacemaker. However, after atrial lead implantation, they were more symptomatic of atrial arrhythmias, suggesting that atrial fibrosis could disturb pacemaker capture as was suggested by Chiang et al. Sometimes pacemaker capture difficulties were seen in both the atrium and the ventricle depending on *SCN5A* mutation if it also causes fibrosis on the ventricle (Chiang et al., 2015).

6.1.2 QRS fragmentation

When looking for an electrical link between the genotype and the phenotype in our patients, we noted a distinct ECG pattern with fragmentation of the R wave especially in leads II, III, and aVF. This fragmentation is a low-amplitude signal at the beginning of the QRS complex with similarities to the epsilon wave in patients with ARVC (Nasir et al., 2004; Muhappan & Calkins, 2008), although in a different location. In the normal heart, an activated cell of the conduction system moves to positive potential because of the rapid inward surge of Na^+-ions. Na^+ contributes the electrical energy for impulse conduction. (Ritchie et al., 2013.) Anomalous Na^+ current handling in *SCN5A D1275N* gene mutation may affect the initial part of the QRS complex, resulting in the observed R-wave fragmentation. We have no definite explanation for the fact that this fragmented signal appeared to be larger during junctional rhythm. Functional change is possible as described in patients with progressive ARVC in which epsilon waves are caused by slow conduction in the right ventricle (Marcus & Zareba, 2009). Interestingly, all our *SCN5A* D1275N patients, who progressed from sinus rhythm to atrial standstill, showed an increase in the amplitude of the initial fragmentation of the QRS complex. It could be speculated that progressive increase of this "spike" in the initial part of the QRS may signal disorders in atrial conduction and there by predict cardiac thromboembolic diseases. In 2018, *SCN5A* D1275N mutation was reported as associative with left atrial dysfunction and stroke even in children (Moreau et al., 2018). Also 31% of our *SCN5A* D1275N mutation carriers have thromboembolic stroke at the age of 31 or younger (Laitinen-Forsblom et al., 2006).

All our gene mutation carriers also had fragmentation of the S wave and a prolonged upstroke in the right precordial leads in the 12-lead ECG. FQRS has been defined as a marker of ARVC and the Brugada syndrome, both of which have been linked to Na^+ channel dysfunction (Surawicz & Knilans, 2008; Rizzo et al., 2012). Prolonged S-wave upstroke in leads V1 through V3 is also a common ECG feature of ARVC (Nasir et al., 2004; Muhappan & Calkins, 2008). Morita et al. showed an over-representation of patients with the *SCN5A* mutation in patients with fQRS (33% versus 5% in non-affected individuals) (Morita et al., 2008). All of our patients have fQRS, and QRS fragmentation is said to be a prognostic tool in structural heart diseases (Haukilahti et al., 2016).

6.1.3 QRS-duration changes

In the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) consortium QRS genome-wide association study meta-analysis, QRS duration was associated with future development of atrial fibrillation (Ritchie et al., 2013). Our *SCN5A* D1275N family members had prolonged QRS duration; many of them had different atrial arrhythmias and some of them had BBB. It is possible that *SCN5A* D1275N mutation also causes degenerative intraventricular conduction defects. Types of intraventricular conduction defects (such as LBBB, LAFB, and RBBB) were present in the family with *SCN5A* mutation in the classical description of idiopathic Lev–Lenegre disease (Probst et al., 2003). Te Riele et al. speculated that $Na_v1.5$ is a multifunctional protein variant with their caused a variety of clinical phenotypes depending on genetic changes in Nav1.5. They showed using hiPSC-CMs from an ARVC patient with *SCN5A* variants that QRS duration was significantly prolonged. (te Riele et al., 2017.) Mutations in the cardiac Na^+ channel encoded by the gene *SCN5A* can result in many different phenotypes. The wide array of clinical phenotypes explains the variable outcomes of the same mutation even within one family because many different factors affect the expression of the gene. (Moreau & Chahine, 2018.)

6.1.4 Impact of the study on treatment

Atrial standstill, a severe form of atrial cardiomyopathy, is associated with combined heterozygous mutations of *SCN5A* and *Cx-40* genes (Groenewegen et al., 2003; Goette et al., 2016), and our *SCN5A* D1275N mutation patients have atrial cardiomyopathy without *Cx-40* mutation. *SCN5A* D1275N mutation carrier may need medication optimization including anticoagulation and antiarrhythmic medication and pacemaker reviews. Patients with *SCN5A* mutation are seen pacemaker capture difficulties in both the atrium and the ventricle (Chiang et al., 2015).

We found progressive ECG changes in patients with *SCN5A* D1275N mutation. Consequently, we agree with Probst et al. that carriers of a *SCN5A* mutation need a clinical and ECG follow-up because of the risk associated with severe conduction defects (Probst et al., 2006).

6.2 The *JPH2* gene p.(Thr161Lys) mutation causing hypertrophic cardiomyopathy

We have identified the *JPH2* p.(Thr161Lys) variant in nine Finnish index patients with HCM and have shown cosegregation of the variant with cardiomyopathy in six of these families. This is the first *JPH2* variant shown to be causative for HCM. Altogether 26 heterozygotes individuals with the *JPH2* c.482C>A, p.(Thr161Lys) (NM_020433.4) variant were found and the penetrance was 71% by age 60 and 100% by age 80.

Several studies have highlighted the importance of JPH2 for normal cardiac physiology. JPH2 is a cardiac specific member of the junctophilins and it has emerged as a potentially important regulator of excitation-contraction coupling in cardiomyocytes. (Takeshima et al., 2000; Landstrom et al., 2007.) However, little is known about the significance of *JPH2* as a causative gene for cardiomyopathy.

6.2.1 *JPH2* p.(Thr161Lys) versus two Finnish founder mutations

Although it has been difficult to demonstrate phenotypic correlations between different genes and different mutations, there is a significantly worse outcome in patients tested positive versus negative for mutations in sarcomere genes (Lopes et al., 2013). Cardiac phenotype related to these variants differs somewhat from typical HCM. In Finland, three mutations in sarcomere genes, myosin binding protein C *(MYBPC3*-Q1061X), alpha tropomyocin (*TPM1*-D175N), and myosin heavy chain 7 (*MYH7*-R1053Q*)*, account for about 23% of all cases with HCM (Kuusisto et al., 2016). Two Finnish founder mutations for HCM have previously been published, one in myosin binding protein C *(MYBPC3)* gene and the other in alpha tropomyocin (*TPM1)* gene (Hedman et al., 2004; Jääskeläinen et al., 2013). The *JPH2* p.(Thr161Lys) variant presented here differs from the other two founder mutations in clinical presentations. The *JPH2* variant associates with earlier disease onset (27 years vs. 52 and 49 years) than the *MYBPC3* p.(Gln1061*) or *TPM1* p.(Asp175Asn), respectively, although no patients under age of 16 were included in the previous study (Jääskeläinen et al., 2013). Systolic heart failure was observed in 5 (25%) and LV dysfunction was present in half of the affected patients with *JPH2* variant when defined by EF<47% or elevated proBNP. Of the probands with *MYBPC3* p.(Gln1061*), none has had congestive heart failure. However, dyspnea was present in 31%. Also none of the index patients with the *TPM1* p.(Asp175Asn) mutation

had significant systolic dysfunction (EF<46%) and only one (5%) had a history of systolic heart failure. Severe conduction defects defined by 3-degree AV-block or R/LBBB was observed in nine (45%) and AF in nine (45%) of the affected individuals or obligate carriers with *JPH2* variant, but no SCDs were detected in our families. AF is generally suggested because of increased atrial pressures and remodeling as an adaptation to diastolic dysfunction, possible LVOT obstruction, and atrial myopathy (Cooper et al., 2017).

Of the individuals with *MYBPC3* p.(Gln1061*) variant, 26% presented syncope/presyncope, 23% had either chronic or paroxysmal AF, 9% have had sustained VT or VF and 26% had family history of SCD. Three out of 34 (9%) HCM patients carrying the *TPM1* p.(Asp175Asn) presented with a documented SCD at young or middle age. Of the probands with *TPM1* or *MYBPC3* founder mutation, ICD was implanted in 2 (10%) and 7 (20%) patients respectively and no significant conduction problems necessitating pacemaker implantation were described in the index publications. (Hedman et al., 2004.) These observations highlight the significant differences in clinical presentation of the patients with the previously characterized founder mutations compared to proposed *JPH2* mutation described in this study. However, the degree of LV hypertrophy in patients with *JPH2* mutation may be similar in patients with these founder mutations (22±5 mm, 18±6 mm and 20±5 mm with variant in *MYPBC3*, *TPM1* or *JPH2*, respectively) (Hedman et al., 2004; Jääskeläinen et al., 2013), although the *JPH2* variants carriers were younger at a time of evaluation compared to carriers of other founder mutations. It is known that significant variation in clinical presentation within and between the families with the same mutation exists in all types of HCM (Roma-Rodrigues & Fernandes, 2014).

We identified one family (Family 6) where all three affected individuals carry the well-established pathogenic *MYBPC3* variant, p.(Gln1061*), in addition to the *JPH2* p.(Thr161Lys) variant. Their phenotypic presentation did not differ significantly from the other families that are unexpected as patients with homozygosity/compound heterozygosity or digenic pathogenic variants in sarcomere genes, which may present at very early age (Nanni et al., 2003; Saltzman et al., 2010). In literature, at least nine patients have been described to have homozygous or confirmed compound heterozygous disease-causing variant in *MYBPC3* and at least the other not-truncating variant. In these patients, the mean age at onset was 4.1 years. Three of these cases presented at neonatal phase, of which all died before age of two months. (Dellefave et al., 2009; Marziliano et al., 2012.) All patients with homozygous or compound heterozygous truncating pathogenic mutations in *MYBPC3* reported so far (n = 21) were diagnosed with severe cardiomyopathy and/or died within the first

few months of life (Marziliano et al., 2012). At the moment, it is unclear whether compound mutation present in sarcomere and non-sarcomere gene simultaneously has additive detrimental effect on disease onset and/or progression.

6.2.2 Pathogenicity of *JPH2* mutations

Quick et al. have described a *JPH2* p.(Ala405Ser) variant in a single patient with basal septal hypertrophy and diastolic dysfunction (Quick et al., 2017). Most of the previously published variants are absent or rare in the Exome Aggregation Consortium (ExAC) or the Genome Aggregation database (gnomAD) reference populations and therefore they have potential to be disease causing. Truncating *JPH2* variants are relatively rare in ExAC reference population (carrier frequency 1 per 6,030 individuals) but due to the small size of the gene, the probability of being loss of function (LoF) intolerant (pLI) value is 0.01, which does not suggest that loss of function alterations would be poorly tolerated. The clinical data on HCM related to the previously published *JPH2* missense variants is limited. Mostly, clinical HCM appears to be diagnosed after teenage but at least the patient with p.(Glu169Lys) manifested HCM at the age of 5 months (Beavers et al., 2013), similarly as two patients in our *JPH2* study.

We showed that the *JPH2* p.(Thr161Lys) is classified as pathogenic based on the ACMG (the American College of Medical Genetics and Genomics) classification scheme (Richards et al., 2015), as the variant resides in a conserved position, is predicted to be deleterious by *in silico* prediction tools, is absent in control populations and cosegregates with dominant HCM in six families.

6.3 Mutations in different localizations in the *RyR2* gene affect the drug responsiveness in CPVT

We studied the antiarrhythmic potential of dantrolene in the treatment of CPVT1 assessing the efficacy of intravenously administered dantrolene in patients carrying various *RyR2* mutations and compared these effects to *in vitro* studies using iPSC derived CMs generated from the same patients. Our findings demonstrate that intravenous dantrolene abolished or markedly reduced arrhythmias in a subgroup of

CPVT1 patients with specific *RyR2* mutations. By combining evidence from *in vivo* and *in vitro* studies, we suggest that the location of the *RyR2* mutation affects the antiarrhythmic effect of dantrolene in CPVT1 (Figure 18).

6.3.1 CPVT1 patient-specific iPSC derived cardiomyocytes

Several studies using induced pluripotent stem cell (iPSC) technology (Takahashi et al., 2007) have shown the ability of CPVT1 patient-specific iPSC derived cardiomyocytes (CMs) to replicate the disease phenotype in cell culture (Novak et al., 2011; Fatima et al., 2011; Jung et al., 2012; Kujala K. et al., 2012; Itzhaki et al., 2012; Di Pasquale et al., 2013). Dantrolene was reported to rescue the disease phenotype in iPSCs derived CMs from a single *RyR2* S406L mutation carrier (Jung et al., 2012), but no *in vivo* data exists on its effects on CPVT1 patients. Usually dantrolene is a drug used to treat another ryanodine receptor disorder, for example malignant hyperthermia (Kobayashi et al., 2010).

It is also a significant aspect that even if a drug is found to be beneficial in the patient-derived iPSC-CMs, it cannot be automatically concluded that this will translate into a clinical benefit for patients with same disease. Here we show that dantrolene, if given intravenously, has an antiarrhythmic effect also in some but not in all patients with CPVT1. This antiarrhythmic effect was observed only in patients with *RyR2* mutations in the N-terminal or central regions of *RyR2* protein (clusters 1–3), whereas virtually no effect was seen in patients carrying mutations at the end of cluster 3 or in the transmembrane region (cluster 4). Although a dose-dependent effect cannot be excluded, similar observations on mutation-specific drug responses have been obtained in some other genetic disorders including long QT syndrome type 3 (Ruan et al., 2007), cystic fibrosis (O'Reilly et al., 2013), Wilson disease (Overeem et al., 2019), as well as in certain neoplastic diseases (Willyard, 2011).

More than 150 mutations in *RyR2* gene have been reported before our study and they are clustered in four hotspots (Priori & Chen, 2011). One third of the reported mutations are in clusters 1 and 2, and the rest are equally distributed between clusters 3 and 4. Only 10% of *RyR2* mutations have been found outside these clusters (Priori & Chen, 2011). The location of the *RyR2* mutation appears to be critical for a favorable effect of dantrolene. The binding site for dantrolene is localized in the N-terminus of *RyR2* between amino acid 601 and 620. (Paul-Pletzer et al., 2005; Kobayashi et al., 2009.) In previous studies (Kobayashi et al., 2005; Paul-Pletzer et al., 2005; Kobayashi et al., 2009), it has been demonstrated that the dantrolene-binding

sequence is considered to constitute part of the domain switch region, suggesting that dantrolene is involved in the correction of defective unzipping and allosteric stabilization of interdomain interactions between the N- terminal and central regions of *RyR2*, resulting in inhibition of Ca^{2+} leak (Kobayashi et al., 2005; Suetomi et al., 2011; Wang et al., 2011). We also show that dantrolene abolished arrhythmias in CPVT1 patients with mutations of N terminal or central domain, suggesting that a defective interdomain interaction within the *RyR2* could be the underlying arrhythmogenic mechanism in the exon 3 deletion, P2328S, T2538R and L4115F. However, dantrolene did not suppress T2538R-related arrhythmias to the same extent as arrhythmias caused by other central region mutations. It has been speculated that differences in the mode of interdomain interaction in dantrolene-binding regions may result in differences in its antiarrhythmic efficacy (Suetomi et al., 2011). Furthermore, other drug-binding regions in the carboxyl-terminal half of the *RyR2* or additional low affinity drug binding sites in the N-terminal area could exist (Kobayashi et al., 2009). No previous studies on the effects of dantrolene on *RyR2* mutations in or close to the transmembrane are available; here we demonstrate that dantrolene has no or only minimal effect on arrhythmias if mutations are located in these areas. It is intriguing that the patient with Q4201R mutation did not respond to dantrolene even though this mutation is located in cytosolic portion of *RyR2* and in cluster 3 although in its terminal part. This finding implies that the location of the mutation in a certain mutation cluster does not necessarily determine the antiarrhythmic response and highlights the utility of the iPSC model for individual functional analysis.

6.3.2 Dantrolene effects in the clinical setting and corresponding iPSC-CM models

Our study showing similar patient-to-patient variation in dantrolene effects in the clinical setting and corresponding iPSC-CM models propose that, at least in theory, it may be possible to tailor an individual's medication in cell culture without predisposing the individual to the potentially serious side effects of a drug. Dantrolene did not affect normally beating CMs. This is consistent with previous reports showing that dantrolene inhibits only abnormal Ca^{2+} release and has no effect on the normal Ca^{2+} transients, suggesting that the native conformation of *RyR2* may restrict binding of the drug and that dantrolene binding to *RyR2* might be dependent on a specific conformational state present only in mutated cells. (Paul-Pletzer et al., 2005;

Kobayashi et al., 2009.) Defective calmodulin binding caused by *RyR2* domain unzipping has also been shown to be restored by dantrolene (Xu et al., 2010; Ono et al., 2010), which may as well explain why dantrolene exerts effects on diseased but not healthy hearts. Although dantrolene as such would not be suitable for long-term treatment of CPVT1 due to its side effects, and although only a subset of patients would benefit from it, our data shows its antiarrhythmic potential for some patients. Although in our study of *RyR2* mutations we showed that even patients with different mutations in the same gene benefit from dantrolene diffe-rently.

Drug responses were similar in patients and their iPSC-CMs, in other words CPVT1 *in vitro* phenotypes depending on the nature of the mutation. Immature phenotype of the iPSC-CMs may too produce variation in arrhythmias. However, in a previous study of the same research group (Kujala K. et al., 2012) the electrophysiology of CPVT1 iPSC-CMs appeared fairly mature, which also reflects that arrhythmias are more consistent in CPVT1 CMs than in control CMs. In addition, the beating frequency of CPVT1 CMs was lower than that in control CMs. This is in line what has been reported also with CPVT1 patients (Lobo et al., 2011; van der Werf & Wilde, 2013). All the CPVT1 CMs showed similar disturbances in intracellular Ca^{2+} cycling. Ca^{2+} transient abnormalities were somewhat more common in the cluster 4 mutation than in cluster 1, 2 and 3 mutations. Exon 3 deletion differed from all the other mutations by having lower diastolic Ca^{2+} levels and beating frequency both at baseline and during adrenaline perfusion. Exon 3 encodes secondary structure elements that are crucial for folding of the N-terminal domain. It has been proposed that *RyR2* with exon 3 deletion has evolved additional means to regulate Ca^{2+} release, by altering the conformation of the domain (Lobo et al., 2011), which may result in the observed differences in Ca^{2+} transients.

6.4 Comparison of 12-lead ECG and *in Vitro* FP of hPSC-CM

It is important that we have methods to search cardiomyocytes function, which correlates to *in vitro* models of the native human myocardium. There are no direct reports about the relationship of intricate electrophysiological characteristics between the human heart and hPSC-CMs. In our study IV, we investigated the relationship of electrical activation and beating rate clinically using ECG and MEA recordings *in vitro* of hPSC-CMs and found that the results correlated well with each other.

The electrical properties of the heart are due to ion transfer across the cell membrane. *In vivo*, the transfer rate of potassium ions is dependent of the beating rate (Aziz et al., 2018). The repolarization time presented as the QT interval in the ECG is, thus dependent on how fast the heart beats (Karjalainen et al., 1994). Because heart rate is normally the main determinant of repolarization length, there are several formulas how to correct the measured QT interval so that QT intervals with different beating frequencies can be compared (Surawicz & Knilans, 2008). The simplest and most widespread approach to correct the QT interval is to divide its value by the square root of the preceding RR interval expressed in seconds ($QTc = QT/\sqrt{RR}$), i.e., by using Bazett's formula (Bazett, 1920; Funck-Brentano & Jaillon, 1993).

With hPSC-CMs, these same correction formulas have been used without any evidence whether the same formulas are effective during *in vitro* situations (Stett et al., 2003). In our QT/FPD study, we chose control individuals with a low baseline beating rate and performed a stress exercise test to obtain QT variation over the same beating frequency spectrum as obtained with spontaneously beating hPSC-CMs. The cells were cultured on MEA platform, and the FPD corresponding to QT in ECG was measured. A strong correlation was observed between QT intervals and FPDs, and the correction of these parameters using Bazett's formula gave similar results, thus confirming that the ion fluxes in cell culture present with corresponding performance as they do *in vivo* in the heart.

Normally the QT interval of the heart increases with decreasing heart rate (Karjalainen et al., 1994) due to decrease in potassium flux across the cell membrane (Aziz et al., 2018). The hPSC-CM beating rate in our cell cultures varied from 55 (minimum) to 100 (maximum) bpm with the average of 73 bpm. We made comparison with the cardiac parameters between ECG and MEA recordings and could show that QT and RR parameters correlate excellently with FPD and PPI results. In the volunteers who were used for the comparison, the heart rate varied between 39 and 120 bpm with an average of 76 bpm.

6.4.1 QT/RR versus FPD/PPI

AHA/ACCF/HRS recommends that linear regression functions rather than the Bazett's formula be used for QT-rate correction although Bazett's formula is the most widely used for QT-rate correction (Rautaharju et al., 2009). QT/RR value means how quickly and how much QT interval changes when heart rate changes (Malik et al., 2018) corresponding to FPD/PPI values in MEA. The over- or undercorrection

of QTc may lead to significant and systematic bias with both false positive and false negative findings, whereas QT/RR patterns in all different subjects will be characterized by the same mathematical form. Population risk stratification studies investigating QT/RR patterns would also benefit from this mathematical description avoiding the heart rate influence. (Malik et al., 2013.) QT/RR mean value of our volunteers was 0.433, ranging from 0.40 to 0.46. The mean FPD/PPI value of 0.44 indicates that the basal FPD in relation to the time between individual beats corresponds with QT/RR values.

The coefficient of determination value of 0.71 is good for biological samples. Our results indicate that 71% of the FPD prolongation is explained by concurrent prolongation of the PPI. However, the FPD generally increased when PPI was prolonged corresponding to changes of QT time and RR cycle. Taken together with the observation that hPSC-CMs demonstrated fairly stable PPI dynamics within recordings, we conclude that they are rather reliable models in terms of their electrophysiological aspects, especially when corrected for beating-rate-dependent FPD modulation. It is also known that in different individuals the predicted QT times may normally vary up to 90 ms even if the RR cycle lengths are the same (Surawicz & Knilans, 2008). QT time variation between our volunteers was less than 60 ms at various RR cycle lengths. The coefficient of determination of our volunteers was about 0.68 for ECG (Figure 19).

6.4.2 QTc= QT/√RR versus cFPD= FPD/√PPI

The cFPD value of 400 ms for hPSC-CMs is within the physiological normal range for QTc values. Because uncorrected FPD was 375 ms at 73 bpm, the Bazett's formula is adequately compensating for the rate-induced FPD shortening also in hESC-CMs. Analogously, the volunteers had a mean QTc of 432 ms and their uncorrected mean QT was 397 ms with a mean heart rate of 76 bpm. However, a heart rate of 60 bpm is the most optimal when using Bazett's formula (Funck-Brentano & Jaillon, 1993). Usually the relationship between QT/RR adaptation and mean QTc values means that those subjects who show longer QTc intervals have steeper QT/RR patterns (Malik et al., 2013). In our study, we demonstrated that the relationship FPD/PPI adaptation and cFPD values functioned similarly to the relationship between QT/RR adaptation and QTc values. It is also known that Bazett's formula overcorrects the QT interval at high heart rates (Surawicz & Knilans, 2008). This

probably is the reason for this small, normal variations range difference between our cFPD and QTc values.

However, there is no consensus regarding, which would be clinically the optimal formula (Fridericia's or Bazett's formula) (Izumi-Nakaseko et al., 2017). Still, Bazett's formula is easy to use and widely applied and thus it was chosen in this study to compare *in vitro* and *in vivo* electrical parameters in different beating rates.

6.5 Future perspectives

As we learn to understand the cellular and molecular mechanisms in healthy heart and in disease states, we can find better methods for diagnostic and treatment purposes.

The hope is that molecular genetic approach can be applied to common types of arrhythmia, such as atrial fibrillation and SCD. Studies of large populations to identify common genetic variants that predispose individuals to arrhythmias hold similar promise for early detection and intervention in asymptomatic patients at high risk. Arrhythmias and cardiomyopathies are an important public health challenge. (Mizusawa, 2016.)

Some authors think that for example severe septal-leaflet contact is enough for HCM diagnosis (Sen-Chowdhry et al., 2016). In the future, molecular genetic diagnosis helps us to do diagnosis to patients with another anatomical abnormality of HCM without wall thickness. Further research is warranted to evaluate *JPH2* variants and other genes related to Ca^{2+} handling in the cardiomyocyte to further shed light on the genetic background of HCM also using hPSC-CM techniques. Variant interpretation and correlation to phenotype is still exacting as earlier studies form major pitfalls by false classifications related to small reference populations, coincidental segregations and evaluation of only a small subset of the potentially significant genes behind a patient's phenotype (Sedaghat-Hamedani et al., 2018). Large-scale genetic research will eventually bring more consistency to the evaluation of families with inherited cardiac diseases. Variant sharing with relevant information of the phenotype in mutation databases is important to develop the field further. (Garcia et al., 2016.)

Genetic diagnosis enables cost-effective screening of first-degree family members and eliminates healthcare expenditures for relatives without pathogenic mutations, resulting in substantial health care cost savings. Relatives who do not carry the

mutation can be excluded from regular cardiac follow-up and can be reassured that there is no increased risk in their offspring. (Zipes et al., 2019.)

Maybe in the future we can use whole-exome sequencing (WES) and other next-generation sequencing (NGS) approaches and to successfully identify genetic variants potentially contributing to the cardiomyopathies and life-threatening arrhythmias better than today (Miles & Behr, 2016). We also believe that someday we can use the novel classification of inherited cardiomyopathies, which is currently based upon the phenotypic expression. The new genomic classification should take into account the underlying gene mutations and the cellular level of expression of encoded proteins, thus distinguishing cytoskeleton (cytoskeletalopathies), sarcomeric (sarcomyopathies), desmosomal (desmosomalopathies), junctophilin (junctophilinopathies) cardiomyopathies, and ion channelopathies (Corrado et al., 2005).

Then we can additionally choose optimal treatments individually for patients by combining evidence from *in vivo* and *in vitro* studies using iPS-cell techniques (Mercola et al., 2013; Yamazaki et al., 2018; Pölönen et al., 2018). Even the recognition of potential mutation-specific responses will be important for future drug development; one drug may not work for all patients even if the phenotype is the same. The genetic discoveries have enhanced understanding of the molecular pathogenesis of heart diseases and have stimulated efforts designed to identify new therapeutic agents. The multifunctional roles for cardiac proteins in cardiomyocytes may have implications for the development of novel therapeutic approaches for cardiomyopathies, cardiac arrhythmias and heart failure.

6.6 Limitations of the studies

In all the studies presented, the number of the patients is low due to the rarity of the diseases or mutations.

6.6.1 Study I

Our findings represent one large Finnish family with a specific gene mutation. These findings may not necessarily apply to other variants with mutations in the same gene. More studies are needed to explore the complete ECG pattern of *SCN5A* gene mutations.

6.6.2 Study II

Most of the submissions of the *JPH2* variants in ClinVar or other databases reviewed in this work did not include phenotype data and thus provide limited information for the clinical society.

6.6.3 Study III

According to the study design, we were permitted to study only acute effects of intravenously administered dantrolene. Although we titrated the dose of dantrolene according to the weights of the patients, serum levels of the drug were not measured and could have varied from patient to patient, resulting in concentration-dependent variation in clinical responses. In addition, we used only a fixed concentration of dantrolene, selected on the basis of the work by Jung et al. (Jung et al., 2012) in our iPSC studies.

6.6.4 Study IV

Only one stem cell line was used in this study, but already with that we found a correlation with the corresponding parameters in 12-lead ECG thus increasing the number of cell lines would not have an effect of the results of the study.

7 SUMMARY AND CONCLUSIONS

Study I clearly demonstrated that 12-lead ECG can be used to predict arrhythmias in individuals carrying the *SCN5A* D1275N mutation.

In a Finnish family with the *SCN5A* D1275N mutation, ECG changes were very frequent. In addition to P-wave and PR changes, typical abnormalities were initial fragmented R waves in the inferior leads II, III, and aVF as well as the S wave in leads V1–V3 accompanied by slow S-wave upstroke and prolonged QRS duration. The progressively growing initial fragmentation of QRS complex in inferior leads before junctional escape rhythm was clearly visible. Atrial electrical changes (P-wave and PR duration) probably predispose to arrhythmias, such as junctional rhythm, atrial standstill, and atrial fibrillation with increased cardioembolic stroke risk.

Our discoveries confirm that specific findings are present in the standard 12-lead ECG in individuals with the *SCN5A* mutations before clinical symptoms appear, and therefore ECG follow-up of asymptomatic individuals is recommended.

Study II, we demonstrated that *JPH2* p.(Thr161Lys) is a pathogenic mutation based on the ACMG classification scheme.

We observed several Finnish families with *JPH2* p.(Thr161Lys) and analyzed their cardiac findings. The main clinical features were left ventricular hypertrophy, arrhythmia vulnerability and conduction abnormalities including third degree AV-block. Some patients had end-stage severe left ventricular heart failure with normal or mildly enlarged diastolic dimensions.

Based on several clinical parameters, we propose that the heterozygous *JPH2* p.(Thr161Lys) variant is a new Finnish mutation causing atypical HCM, thus strengthening the role of non-sarcomeric *JPH2* as a causative gene for HCM.

Study III, we conclude that hiPSC-derived CMs could serve as a platform for drug development and to design personalized medication.

We show here the proof of principle that intravenously administered dantrolene suppresses ventricular arrhythmias in patients with congenital *RyR2* defect and that

the location of the *RyR2* mutation affects the antiarrhythmic effect of this drug. We also demonstrated that hiPSC-derived CMs correctly predict the clinical response to dantrolene in CPVT1 patients in mutation-specific way. These findings illustrate the potential of hiPSC models to individualize the drug therapy of inherited diseases.

Study IV, our results further validate the hESC-CM clusters as proper *in vitro* models of the native human myocardium. In this study, for the first time the relationship of ECG and cell culture MEA recordings were systematically compared. QT interval is beating-rate-dependent and QTc is the rate-corrected QT interval. The same correction formula (Bazett) was observed to be equally applicable to corresponding repolarization parameter (FPD and cFPD) in hPSC-CMs and thus the same rate correction formula can be used for electrical recordings in cell cultures.

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ORIGINAL PUBLICATIONS

PUBLICATION

I

Electrocardiogram changes and atrial arrhythmias in individuals carrying sodium channel *SCN5A* D1275N mutation.

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Original article

a) Electrocardiogram changes and atrial arrhythmias in individuals carrying sodium channel *SCN5A D1275N* mutation

b) 12-lead ECG changes in *SCN5A D1275N* mutation carriers

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Abstract

Introduction: The cardiac sodium channel *SCN5A* regulates atrioventricular and ventricular depolarization as well as cardiac conduction. Patients with cardiac electrical abnormalities have an increased risk of sudden cardiac death (SCD) and cardio-embolic stroke. Optimal management of cardiac disease includes the understanding of association between the causative mutations and the clinical phenotype. A 12-lead electrocardiogram (ECG) is an easy and inexpensive tool for finding risk patients.

Materials and methods: A blood sample for DNA extraction was obtained in a Finnish family with 43 members; systematic 12-lead ECG analysis was performed in 13 of the family members carrying an *SCN5A D1275N* mutation. Conduction defects and supraventricular arrhythmias, including atrial fibrillation/flutter, atrioventricular nodal re-entry tachycardia (AVNRT) and junctional rhythm were searched for.

Results: Five (38%) mutation carriers had fascicular or bundle branch block, ten had atrial arrhythmias; no ventricular arrhythmias were found. Notching of the R- and S waves – including initial QRS fragmentation - and prolonged S-wave upstroke were present in all the affected family members. Notably, four (31%) affected family members had a stroke before the age of 31 and two experienced premature death.

Conclusions: A 12-lead ECG can be used to predict arrhythmias in *SCN5A D1275N* mutation carriers.

KEYWORDS
SCN5A D1275N mutation; ECG; arrhythmias; conduction defects

KEY MESSAGES

- The 12-lead ECG may reveal cardiac abnormalities even before clinical symptoms occur.
- Specific ECG findings – initial QRS fragmentation, prolonged S-wave upstroke as well as supraventricular arrhythmias - were frequently encountered in all *SCN5A D1257N* mutation carriers.
- ECG follow-up is recommended for all *SCN5A D1275N* mutation carriers.

Introduction

Regulation of the expression and proper function of voltage-gated ion channels at the plasma membrane of excitable cells is essential in maintaining cellular excitability and electrical impulse propagation. In the heart, voltage-gated sodium (Na^+) channels determine the amplitude and slope of the action potential upstroke, which are especially important in the control of impulse conduction velocity, and in the maintenance of appropriate waves of excitation through the working myocardium. Dysfunction of these channels can lead to life-threatening cardiac arrhythmias (1,2).
Some mutations associated with cardiac disease have been identified in the gene encoding cardiac sodium channel α-subunit *SCN5A*, which is responsible for the generation and propagation of action potentials in the atrium, ventricle and specialized conduction system (3).
Genetic defects associated with cardiac electrical disorders are usually found in genes encoding for cardiac ion channels. Cardiac Na^+ channel dysfunction caused by mutations in the *SCN5A* gene is associated with a number of relative uncommon arrhythmic diseases, including long QT syndrome type 3 (LQT3), Brugada syndrome, and short QT interval with Brugada-like electrocardiogram (ECG) (2,4), progressive cardiac conduction disease (PCCD), sick sinus syndrome (SSS), and atrial standstill, which all potentially can lead to fatal arrhythmias at relatively young age. Various *SCN5A* mutations are known to present with mixed cardiac phenotypes including arrhythmias and dilated cardiomyopathy (5). *SCN5A* mutations are also involved in overlap syndromes of cardiac sodium channelopathy. Remme and co-workers provided an overview of the current knowledge on *SCN5A* mutations associated with sodium channel overlap syndromes (6,7). They also discussed the possible role of modifiers in determining disease expressivity in an individual patient (6,8,9,10,11,12).

Cardiac conduction, as assessed by the PR interval and QRS duration, is an important electrophysiological trait and a determinant of arrhythmic risk (13). Atrial standstill is a rare cardiac disorder characterized by the absence of electrical and mechanical atrial activity. Atrial standstill can be persistent or transient, and either diffuse or partial in the atrial wall involving either one or both atria (14,15). Atrial standstill is characterized by bradycardia, diminution or absence of the P wave, junctional (usually narrow complex) escape rhythm and failure of atrial excitation, either spontaneously or by pacemaker stimulation. Atrial standstill is associated with an increased risk of sudden death due to occurrence of very low escape rhythm and an increased risk of thromboembolic stroke due to the high incidence of atrial fibrillation/flutter (3).
Familiar atrial standstill has been associated with the *SCN5A D1275N* mutation combined with rare polymorphisms in an atrial-specific connexin40 (Cx40) gene (16,17,18,19). In a large Finnish family cardiac conduction defects and atrial arrhythmias were also associated with the same *SCN5A D1275N* mutation, although not linked to the polymorphism in the Cx40 gene (20).
Although no sudden cardiac death was recorded in the family, at least four affected members suffered from cardio-embolic stroke by the age of 31 and two of them died of stroke (20). In this report we present the ECG findings, including atrial arrhythmias in individuals carrying the *SCN5A D1275N* mutation.

Materials and methods

Subjects

A Finnish family with 45 members was evaluated at the Heart Center, Tampere University Hospital. All participants gave their informed consent for study participation and the local Ethics Committee gave their approval for the study (R01128). A blood sample for DNA extraction was obtained from 43 family members. In this study we report the ECG findings of eleven *SCN5A D1275N* mutation carriers and two deceased obligate carriers. The oldest family member had nine children and only the ECGs of the *SCN5A D1275N* mutation carriers, including the two obligate carriers were analysed (Figure 1).

Genetic analysis

The genotype analysis of the family members has been previously described in detail by Laitinen-Forsblom et al (20). Based on candidate gene approach, a point mutation (guanine to adenine at position 3823 in exon 21) in the *SCN5A* gene was observed causing a substitution of aspartic acid with aspargine *(D1275N).*

ECG analysis

A 12-lead surface ECG was recorded at rest and the study participants were not allowed to smoke or drink coffee at least six hours prior to the ECG recording. The ECG electrodes were positioned according to standardized protocol at a paper speed of 50 mm/s and 1 mV/cm standardization.
Heart rate, P-wave duration and amplitude, PR interval and deviation from the baseline, QRS duration, S-wave amplitude and up-stroke, QT, and corrected QT (QTc; Bazett`s formula) intervals were measured manually by one of the investigators (SV).
Normal P-wave duration was defined as 70-140 ms (21). The P wave was described as flat, if the amplitude was $\leq$ 0.05 mV in the limb leads.
We used 120 to 200 ms as normal values for the PR interval. The interval was measured in the lead with the largest and widest P wave and the longest QRS duration. The P and QRS axis was considered normal when it was between - 30° and + 90°. We defined PR-segment depression as $\geq$ 0.08mV, and elevation as $\geq$0.05 mV (21).
For QRS-complex fragmentation (fQRS), we used the definition by Das et al: additional R wave (R´ prime), notching in nadir of the S wave, notching of R wave, or the presence of more than one R prime (fragmentation). Typical bundle branch block (BBB) pattern (QRS $\geq$ 120 ms) - right or left – and incomplete RBBB were excluded from the definition of fQRS (22, 23). In left anterior fascicular block (LAFB) QRS duration may be normal or slightly prolonged (21). The upper limits of the S-wave amplitude

in leads V1-V3 were 1.8, 2.6, and 2.1 mV, respectively (20). The QT- interval was measured according to established methodology (21).
The diagnosis junctional escape rhythm required at least three successive complexes. Both the typical finding with a narrow QRS and possible cases with broad QRS due to pre-existing intraventricular conduction defect were included (21).
Atrial standstill is characterized by bradycardia (HR< 50/bpm), the absence of P waves and a junctional narrow or slightly wide QRS complex and regular escape rhythm (16,17,21).

Results

Subjects

In genetic analysis, we found eleven *SCN5A D1275N* mutation carriers and two obligate carriers. The mutation in the *SCN5A* gene present in this family result in the substitution of asparagine for aspartic acid at aa *1275 (D1275N)*. One of the obligate carriers was the oldest family member (patient I:1; Figure 1) of this pedigree and another one (patient II:2) was the father of two mutation carriers (patients III:5, III:6). Both of these obligate mutation carriers had passed away before this study was initiated. Four of nine children of the oldest obligate mutation carrier (patient I:1) had the *SCN5A* mutation. Only the mutation carriers of these children are presented in the figure 1. Four mutation carriers (patients II:2, II:3, II:4, and III:6) of the family had encountered thromboembolic stroke by the age of 31 years, two of them (father II:2 and son III: 6) died at young age; the father at the age of 30 and the son even at the age of 21 (Figure 1).

Electrocardiografic characteristics of the family.

Five of eleven (38%) mutation carriers had a VVIR-type of pacemaker implanted due to a bradycardic junctional rhythm (patients III:6 and III:7, Figure 1) or atrial fibrillation with symptomatic bradycardia (patients I:1, II:4, and III:5). The oldest family member (patient I:1) had atrial fibrillation with slow ventricular response at the age of 38 years and her ECG showed LAFB and RBBB before pacemaker implantation. She had anticoagulation therapy because of atrial fibrillation, but no history of stroke. Nine (69%) *SCN5A D1275N* mutation carriers had atrial fibrillation or flutter.
Transient atrial standstill was observed in one patient (III:7) (8%); he also had paroxysmal RBBB and LAFB. Two other patients - father and son - (II:2 and III:6) with junctional rhythm had no documented atrial tachyarrhythmias before premature death due to stroke. The son (III:6) also had RBBB and LAFB.
In all patients carrying the *SCN5A D1275D* mutation, we found a distinct initial pattern of QRS fragmentation with notching of the R wave (Figure 2). This change was prominent in leads II, III or aVF. The fragmentation seemed to be more prominent during junctional rhythm than during sinus rhythm (Figure 3). All the *SCN5A D1275N* patients, who progressed from sinus rhythm to atrial standstill, showed an increase in the amplitude of the initial fragmentation of the QRS complex.
All 13 (100%) patients had fragmentation of the nadir of the S wave in at least one of the leads V1-V3 and also a prolonged S-wave upstroke.
The P wave was generally flat in all mutation carriers in conjunction with remarkable PR depression in three (23%) individuals (II:2, III:4, III:7). The P wave could not be analyzed in three patients (II:2, III:5 and III:7) due to junctional rhythm.
The QTc interval was normal in the 12-lead ECG in all mutation carriers.
(Figure 2 and table I).

Discussion

We describe a large family with a mutation in the *SCN5A* gene *(D1275N)* with variable clinical phenotypes, including supraventricular arrhythmias, atrial standstill and sudden death due to thromboembolic stroke. The

12-lead ECG proved to be abnormal in all affected family members. Atrial arrhythmias and atrial standstill were probable etiologic factors for thromboembolic stroke, and the deaths occurred at the age of 31 or younger. Typical changes of the 12-lead ECG predict conduction defects and atrial arrhythmias in the patients with the mutation *SCN5A (D1275N)*. Many rare and common SCN5A variants are associated with cardiac conduction defects (24).
Cardiac conduction, as assessed by the PR interval and QRS duration, is an important electrophysiological trait and a determinant of arrhythmic risk (13). The ECG is characterized by different waves that represent specific phases of the cardiac activation sequence: the P wave reflects atrial depolarization, the QRS complex represents ventricular depolarization, and the T wave indicates ventricular repolarization (25).

PR-segment changes
A wide, notched or low-amplitude Ta can predict an interatrial conduction disturbance with or without atrial enlargement. The Ta wave represents atrial repolarization and the interval from the beginning of the P wave to the end of Ta is the atrial equivalent of the ventricular QT interval (26). Theoretically, the analysis of atrial repolarization may reveal important information regarding arrhythmic propensity, as does QT-interval analysis in case of ventricular arrhythmias. PR depression may be considered as a marker of atrial repolarization abnormality with similar arrhythmogenic potential as the ST elevations for ventricular arrhythmias.
Conditions that significantly reduce Na^+-current (i.e Brugada syndrome mutations) may selectively shorten the duration of the epicardial action potential. This condition creates a temporal imbalance between endocardial and epicardial repolarization, and such electrical heterogeneity may underlie the ST-segment elevation and pro-arrhythmic manifestations of the Brugada syndrome (27). Three of our *SCN5A* gene mutation *D1275N* carriers in sinus rhythm had a remarkable PR depression in many leads just before junctional rhythm episodes.
Therefore, we propose that abnormal PR depression in many inferolateral leads may predict atrial conduction defects associated with P-wave prolongation. Therefore, PR depression may be a precursor of atrial arrhythmias including conduction disorders, atrial fibrillation and atrial standstill. Also, Makita et al (17) showed progressive atrial dysfunction leading to the atrial standstill in *SCN5A* mutation *L212P.*

P wave and PR interval
SCN5A mutations with loss-of-function properties have been identified in patients with cardiac conduction defects, SSS (17) and atrial standstill (16,18). Herfst and co-workers have shown that P-wave broadening and a tendency to PR-prolongation are manifested in the ECG of individuals with the *SCN5A/5280delG* mutation, presumably due to a reduction in Na^+ current (1). PR prolongation was also associated with increased risk of atrial fibrillation (28), need for pacemaker implantation and excess mortality (29). We also found these changes in some of our *SCN5A* family members even though the mutation was different.
One of our patients, who underwent an electrophysiology study, proved to have fibrosis of the right atrial lateral wall. Loss of I_{Na} function could induce atrial fibrosis, which may cause atrial fibrillation in individuals with associated structural abnormalities including increased diastolic ventricular pressure in the setting of dilated cardiomyopathy with subsequent enlargement of the atria (10). Watanabe et al. studied the same *SCN5A* mutation as in our family and they observed slowed and disordered cardiac conduction and decreased contractile function in mice bearing an *SCN5A* mutation; mice with two *D1275N* alleles displayed worse phenotypes than those with one variant allele. In vitro electrophysiological studies identified reduced peak cardiac Na^+ current as a key defect and this is consistent with the observed reduced conduction velocity (30). Two of our patients needed upgrading from a VVI-type to a physiologic pacemaker. However, after atrial lead implantation they were more symptomatic of atrial arrhythmias, suggesting that atrial fibrosis could disturb pacemaker capture as was suggested by Chiang and co-workers (31).

QRS fragmentation
Many investigators considered fragmented QRS (fQRS) as an independent predictor of cardiac events (32,33,34,35,36). When looking for an electrical link between the genotype and the phenotype in our patients, we noted a distinct ECG pattern with fragmentation of the R wave especially in leads II, III and aVF. This fragmentation is a low-amplitude signal at the beginning of the QRS complex with similarities

to the epsilon wave in arrhytmogenic right ventricular cardiomyopathy (37), although in a different location. In the normal heart, an activated cell of the conduction system moves to positive potential because of the rapid inward surge of Na^+-ions. Na^+ provides the electrical energy for impulse conduction (38). Anomalous Na^+ current handling in SCN5A *D1275N* gene mutation may affect the initial part of the QRS complex, resulting in the observed R-wave fragmentation. We have no definite explanation for the fact that this fragmented signal appeared to be larger during junctional rhythm. Interestingly, all our *SCN5A D1275N* patients, who progressed from sinus rhythm to atrial standstill, showed an increase in the amplitude of the initial fragmentation of the QRS complex. It could be speculated that progressive increase of this "spike" in the initial part of the QRS may signal disorders in atrial conduction and there by predict cardiac thromboembolic diseases.
All our gene mutation carriers also had fragmentation of the S wave and a prolonged upstroke in the right precordial leads in the 12-lead ECG. FQRS has been defined as a marker of arrhythmogenic right ventricular cardiomyopathy and the Brugada syndrome, both of which have been linked to Na^+ channel dysfunction (21). Prolonged S-wave upstroke in leads V1 through V3 is a common ECG feature of arrhythmogenic right ventricular cardiomyopathy (37). Morita et al showed an over-representation of patients with the *SCN5A* mutation in patients with fQRS (33% vs. 5% in non-affected individuals) (36) and our results are in line with these previous observations.

QRS duration
In the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) consortium QRS genome-wide association study meta-analysis, QRS duration was associated with future development of atrial fibrillation (38). Probst and coworkers described progressively increased QRS duration in patients with *SCN5A*-linked hereditary Lev-Lenegre disease affecting the His bundle and its branches (39). Our *SCN5A D1275N* family members had prolonged QRS duration; many of them had different atrial arrhythmias and some of them had BBB. It is possible that *SCN5A D1275N* mutation also causes degenerative intraventricular conduction defects. Types of intraventricular conduction defects (such as LBBB, LAHB and RBBB) were present in the family with *SCN5A* mutation in the classical description of idiopathic Lev-Lenegre disease (39).
Progressively increased conduction defects predict atrial paralysis and atrial arrhythmias, which are possible reasons for cardio-embolic stroke without anticoagulation (39,40). Prost et al demonstrated that carriers of a *SCN5A* mutation need a clinical and ECG follow-up because of the risk associated with severe conduction defects (41). Mutations in the cardiac Na^+ channel encoded by the gene SCN5A can result in many different phenotypes. The wide array of clinical phenotypes explains the variable outcomes of the same mutation even within one family.

Limitations
Our study did not include long-term follow-up. Therefore, we have no solid data on the consistency of the ECG findings.
We were not able to perform reliable analysis of the P waves during stress test in the study population. Therefore, exercise test data was not included.
The findings presented represent one family with a specific gene mutation. These findings may not necessarily apply to other families with mutations in the same gene. More studies are needed to explore the complete ECG pattern of *SCN5A* gene mutations.

Conclusions
In a Finnish family with the *SCN5A D1275N* mutation, ECG changes were very frequent in the mutation carriers. In addition to P-wave and PR changes, typical abnormalities were fragmented R waves in the inferior leads II, III and aVF as well as of the S wave in the leads V1-V3 accompanied by slow S-wave upstroke and prolonged QRS duration. Atrial electrical changes probably predispose to arrhythmias, including junctional rhythm, atrial standstill and atrial fibrillation with increased cardio-embolic stroke risk at young age. Our findings confirm that complex and variable findings are present in the standard 12-lead ECG in individuals with a single *SCN5A* mutations. Therefore, ECG follow-up of asymptomatic individuals is recommended.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Table I. Distribution of 12-leads ECG parameters of the *SCN5A D1275N* patients.

ECG parameters	n = 13 (%)	Individual
Junctional rhythm	4 (31%)	(II:1, II:2, III:6, III:7)
Atrial fibrillation or flutter	9 (69%)	(I:1, II:1, II:3, II:4, III:1, III:3, III:4, III:5, III:7)
Atrial tachycardia	1 (8%)	(III:5)
Flat P wave in lead II	4 (31%)	(II:1, II:2, III:2, III:7)
P wave > 140 ms	2 (15%)	(II:1, III:2)
PR interval > 200 ms	4 (31 %)	(II:1, III:1, III:2, III:5)
Fragmentation of initial QRS	13 (100%)	
S-wave upstroke ≥ 55 ms in leads V2-3	13 (100%)	
Notching in nadir of the S wave in leads V1, V2, V3	13 (100%)	
QRS ≥ 120 ms	10 (77%)	(all except II:3, III:1, III:3)
RBBB	4 (31%)	(I:1, III:6, III:7, IV:1)
LAFB	4 (31%)	(I:1, II:3, III:6, III:7)
Pacemaker	5 (38%)	(I:1, II:4, III:5, III:6, III:7)

Figure legends

Figure 1. Pedigree indicating the ECG findings. Four of nine children of the oldest obligate mutation carrier (patient I:1) had the *SCN5A* mutation, only these individuals significant to genetic analysis and their children are included. AF = atrial fibrillation or flutter, AT = atrial tachycardia, AV I = grade I atrioventricular block, AVNRT = atrioventricular nodal re-entry tachycardia, c = chronic, DCM = dilated cardiomyopathy, LAFB = left anterior fascicular block, p = paroxysmal, PAMA = implanted pacemaker, pr Pd = prolonged P duration, RBBB = right bundle branch block, SSS = sick sinus syndrome, TIA = transient ischemic attack, y = years

Figure 2. A typical 12-lead ECG in an asymptomatic patient with the *SCN5A D1275N* mutation.
Wide arrow: low amplitude signal at the beginning QRS complex.
Narrow arrow: notch in S wave
: deep S wave in chest leads
: Prolonged S-wave upstroke ≥ 55 ms
: flat P wave
: PR depression

Figure 3. Typical large initial fragmentation of the QRS complex with junctional escape rhythm in an *SCN5A* gene *D1275N* mutation carrier. The arrow shows modification in the progressive growing initial fragmentation of QRS complex in inferior leads.

Table I. Distribution of 12-leads ECG parameters of the *SCN5A D1275N* patients.

Figure 1

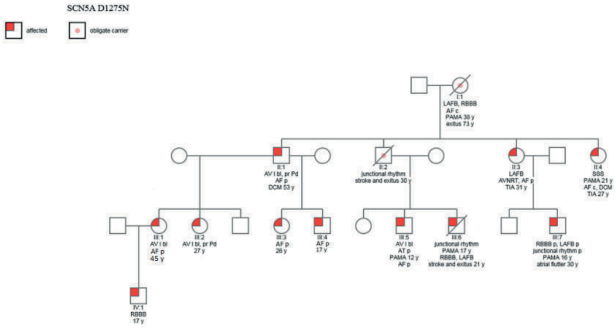


Figure 2

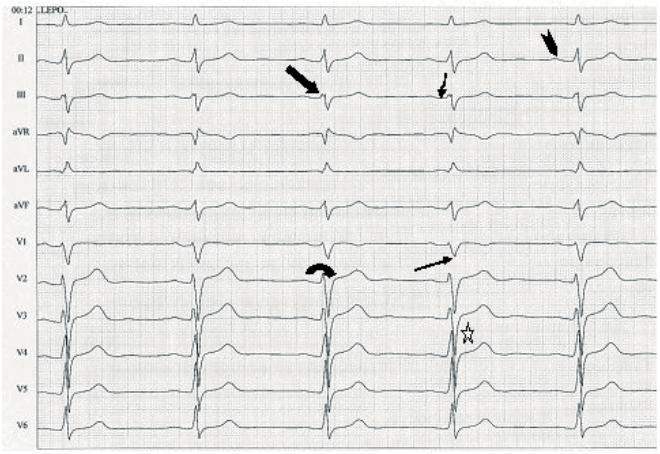
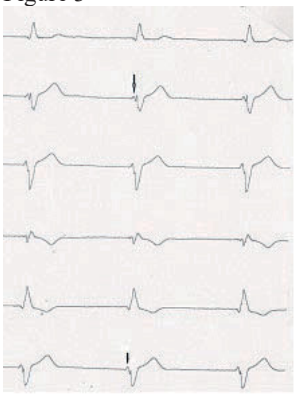


Figure 3



PUBLICATION

II

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RESEARCH ARTICLE

Heterozygous junctophilin-2 (*JPH2*) p.(Thr161Lys) is a monogenic cause for HCM with heart failure

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Abstract

During the last two decades, mutations in sarcomere genes have found to comprise the most common cause for hypertrophic cardiomyopathy (HCM), but still significant number of patients with dominant HCM in the family are left without molecular genetic diagnosis. Next generation sequencing (NGS) does not only enable evaluation of established HCM genes but also candidate genes for cardiomyopathy are frequently tested which may lead to a situation where conclusive interpretation of the variant requires extensive family studies. We aimed to characterize the phenotype related to a variant in the junctophilin-2 *(JPH2)* gene, which is less known non-sarcomeric candidate gene. In addition, we did extensive review of the literature and databases about *JPH2* variation in association with cardiac disease. We characterize nine Finnish index patients with HCM and heterozygous for *JPH2* c.482C>A, p.(Thr161Lys) variant were included and segregation studies were performed. We identified 20 individuals affected with HCM with or without systolic heart failure and conduction abnormalities in the nine Finnish families with *JPH2* p.(Thr161Lys) variant. We found 26 heterozygotes with the variant and penetrance was 71% by age 60 and 100% by age 80. Co-segregation of the variant with HCM phenotype was observed in six families. Main clinical features were left ventricular hypertrophy, arrhythmia vulnerability and conduction abnormalities including third degree AV-block. In some patients end-stage severe left ventricular heart failure with normal or mildly enlarged diastolic dimensions was detected. In conclusion, we propose that the heterozygous *JPH2* p.(Thr161Lys) variant is a new Finnish mutation causing atypical HCM.

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Introduction

Hypertrophic cardiomyopathy (HCM) is considered the most common form of inherited cardiomyopathies estimated to affect one in 500 in general population [1]. Diagnosis of HCM is made by two-dimensional echocardiography showing hypertrophied, non-dilated left ventricle (LV) in the absence of other cardiac or systemic causes of hypertrophy such as aortic valve stenosis or hypertension [2]. HCM may manifest at any age but typically in the adulthood. The clinical course of the disease varies significantly from person to person. Some patients remain asymptomatic through their life whereas others suffer from arrhythmias or embolic stroke, develop severe heart failure or experience sudden cardiac death (SCD) even at early age [3–5].

Hereditary HCM is a dominant disorder, commonly associated with mutations in sarcomere genes. Phenocopies of HCM include Anderson-Fabry disease (galactosidase alpha, *GLA*), Danon disease (lysosomal-associated membrane protein 2, *LAMP2)*, *PRKAG2* related glycogen storage disease (protein kinase AMP-activated non-catalytic subunit gamma 2, *PRKAG*2), cardiac amyloidosis, neuromuscular diseases and malformation syndromes (Noonan spectrum syndromes).[6] Genetic diagnostics has proven as an effective strategy to differentiate between potential underlying causes and to rule out phenocopies. Accurate molecular genetic diagnosis helps to detect the genetic cause of those phenocopies that might require special therapy (e.g. enzyme replacement therapy) [7–9].

The most established genes associating with HCM are myosin binding protein C (*MYBPC3)*, myosin heavy chain 7 *(MYH7)*, troponin I3, cardiac type *(TNNI3)*, troponin T2, *cardiac type (TNNT2)*, tropomyosin 1 *(TPM1)*, myosin light chain 2 *(MYL2)*, myosin light chain 3 *(MYL3) and* actin, alpha, cardiac muscle 1 *(ACTC1) [10]*. The role of candidate genes such as troponin C1, slow skeletal and cardiac type (*TNNC1)* [11] and actinin alpha 2 *(ACTN2)* [12] has remained obscure. Diagnostic yield of molecular genetic testing in daily practice is 25–40% in HCM [10]. This demonstrates the challenge of differentiating genetic disease from acquired conditions or other systemic diseases and indicates that there are novel disease genes and more complex genetic variations left to be discovered. Although it has been difficult to demonstrate phenotypic correlations between different genes and different mutations, there is a significantly worse outcome in patients tested positive vs. negative for mutations in sarcomere genes [13]. A large proportion of the mutations are unique for a family and have not been reported before. In certain populations, however, founder mutations comprise a large part of detected mutations. To date HCM founder mutations have been identified in *MYBPC3*, *MYH7* and *TPM1* genes, and reported in the Netherlands [14], Spain [15], South Africa [16], Finland [17], Italy [18], Japan [19], South Asia [20] and in the Amish population [21].

Junctophilin-2 gene (*JPH2*) is the major structural protein in cardiomyocytes for coupling of transverse (T) tubule-associated L-type Ca^{2+} channels and type-2 ryanodine receptors on the sarcoplasmic reticulum within junctional membrane complexes (JMC) [22, 23]. Signaling between these two Ca^{2+} channels is required for normal cardiac contractility. Disruption of the JMC is a common finding in failing hearts. Downregulation of *JPH2* gene has been associated with heart failure and mutations in this gene have been suggested to associate with HCM. *JPH2* was initially published as candidate gene for HCM in 2007 when Matsushita *et al.* found p.(Gly505Ser) in four probands among 148 Japanese HCM patients [24] but this variant was later found to be a common polymorphism present in up 4–6% in Asian/African populations. Role of the JPH2 in cardiomyopathies has been obscure as only one rare variant segregating with any type of cardiomyopathy has been published [25].

In this study, we characterize the cardiac phenotype related to *JPH2* c.482C>A, p.(Thr161Lys) variant in nine Finnish index patients and their family members. We also review

evidence gathered from the literature and variant databases that supports or is against the pathogenicity of the mutations in *JPH2* to highlight challenges we have when rare gene variants from candidate genes are detected.

Subjects and methods

The methods for this trial is available as supporting information; see S1 Protocol http://dx.doi.org/10.17504/protocols.io.[PROTOCOL]

Index patients with the *JPH2* variant c.482C>A, p.(Thr161Lys) and their relatives from four Finnish hospitals were included. A written informed consent was obtained from all participants. This study has been approved by the Ethical Review Committee of the Department of Medicine, University of Helsinki (Dnro 307/13/03/01/11) and by the Ethical Review Committee of the Department of Medicine, University of Tampere (R08070) and conforms to the ethical principles outlined in the Declaration of Helsinki.

HCM was clinically diagnosed according to ESC Guidelines [2]. Hypertrophic cardiomyopathy (HCM) is defined by the presence of increased LV wall thickness that is not solely explained by abnormal loading conditions. In adults, the diagnosis of HCM requires LV wall thickness $\geq$15 mm as measured by any imaging technique. Correspondingly, the clinical diagnosis of HCM in first-degree relatives of patients with LVH $\geq$15 mm is based on the presence of otherwise unexplained increased LV wall thickness $\geq$13 mm. In children, the diagnosis of HCM requires LV wall thickness more than two standard deviations greater than the predicted mean.

Family history was obtained and pedigrees were drawn. The adult participants were assessed clinically at Heart and Lung Center, Helsinki University Hospital, at the Heart Hospital, Tampere University Hospital or at the Seinäjoki Central Hospital by physical examination, resting 12-lead ECG, appropriate laboratory tests and transthoracic echocardiography (TTE). Cardiac MRI was performed in some cases especially in patients with borderline diagnostic findings at echocardiography. All participants are of Finnish ethnicity.

Molecular genetic studies

Genetic testing was carried out from genomic DNA using the OS-Seq™ (oligonucleotide-selective sequencing) next-generation sequencing method [26, 27]. The genetic evaluation of the index patients was performed using the Blueprint Genetics Core Cardiomyopathy or Pan Cardiomyopathy Panels covering 69 and 103 genes, respectively, associated with cardiomyopathies and their genetic phenocopies (Supplementary file 1). The presence of the variant in probands' relatives was studied by bi-directional Sanger sequencing.

Results

Genetic studies

A heterozygous *JPH2* c.482C>A, p.(Thr161Lys) (NM_020433.4) variant was observed in nine unrelated Finnish probands with cardiomyopathy. Altogether the p.(Thr161Lys) was detected in 20 affected individuals. The variant co-segregated with HCM in six families (Families 2–4, 6–8, Fig 1) and in three families the mutation was found only in the probands (Family 9), in probands and young family member without HCM (Family 1) or in probands and in another family member without HCM (Family 5). Systolic heart failure or conduction abnormalities were observed in every family. These features were present in 12/20 (60%) of the affected patients including ten heterozygous affected individuals and two obligate carriers. The p.(Thr161Lys) was absent in three family members without LV hypertrophy who were over 20

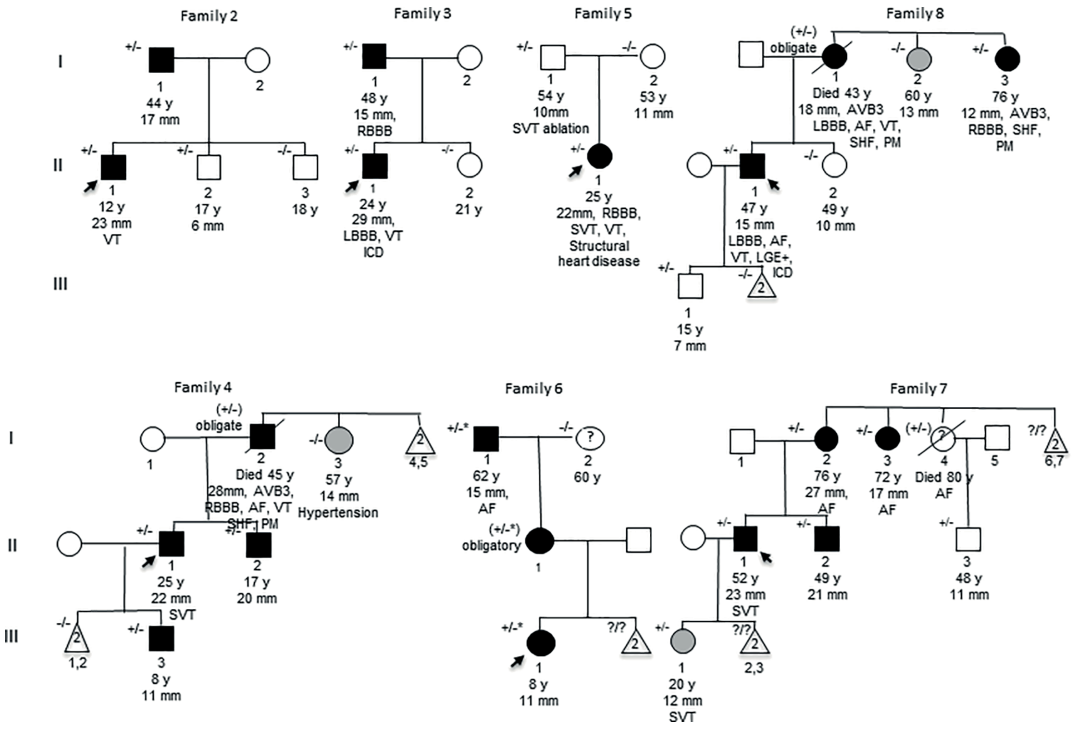

Fig 1. Pedigrees of seven families affected with the *JPH2* c.482C>A, p.(Thr161Lys), (rs587782951, NM_020433.4) variant. Pedigrees of three families affected with the *JPH2* c.482C>A, p.(Thr161Lys), (rs587782951, NM_020433.4) variant. Circles represent women, squares men and triangles gender blinded. Black-filled symbols represent individuals who fulfill ESC 2008 diagnostic criteria for HCM with [2]. We also considered as affected with HCM one family member (Family 8:I.3) who had imminent cardiomyopathy with borderline LVH (12 mm), 3-degree AV block, RBBB and severe systolic heart failure. Genotypes: +/- heterozygous for the *JPH2* p.(Thr161Lys), -/- wild type allele, * *MYBPC3* p.(Gln1061*). Age of the family members at last follow-up, maximum LV wall thickness and some other key signs of clinical disease are listed below the symbols. Arrows indicate index patients. Abbreviations listed in Table 1.

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years of age (3.I.2, 5.I.2, 8.II.2). It was also absent in asymptomatic 60-year old female (Family 6.I.2) who was not evaluated clinically and two individuals with LVH likely explained by severe hypertension (Family 4:I.3 and Family 8:I.2). Pedigrees of the families 2–8 are presented in Fig 1. This heterozygous missense variant has not been observed in the Exome Aggregation Consortium (ExAC) data set, comprised in total of over 60,000 unrelated individuals or in the Genome Aggregation Database (gnomAD; total of 126,216 exomes and 15,137 genomes). *In silico* bioinformatic tools Polyphen, SIFT and Mutation Taster predict it to be deleterious. Threonine is conserved amino acid at this position among mammals.

The main clinical characteristics of the probands and family members are shown in Table 1. These include left ventricular hypertrophy (LVH), and in some cases end-stage severe left ventricular (LV) failure with normal or mildly dilated LV. Average at diagnosis was 26.9 ±20.6 years in the nine probands and their maximum LV wall thickness was 20.4±5.2 mm at age of 34.1. Atrial or ventricular arrhythmias considered abnormal were observed in 13/20 (65%) of the heterozygotes. Pacemaker (PM) or ICD was implanted in 6/20 (30%) and reduced

Table 1. Clinical characteristics of the probands and their family members.

Family	Age (M/F)	Genotype	Conduction defect	Arrhythmias	PM, ICD	LV-WT	LVEDD (mm)/ EF (%)	proBNP (ng/l)	Age at dg	Phenotype	Other
Family 1											
I.1	52M	+/-	no	AF	ICD	20	46/56%	400	47	HCM	
Family 2											
I.1	44M	+/-	no	no	no	16	46/72%	76	44	HCM	
II.1	12M	+/-	no	VT	no	23	48/77%	315	12	HCM	
II.2	16M	+/-	pRBBB	no	no	6	51/63%	NA	-	normal	
Family 3											
I.1	48M	+/-	RBBB	no	no	15	49/72%	44	41	HCM	
II.1	24M	+/-	LBBB	VT	ICD	29	44/75%	145	13	HOCM	Pk-grad 81mmHg
Family 4											
I.2	45M	n.a.	AVB3, LAHB, RBBB	AF, VT	PM	28	55/20%	8425	17	HOCM, SHF	Pk-grad 50mmHg Died 45 y
I.3	57F	-/-	no	no	no	14	43/60%	33	-	LVH/ Hypertens	BP 160/110 mmHg
II.1	25M	+/-	no	SVT	no	22	45/67%	104	9	HCM	
II.2	17M	+/-	LAHB	no	no	20	49/55%	NA	1	HCM	
III.3	8M	+/-	no	no	no	11	35/>50%	NA	1	HCM	
Family 5											
I.1	54M	+/-	no	SVT	no	10	50/70%	NA	-	normal	SVT ablation
I.2	53F	-/-	no	no	no	11	44/83%	164	-	normal	
II.1	25F	+/-	RBBB, LAHB	VES, VT, SVT	no	22	43/60%	5700	12	HCM, SHF	Multiple VSDs, PDA operated aged 2 years. During pregnancy LVEF 45%
Family 6											
I.1	62M	+/-,*	LAHB	AF	no	15	40/52%	998	61	HCM	
I.2	60F	-/-	NA	NA	NA	NA	NA	NA	-	NA	
II.1	F	(+/-,*)	NA	NA	NA	NA	NA	NA	-	HCM	
III.1	8F	+/-,*	NA	no	no	11	33/>60%	3928	7	HCM	
Family 7											
I.2	76F	+/-	LAHB	AF	no	27	49/56%	NA	?	HCM	
I.3	72F	+/-	no	AF	no	17	45/60%	NA	?	HCM	
I.4	80F	(+/-)	no	AF	no	NA	NA	NA	?	?	Died age 80 y
II.1	52M	+/-	AVB1	VES, SVT	no	23	49/63%	NA	43	HCM	
II.2	49M	+/-	AVB1	no	no	21	60/50%	NA	?	HCM	
II.3	48M	+/-	no	no	no	11	NA	NA	-	normal	
III.1	20F	+/-	no	SVT	no	12	44/66%	NA	19	normal	
Family 8											
I.1	43F	(+/-)	AVB3, LBBB	AF, VT	PM	18	70/30%	6637	25	HCM, SHF	Died aged 43. HF. WT at autopsy 15 mm.
I.2	60F	-/-	no	no	no	13	40/71%	160	-	LVH/ Hypertens	
I.3	76F	+/-	AVB3, LAHB, RBBB	no	PM	12	46/40%	5267	67	HCM	Mixed cardiomyopathy
II.1	47M	+/-	AVB1, LBBB	AF, VT	ICD	15	51/37%	4426	36	HCM, SHF	
II.2	49F	-/-	no	no	no	10	49/66%	31	-	normal	
III.1	15M	+/-	no	no	no	7	46/66%	NA	-	normal	
Family 9											

(*Continued*)

Table 1. (Continued)

Family	Age (M/F)	Genotype	Conduction defect	Arrhythmias	PM, ICD	LV-WT	LVEDD (mm)/ EF (%)	proBNP (ng/l)	Age at dg	Phenotype	Other
I.1	63F	+/-	no	AF	PM	19	45/60%	4082	63	HCM	

Index patients are marked in bold. Symbols and abbreviations: Age (M/F)–age and gender (M, male; F, female); Genotype–+/- is heterozygous and (+/-) obligatory heterozygous for p.(Thr161Lys) in *JPH2* and -/- is wild type, * heterozygous for *MYBPC3* Gln1061*; R/LBBB–presence of right/left bundle branch block; LAHB–left anterior hemiblock; AVB1-3 –atrioventricular block types 1–3; Arrhythmias–AF for atrial fibrillation/flutter, SVT for supraventricular tachycardia (>10 episodes short episodes per day or SVT requiring cardioversion), VT for ventricular tachycardia ≥ 3 beats with frequency >100/min, VES for ventricular extrasystoles >1000 per day, SVES for supraventricular extrasystoles >5000 per day; PM, ICD, CRT-P/D–pacemaker, implantable cardioverter-defibrillator, cardiac resynchronization therapy device; LV-WT-maximal left ventricular wall thickness measured by echocardiography or cardiac MRI; LVEDD & EF–left ventricular end-diastolic diameter (mm) and ejection fraction (%); Age at dg-age at diagnosis of cardiomyopathy; Phenotype–phenotype at diagnosis, HCM–hypertrophic cardiomyopathy, LVH–left ventricular hypertrophy; SHF–systolic hear failure; Other–other significant clinical features

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LVEF (<47%) or elevated proBNP concentration (>300 ng/l) was observed in 9/20 (45%) of the patients. Penetrance of HCM was 48%, 71% and 100% by age of 40, 60 and 80, respectively.

Family 1. Proband of this family was diagnosed HCM at age 47. His genotype positive (not drawn on pedigree) son was phenotype negative with maximal wall thickness of 10 mm at age 22, which is in line with age-dependent penetrance of cardiomyopathies.

Family 2. Proband (II.1) of family 2 developed HCM at age of 12 and has significant burden of arrhythmias. Subsequently, his genotype positive father was found to have also HCM and arrhythmias. Proband's brother was genotype positive yet phenotype negative at age of 16.

Family 3. Proband (II.1) developed HCM at age of 13 and LV wall thickness increased up to 29 mm by age 24. He had also LVOT obstruction with 81mmHg systolic gradient. The patient was prescribed beta blockers and disopyramide, and LVOT gradient decreased to 20mmHg. His symptoms, chest pain and shortness of breath presenting during light walking, were suppressed by the medication. His genotype positive father has also HCM but no arrhythmias. The proband has ventricular arrhythmias. Both affected individuals in this family have preserved systolic function.

Family 4. Proband (II.1) is a male with clinical diagnosis of HCM at age 9. He has had recurrent episodes of sustained atrial tachyarrhythmias. On echocardiography, maximal LV wall thickness was 22mm. Proband's 17-year-old genotype positive brother (II.2.) had maximal LV thickness of 20mm. He and proband's son were diagnosed at very early age. Proband's father (I.2) was diagnosed with HCM aged 17 years as his ECG showed inferolateral T-inversions like the ECGs of his both sons and echocardiography revealed septal thickness of 17mm. Later he developed variable 2–3 degree AV block and received pacemaker. He had also LVOT obstruction with 50mmHg systolic gradient. The patient was prescribed beta blockers and disopyramide and LVOT gradient decreased to 21 mmHg. At age 26, his LVEF was 70%. During the follow-up, maximal septal thickness increased to 28mm and posterior wall to 20mm. The patient had paroxysmal atrial flutter and later chronic atrial fibrillation (AF). By the age of 40 years, the patient developed NYHA 3 dyspnea and his LVEDD was 58mm, LVEF 40% and septal thickness 18mm. He had right heart failure and ascites, proBNP was 8425ng/l. Aged 45 years LVEDD was 55mm and LVEF 20–25%. The patient was listed for heart transplantation but died aged 45 years due to heart failure and sepsis. He deceased before the NGS technology became to clinical use and his genotype remains unknown. His disease fits well with the phenotype described here in genotype positive individuals. Genotype of proband's mother is also unknown, thus we were not able to confirm that the *JPH2* variant was inherited from the affected parent. Index patient's father's mother had also HCM and died aged 42 years while

sleeping and her father (proband's father's maternal grandfather) died aged 45 years due to an assumed cardiogenic reason (not drawn in pedigree). As a whole probands and two adult family members heterozygous for the variant fulfilled the criteria for HCM, whereas one individual (I.3), who is negative for the variant has LVH likely explained by severe hypertension (blood pressure 160/110mmHg) and obesity (BMI 32 kg/m^2).

Family 5. Proband (II.1) had multiple VSDs, mild mitral stenosis and patent ductus arteriosus necessitating surgery aged 2 years. HCM was diagnosed when she was 12. ECG showed RBBB likely due to previous cardiac surgery, abnormal amount of ventricular and atrial premature complexes and she also suffers from SVTs and VTs. She had high levels of pro-BNP (5700 ng/L) although having normal systolic function at echocardiography. Her genotype positive father has no morphological HCM phenotype but he underwent ablation for SVT with good response.

Family 6. Proband (III.1) is a girl who developed HCM by the age of 7 years. She has also a well-established pathogenic nonsense mutation in *MYBPC3* (c.3181C>T, p.(Gln1061*)). Her pro-BNP levels are high (3928 ng/L). She has inherited both of the variants from his mildly affected grandfather through her mother who has HCM and is obligate carrier of both variants.

Family 7. In this family, the index patient (II.1), his brother (II.2), mother (I.2) and mother's sister (I.3) were heterozygous for the *JPH2* p.(Thr161Lys) variant. They all fulfilled the imaging criteria of HCM with maximal LV wall thicknesses of 17-27mm. None of these patients had heart failure. Mother's other sister (I.4) who was an obligatory carrier died at age 80 due to mesenterial thrombosis. Her son is heterozygous for the *JPH2* variant. Twenty-year-old daughter (III.1) of the index has borderline phenotype (septal thickness 12 mm) and her ECG showed abnormal Q-waves at I, II, aVF, V5-6 leads and episodes of ectopic atrial rhythm.

Family 8. The index patient (II.1) is heterozygous for the *JPH2* p.(Thr161Lys) variant. He was a previously sportive male who presented with decreased exercise tolerance at the age of 36 years. ECG showed LVH and on echocardiography LV was 51/36mm, EF 55% and septum 14mm at maximum. Five years later septum measured 15mm fulfilling the HCM criteria. At age of 45, ECG showed sinus rhythm and trifascicular block (LBBB + type 1 AV block). TnT concentration was constantly increased up to 48 ng/l (normal range < 15ng/l) and proBNP concentration has increased up to 4426 ng/l. He has had paroxysmal atrial fibrillation (AF)/flutter and monomorphic ventricular tachycardia (VT). Coronary angiography was normal. Cardiac MRI demonstrated extensive late gadolinium enhancement (LGE) especially at septal and anterior regions. FDG-PET did not detect signs of inflammation. Endomyocardial biopsies from left and right ventricles remained non-diagnostic and showed mainly fibrosis. Electrophysiologic study demonstrated VT. On echocardiography, LV was 51mm, septum 14mm, PW 12mm and EF 37%. Hemodynamics were restrictive. The patient has received an ICD. His mother (I.1) was an obligatory carrier of the variant. She presented with loss of weight, fainting and palpitations aged 28 years and ECG showed LBBB. In cardiac catheterization, LVEDP was elevated and left ventricular cineangiography (LV-cine) showed stiff septum, compatible with HCM. Eight years later she was examined due to tachycardias and collapse episodes. Maximum LV wall thickness was 18 mm. In LV-cine, EF was 32%, stroke volume 38ml. The patient received pacemaker due to episodes of syncope and tachyarrhythmia. At age 40 she had atrial fibrillation and 3-degree AV block and at age 42 her LVEDD was 70 mm and EF 30%. She was treated with digoxin, furosemide, amiodarone, atenolol, captopril and warfarin. Cardiac CT showed dilated left atrium and ventricle but normal pericardium. Before death at the age of 43, she had heart failure (HF) and repeated VT's. Autopsy showed fibrotic changes especially at endocardium of posterior and septal walls. There was very thin and fibrotic area with a size of 3x4 cm in the anterior LV wall. Maximum wall thickness in autopsy

was 15mm. Clinical diagnoses comprised cardiomyopathy, endocardial and myocardial fibrosis. No material for genotype analysis was available. Mother's sister (I.3) is also heterozygous for the p.(Thr161Lys) variant and was considered affected as she has imminent cardiomyopathy with borderline LVH (12 mm), AF, 3-degree AV block, RBBB and severe systolic heart failure not explained by other causes. She has received a PM. Proband's non-carrier aunt (I.2) had mild LVH likely due to hypertension. Proband's non-carrier brother (II.2) had normal findings in cardiac evaluations as well as proband's genotype positive son (III-1) who was 15 years old at last follow-up.

Discussion

We have identified the *JPH2* p.(Thr161Lys) variant in nine Finnish index patients with HCM and have shown co-segregation of the variant with cardiomyopathy in six of these families. In the other three, only the index person presented HCM phenotype due to the size of the family or the young age of the variant carriers. This is the first *JPH2* variant shown to be causative for HCM.

For almost twenty years data has accumulated on the role *JPH2* in cardiac physiology. However, little is known about the significance *JPH2* as a causative gene for cardiomyopathy. *JPH2* is a cardiac specific member of the junctophilins and it has emerged as a potentially important regulator of excitation-contraction coupling in cardiomyocytes. Several studies have highlighted the importance of *JPH2* for normal cardiac physiology [23, 28]. Mice with acute conditional cardiac specific knockdown of *JPH2* have a high incidence of mortality with a rapid development of systolic heart failure [29]. These mice demonstrated grossly enlarged hearts with dilated ventricles and reduced systolic function on echocardiogram. In another mouse model study, heterozygous *JPH2* p.(Glu169Lys) mice had a higher incidence of pacing-induced AF secondary to abnormal spontaneous $Ca^{[2+]}$ waves and increased spark frequency [30].

Cardiac phenotype related to this variant differs somewhat from typical HCM. Two Finnish founder mutations for HCM have previously been published one in myosin binding protein C (*MBPC3*) gene and the other in alpha tropomyocin (*TPM1*) gene [17, 31]. The *JPH2* p.(Thr161Lys) variant presented here differs from the other two in clinical presentations. The *JPH2* variant associates with earlier disease onset (27 years vs. 52 and 49 years) than the *MYBPC3* p.(Gln1061*) or *TPM1* p.(Asp175Asn), respectively, although no patients under age of 16 were included in the previous study [17]. Systolic heart failure was observed in 5 (25%) and LV dysfunction was present in half of the affected patients with *JPH2* variant when defined by EF<47% or elevated proBNP. Of the probands with *MYBPC3* p.(Gln1061*), none has had congestive heart failure. However, dyspnea was present in 31%. Also none of the index patients with the *TPM1* p.(Asp175Asn) mutation had significant systolic dysfunction (EF<46%) and only one (5%) had a history of systolic heart failure. Severe conduction defects defined by 3-degree AV-block or R/LBBB was observed in nine (45%) and AF in nine (45%) of the affected individuals or obligate carriers with *JPH2 variant*, but no SCDs were detected in our families. Of the individuals with *MYBPC3* p.(Gln1061*) variant, 26% presented syncope/pre-syncope, 23% had either chronic or paroxysmal atrial fibrillation, 9% have had sustained VT or ventricular fibrillation and 26% had family history of SCD. Three out of 34 (9%) HCM patients carrying the *TPM1* p.(Asp175Asn) presented with a documented SCD at young or middle age [31]. Additionally, life-threatening arrhythmias were induced with programmed ventricular stimulation (PVS) in one third of patients carrying this mutation. Of the probands with *TPM1* or *MYBPC3* founder mutation, ICD was implanted in 2 (10%) and 7 (20%) patients respectively and no significant conduction problems necessitating pacemaker implantation

where described in the index publications. These observations highlight the significant differences in clinical presentation of the patients with the previously characterized founder mutations compared to proposed *JPH2* mutation described in this study. However, the degree of LV hypertrophy may be similar in patients with these founder mutations (22±5 mm, 18±6 mm and 20±5 mm with variant in *MYPBC3*, *TPM1* or *JPH2*, respectively), although the JPH2 variants carriers were younger at a time of evaluation compared to carriers of other founder mutations. It should be still keep in mind that significant variation in clinical presentation within and between the families with the same mutation exists in all types of HCM.

There are altogether 19 *JPH2* variants associating with dilated or hypertrophic cardiomyopathy listed in the HGMD (Qiagen) and ClinVar databases (July 8, 2017) presented in Table 2. Seventeen of them are missense variants. Before this study, no convincing evidence of segregation within large pedigrees except for the p.(Glu85Lys) [25] and no *de novo JPH2* mutations have been reported in patients with HCM. In 2007, Landstrom *et al*. found two rare missense

Table 2. All *JPH2* variants described in literature and rare *JPH2* variants submitted to ClinVar Database with phenotype information.

JPH2 Variant (NM_020433.4)	Pheno	gnomAD	PP	MT	Index (n)	Family (n)	Co-seg	Class	Other	Reference
c.253G>A, Glu85Lys	DCM	0/0	D	D	1	9	Yes	LP	Penetrance 78%	(Sabater-Molina et al. 2016)
c.301A>C, Ser101Arg	HCM	0/0	PD	B	1	0	No	VUS		(Landstrom et al. 2007)
c.421T>C, Tyr141His	HCM	0/0	D	D	1	0	No	VUS		(Landstrom et al. 2007)
c.494C>T, Ser165Phe	HCM	0/0	D	D	1	0	No	VUS		(Landstrom et al. 2007)
c.502A>C, Ser168Arg	HCM	0/0	D	D	1	0	Np	VUS		ClinVar 222654
c.505G>A, Glu169Lys	HCM	0/0	D	D	1	1	?	VUS		(Beavers et al. 2013)
c.559G>A, Gly187Ser	HCM	1/0	PD	B	1	0	No	VUS		ClinVar 222655
c.565G>A, Ala189Thr	SUD	94/0	B	B	1	0	No	LB		(Narula et al. 2015)
c.692G>A, Arg231Gln	Sdr	39/0	D	B	1	0	No	LB		(Farwell et al. 2015)
c.723C>G, Ser241Arg	DCM	0/0	D	D	1	0	No	VUS		ClinVar 155801
c.1013A>G, Glu338Gly	SCD	0/0	D	D	1	0	No	VUS	Enlarged RV	(Neubauer et al. 2016)
c.1213G>T, Ala405Ser	HCM	3/0	D	D	1	0	No	VUS	Ala405Thr 18 het gnomAD, *De novo*?	(Beavers et al. 2013)
c.1227C>G, Asn409Lys	DCM	0/0	PD	D	1	0	No	VUS		ClinVar 222656
c.1282C>T, Gln428*	DCM	13/0	-	-	1	0	No	VUS		ClinVar 222657
c.1513G>A, Gly505Ser	HCM	589/7	B	B	4	0	No	Benign		(Matsushita et al. 2007, Manrai et al. 2016)
c.1540G>A, Gly514Ser	LVNC	1/0*	D	D	1	0	No	VUS		ClinVar 222658
c.1564C>T, Arg522Trp	DCM	0/0	D	D	1	0	No	VUS		ClinVar 222659
c.1750C>A, Gln584Lys	LVNC	1/0*	B	B	1	0	No	VUS		ClinVar 180592
c.2011-1G>T	HCM	0/0	-	-	1	0	No	VUS		(Xu et al. 2015)

Abbreviations: Pheno, phenotype; gnomAD, number of heterozygotes/homozygotes in the gnomad reference population consisting 120,000 individuals; PP, PolyPhen; MT, MutationTaster; D, probably damaging in PolyPhen and disease causing in MutationTaster; PD, possibly damaging; B, benign in PolyPhen and polymorphism in MutationTaster; Index, number of index patients, Family, number of affected family members with the same variant as index patient; Co-seg, co-segregation; Class, classification of the variant by the authors relying on ACMG recommendation; LP, likely pathogenic; VUS, variant of uncertain significance; LB, likely benign; SUD, Sudden unexplained death; Sdr, complex syndrome

*, Non-pass (low quality) variant in gnomAD; LVNC, left ventricular non-compaction cardiomyopathy. Other abbreviations as in Table 1.

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and one frameshift variant in *JPH2* in three probands with HCM but their families were not studied or genotyped [28]. In 2016, Sabater-Molina showed segregation of the *JPH2* p.(Glu85Lys) with dilated cardiomyopathy with or without left ventricular non-compaction cardiomyopathy (LVNC) features in a large family [25]. Furthermore, Quick *et al* have recently published *JPH2* p.(Ala405Ser) variant in a single patient with basal septal hypertrophy and diastolic dysfunction [32]. Most of the previously published variants are absent or rare in ExAC or gnomAD reference populations and thus they have potential to be disease causing. Truncating *JPH2* variants are relatively rare in ExAC reference population (carrier frequency 1 per 6,030 individuals) but due to small size of the gene, the pLI value is 0.01 which does not suggest that loss of function alterations would be poorly tolerated. The clinical data on HCM related to the previously published *JPH2* missense variants is limited, mostly clinical HCM appears to be diagnosed after teenage but at least the patient with p.(Glu169Lys) exhibited HCM already at the age of 5 months [30], similarly as two patients in this study.

We identified one family (Family 6) where all three affected individuals carry the well-established pathogenic *MYBPC3* variant, p.(Gln1061*), in addition to *JPH2* p.(Thr161Lys) variant. Their phenotypic presentation did not differ significantly from the other families which are surprising as patients with homozygosity/compound heterozygosity or digenic pathogenic variants in sarcomere genes generally present at very early age [33–36]. In literature at least nine patients have been described who had homozygous or confirmed compound heterozygous disease causing variant in *MYBPC3* and at least the other variant is not truncating. In these patients the mean age at onset was 4.1 years. Three of these cases presented at neonatal phase, of which all died before age of two months [34, 35]. All patients with homozygous or compound heterozygous truncating pathogenic mutations in *MYBPC3* reported so far (n = 21) were diagnosed with severe cardiomyopathy and/or died within the first few months of life [35]. At the moment, it is unclear whether compound mutation present in sarcomere and non-sarcomere gene simultaneously have additive detrimental effect on disease onset and/or progression.

In conclusion, the *JPH2* p.(Thr161Lys) is classified as pathogenic based on ACMG classification scheme [37], as the variant resides in a conserved position, is predicted to be deleterious by *in silico* prediction tools, is absent in control populations and co-segregates with dominant HCM in six families. These observations strengthen the role of non-sarcomeric *JPH2* as a causative gene for HCM with or without systolic heart failure and conduction abnormalities. Further research is warranted to evaluate *JPH2* variants and other genes related to Ca^{2+} -handling in the cardiomyocyte to further shed light on the genetic background of HCM. Variant interpretation and correlation to phenotype is still challenging as earlier studies form major pitfalls by false classifications related to small reference populations, co-incidental segregations and evaluation of only a small subset of the potentially meaningful genes behind a patient's phenotype. Large-scale genetic research will eventually bring more consistency to the evaluation of families with inherited cardiac diseases. Unfortunately, most of the submissions of the *JPH2* variants in ClinVar reviewed in this work did not include phenotype data and thus provide limited information for the clinical society. Variant sharing with relevant information of the phenotype in mutation databases is critically important to develop the field further on.

Supporting information

S1 Protocol. Genetic panels used for the family probands. Probands of each family were evaluated by either Blueprint Genetics Core Cardiomyopathy Panel (Family 1, 2, 3, 6, 7) or Pan Cardiomyopathy Panel (Family 4, 5, 8, 9). These next generation sequencing (NGS) panels are targeted into all protein coding exons and exon-intron boundaries of all target genes (listed

below). These panels are validated to detect single nucleotide substitutions and small insertions, deletions and indels up to 46 bp. The panels have ISO 15189 and CAP accreditation. (DOCX)

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Supplemental file 1

Probands of each family were evaluated by either Blueprint Genetics Core Cardiomyopathy Panel (Family 1, 2, 3, 6, 7) or Pan Cardiomyopathy Panel (Family 4, 5, 8, 9). These next generation sequencing (NGS) panels are targeted into all protein coding exons and exon-intron boundaries of all target genes (listed below). These panels are validated to detect single nucleotide substitutions and small insertions, deletions and indels up to 46 bp. The panels have ISO 15189 and CAP accreditation.

Core Cardiomyopathy Panel (72 genes): ABCC9, ACTC1, ACTN2, ANKRD1, BAG3, CALR3, CAV3, CRYAB, CSRP3, CTF1, CTNNA3, DES, DMD, DNAJC19, DNM1L, DSC2, DSG2, DSP, DTNA, EMD, EYA4, FHL1, FHL2, FKTN, FXN, GATAD1, GLA, HFE, ILK, JPH2, JUP, LAMA4, LAMP2, LDB3, LMNA, MIB1, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOM1, MYOZ2, MYPN, NEBL, NEXN, PDLIM3, PKP2, PLN, PRKAG2, PSEN1, PSEN2, RBM20, RYR2, SCN5A, SDHA, SGCD, TAZ, TCAP, TGFB3, TMEM43, TMPO, TNNC1, TNNI3, TNNT2, TPM1, TRIM63, TTN, TTR, TXNRD2, VCL.

Pan Cardiomyopathy Panel (103 genes): ABCC9, ACADVL, ACTC1, ACTN2, AGL, ANKRD1, ATP5E, BAG3, BRAF, CALR3, CASQ2, CAV3, CBL, COA5, CRYAB, CSRP3, CTF1, CTNNA3, DES, DMD, DMPK, DNAJC19, DNM1L, DOLK, DSC2, DSG2, DSP, DTNA, EMD, EYA4, FHL1, FHL2, FKTN, FOXRED1, FXN, GAA, GATAD1, GLA, GLB1, GUSB, HFE, HRAS, ILK, JPH2, JUP, KRAS, LAMA4, LAMP2, LDB3, LMNA, MAP2K1, MAP2K2, MRPL3, MIB1, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOM1, MYOZ2, MYPN, NEBL, NEXN, NRAS, PDLIM3, PKP2, PLN, PRKAG2, PSEN1, PSEN2, PTPN11, RAF1, RBM20, RYR2, SCN5A, SCO2, SDHA, SGCD, SHOC2, SLC25A3, SOS1, SPRED1, SYNE1, SYNE2, TAZ, TCAP, TGFB3, TMEM43, TMEM70, TMPO, TNNC1, TNNI3, TNNT2, TPM1, TRIM63, TSFM, TTN, TTR, TXNRD2, VCL, XK

PUBLICATION

III

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RESEARCH ARTICLE

Antiarrhythmic Effects of Dantrolene in Patients with Catecholaminergic Polymorphic Ventricular Tachycardia and Replication of the Responses Using iPSC Models

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Abstract

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a highly malignant inherited arrhythmogenic disorder. Type 1 CPVT (CPVT1) is caused by cardiac ryanodine receptor (*RyR2*) gene mutations resulting in abnormal calcium release from sarcoplasmic reticulum. Dantrolene, an inhibitor of sarcoplasmic Ca^{2+} release, has been shown to rescue this abnormal Ca^{2+} release in vitro. We assessed the antiarrhythmic efficacy of dantrolene in six patients carrying various *RyR2* mutations causing CPVT. The patients underwent exercise stress test before and after dantrolene infusion. Dantrolene reduced the number of premature ventricular complexes (PVCs) on average by 74% (range 33-97) in four patients with N-terminal or central mutations in the cytosolic region of the *RyR2* protein, while dantrolene had no effect in two patients with mutations in or near the transmembrane domain. Induced pluripotent stem cells (iPSCs) were generated from all the patients and differentiated into spontaneously beating cardiomyocytes (CMs). The antiarrhythmic effect of dantrolene was studied in CMs after adrenaline stimulation by Ca^{2+} imaging. In iPSC derived CMs with *RyR2* mutations in the N-terminal or central region, dantrolene suppressed the Ca^{2+} cycling abnormalities in 80% (range 65-97) of cells while with mutations in or near the transmembrane domain only in 23 or 32% of cells. In conclusion, we demonstrate that dantrolene given intravenously shows antiarrhythmic effects in a portion of CPVT1 patients and that iPSC derived CM models replicate these individual drug responses. These findings illustrate the potential of iPSC models to individualize drug therapy of inherited diseases.

Trial Registration

EudraCT Clinical Trial Registry 2012-005292-14

(www.sydantutkimussaatio.fi/?lang = en), and The Sigrid Juselius Foundation (www.sigridjuselius.fi/foundation). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is one of the most malignant inherited arrhythmogenic disorders. It manifests with exercise-induced premature ventricular complexes (PVCs), polymorphic or bidirectional ventricular tachycardia, or sudden death, usually associated with vigorous physical exercise or mental stress.[1–3] Current therapeutic options include beta-antiadrenergic drugs, flecainide, implantable cardioverter-defibrillators (ICD) [4–6] and left cardiac sympathetic denervation.[7,8] Better antiarrhythmic medication is still needed to minimize the need for ICD shock therapies. The most common subtype, type 1 of CPVT (CPVT1) is a dominantly inherited disease caused by mutations in the cardiac ryanodine receptor (*RyR2*) gene.[9,10] The gain-of-function mutations of *RyR2* cause increased calcium (Ca^{2+}) sensitivity which can lead to spontaneous Ca^{2+} release from sarcoplasmic reticulum, generation of afterdepolarizations, and triggered activity.[4,5,11]

The ryanodine receptor isoform *RyR1* is the skeletal muscle counterpart in the gene family. Mutations of *RyR1* result in malignant hyperthermia, a rare but life-threatening complication of general anesthesia occurring upon administration of volatile anesthetics or depolarizing muscle relaxants. Dantrolene is a specific and currently the only effective treatment for malignant hyperthermia.[12] Interestingly, dantrolene has also shown to exert antiarrhythmic effects in animal models of CPVT1.[13–15] Dantrolene has been proposed to act through binding to the N-terminal parts of *RyR1* and *RyR2* and restoring inter-domain interactions critical for the closed state of the *RyR2* Ca^{2+} channel.[16]

Several studies using induced pluripotent stem cell (iPSC) technology [17] have indicated the ability of CPVT1 patient-specific iPSC derived cardiomyocytes (CMs) to replicate the disease phenotype in cell culture.[18–24] Dantrolene was reported to rescue the disease phenotype in iPSCs derived CMs from a single *RyR2* mutation carrier [20] but no *in vivo* data exists on its effects on CPVT1 patients.

In the present study, we report the proof of principle of the antiarrhythmic activity of dantrolene in a cohort of CPVT1 patients. In addition, we demonstrate that the in vivo drug effects are closely reproduced in iPSC-derived patient-specific CMs, and provide evidence for mutation-specific effects of dantrolene in CPVT1.

Materials and Methods

The protocol for this trial is available as supporting information; see S1 Protocol and S2 Protocol.

Clinical Study Scheme

The study was approved by the Ethical Review Committee of the Helsinki University Hospital (HUS 396/13/03/01/12) and was in accordance with the institutional guidelines and the Declaration of Helsinki. A written informed consent was obtained from all patients. Clinical trial was registered with EudraCT (2012-005292-14). Participants were recruited between 1st March 2013 and 29th May 2014. Follow up time of the patients was three days after the dantrolene infusion. Four patients participated the study at the Helsinki University Hospital and two at the Tampere University Hospital, Heart Center. The authors confirm that all ongoing and related trials for this drug are registered.

Patients

The study group consisted of 6 individuals (mean age 50±10 years, range 37–59 years, 5 females), who were molecularly defined heterozygous carriers of different gain-of-function *RyR2*

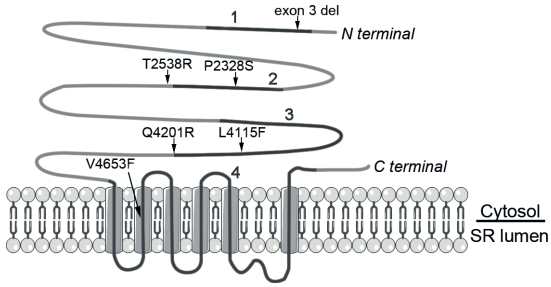

Fig 1. *RyR2* protein, mutations studied in the present study and mutation clusters 1–4. Mutations in this study (arrows) are located in different parts of the *RyR2* protein and mutation clusters. Clusters are represented as black lines numbered from 1 to 4. Cluster 1 comprises of amino acids (AA) 44–466, cluster 2 AA 2246–2534 and cluster 3 AA 3778–4201 and these three clusters are located in the N-terminal and central regions of the protein and form the cytoplasmic domain. Cluster 4 comprises of AA 4497–4959 and forms the transmembrane domain, which is located in the C-terminal region. The figure is modified from [11].

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mutations causing CPVT1. CPVT1 patients carried the following mutations: *c.168-301_c.273+722del1128* mutation (later called as *exon 3 deletion)* or point mutations *p.P2328S (c.6982C>T)*, *p.T2538R (c.7613C>G)*, *p.L4115F (c.12343C>T)*, *p.Q4201R (c.12602A>G)* or *p.V4653F (c.13957G>T)*. Mutation nomenclature was based on *RyR2* reference sequence NM_001035.2. The mutations were located in the four mutation hotspot clusters of the *RyR2* gene (Fig 1).[11] Patients and families carrying mutations *P2328S* [3,10,25], *exon 3 deletion* [3,26], *Q4201R* [10,25] and *V4653F* [10,25] have been described in detail earlier.

All mutations were associated with exercise-induced ventricular arrhythmias and syncopal spells. All except *L4115F* were associated with one or more cases of sudden death at young age in the family. An ICD had been implanted in five of them; one has received an adequate shock therapy. Five out of the six patients had a history of syncopal spells upon a frightening situation or physical exercise, and they all used a beta-adrenergic blocking agent (daily dose of 160 mg of propranolol, 7, 5 to 10 mg of bisoprolol or 95 mg of metoprolol) during all study phases. No other medications were in use. The patients were otherwise healthy without hypertension, diabetes, or evidence of other heart disease. None of them had bundle branch block.

In cardiac ultrasonography, left ventricular end diastolic dimension and systolic function were normal in all patients (data not shown). Basic laboratory parameters including hemoglobin, white blood cell count, plasma sodium, potassium, and creatinine concentrations were analyzed prior to administration of the drug and they were all within the normal range in every study patient (data not shown). Two of the patients with ICD showed atrial pacing at rest prior to exercise; all others had sinus rhythm. Electrocardiographic parameters are presented in Table 1.

Clinical Exercise Stress Test

All RyR2 patients underwent the exercise stress test three times. On the first morning a baseline study was carried out. The test was repeated in the afternoon of the first day after intravenous infusion of dantrolene sodium (Dantrium, 1.5 mg per kg of body weight). The third exercise test was performed on the second day to assess the effects after dantrolene sodium washout and to demonstrate the reproducibility of the basic exercise test.

Exercise tests were performed with a bicycle ergometer. The initial load was 30 W, followed by increments of the load by 15 W each minute. In the baseline study, the patient was

Table 1. Electrocardiographic parameters.

	Before dantrolene (n = 6)	After dantrolene (n = 6)	p-value
Heart rate (min-1)	61±5	60±5	NS
P (ms)	109±14	110±14	NS
PQ (ms)	154±31	163±34	NS
QRS (ms)	86±8	87±5	NS
QT (ms)	419±22	407±18	NS
QTc (ms)	418±35	409±35	NS

Comparison of electrocardiographic parameters of all patients before and after the dantrolene infusion. NS indicates no significant.

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instructed to target to a submaximal workload in order to be able to repeat the exercise stress test. In the consecutive phases of the study, the workload target was the same as that achieved in the first study. A twelve-lead electrocardiogram (ECG) was recorded continuously at paper speed 25 or 50 mms^{-1} and amplification of 0.1 mV/mm throughout the exercise test. After cessation of exercise, ECG was recorded continuously for the first 8 minutes. Both the maximum workload achieved and the heart rate at which ventricular bigeminy first appeared were recorded whenever applicable. Numbers of PVCs during exercise and at recovery phase as well as the maximum number of consecutive PVCs were counted. Plasma creatinine, sodium, potassium and calcium were measured at rest before the first exercise test. The exercise tests and the iPSC studies were done separately and blinded.

Characterization of iPSC Lines

The iPSC study was approved by the ethical committee of Pirkanmaa Hospital District (R08070) and written informed consent was obtained from all the participants. Patient-specific iPSC lines were established as described earlier.[17] Studied iPSC lines were UTA.05605.CPVT generated from patient with *RyR2 exon 3 deletion*, UTA.05208.CPVT from patient with mutation *P2328S*, UTA.07001.CPVT from patient with mutation *T2538R*, UTA.03701.CPVT from patient with mutation *L4115F*, UTA.05503.CPVT from patient with mutation *Q4201R*, UTA.05404.CPVT from patient with mutation *V4653F* and UTA.04602.WT from a healthy control individual.

All the CPVT-iPSC lines were characterized for their karyotypes, mutations, pluripotency, immunocytochemistry, embryoid body (EB) and teratoma formation. Endogenous and exogenous gene expressions were examined by RT-PCR using 1 μl cDNA and 500 nmol/L of each primer in one PCR reaction. β-actin and GAPDH served as the housekeeping genes. Detailed reaction conditions and PCR primers for iPSC characterization have been described earlier. [27] Endogenous pluripotency markers at the protein level were studied with immunocytochemistry. The iPSCs were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, Saint Louis, USA). Primary antibodies anti-SOX2, anti-NANOG and anti-tumor-related antigen (TRA)1-81 (all 1:200, from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-OCT3/4 (1:400, R&D Systems) were used. Cells were mounted with Vectashield (Vector Laboratories, USA) containing DAPI for staining nuclei. To confirm the mutations of the CPVT iPSC lines with sequencing the DNA was isolated using DNA Tissue XS-kit (Macherey-Nagel GmbH & Co., Düren, Germany). The genomic region containing the expected mutation was amplified using PCR. Each PCR product was directly sequenced in both directions using BigDye Terminator v3.1 and ABI 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). In addition to

direct sequencing, *exon 3 deletion* (1128 nucleotides) was also confirmed using PCR and agarose gel electrophoresis. Karyotypes of the cell lines were determined using either standard G-banding chromosome analysis (Medix laboratories, Espoo, Finland) or KaryoLite BoBs assay (Perkin Elmer) based on BACs-on-Beads technology (Molecular and Systems Immunology and Stem Cell Biology, Turku Centre for Biotechnology, University of Turku, Finland). The expression of markers characteristic of ectoderm (*Nestin* or *SOX-1*), endoderm (*AFP* or *SOX-17*), and mesoderm (*VEGF-R2*) development were studied from EBs maintained in EB-medium (KO-DMEM with 20% FBS, Non-Essential Amino Acid (NEAA), L-glutamine and penicillin/streptomycin) for 5 weeks. EB RT-PCR primers can be seen in S1 Table. The teratoma study was approved by ELLA- Animal Experiment Board of Regional State Administrative Agency for Southern Finland (ESAVI/6543/04.10.03/2011). IPSCs were injected into nude mice under the testis capsule and tumor samples collected 8 weeks after injection, followed by fixation with 4% PFA and staining of the sections with hematoxylin and eosin.

Cardiomyocyte Differentiation and Characterization

iPSCs were co-cultured with murine visceral endoderm-like (END-2) cells (Humbrecht Institute, Utrecht, The Netherlands) to differentiate them into spontaneously beating CMs. The beating areas of the cell colonies were mechanically excised and treated with collagenase A (Roche Diagnostics).[28] Single CMs were immunostained with anti-cardiac-troponin-T (1:1500, Abcam, Cambridge, MA, USA), anti-α-actinin (1:1500, Sigma) and anti-connexin-43 (1:1000, Sigma).

Ca^{2+} Imaging

Dissociated spontaneously beating CMs on a coverslip were loaded with 4 μmol/L Fura 2-AM (Life Technologies, Molecular Probes). CMs were continuously perfused with 37°C HEPES based perfusate during measurements and the perfusate consisted of (in mmol/L): 137 NaCl, 5 KCl, 0.44 KH_2PO_4, 20 HEPES, 4.2 $NaHCO_3$, 5 D-glucose, 2 $CaCl_2$, 1.2 $MgCl_2$ and 1 Na-puryvate (pH was adjusted to 7.4 with NaOH). Ca^{2+} measurements were conducted on an inverted IX70 microscope (Olympus Corporation, Hamburg, Germany) and cells were visualized with UApo/340 x20 air objective (Olympus). Images were acquired with an ANDOR iXon 885 CCD camera (Andor Technology, Belfast, Northern Ireland) synchronized with a Polychrome V light source by a real time DSP control unit and TILLvisION or Live Acquisition software (TILL Photonics, Munich, Germany). Fura 2-AM in CMs was excited at 340 nm and 380 nm light and the emission was recorded at 505 nm. For Ca^{2+} analysis, regions of interest were selected for spontaneously beating cells and background noise was subtracted before further data processing. The Ca^{2+} levels are presented as fura ratio units of F340/F380. Ca^{2+} peaks were analyzed with Clampfit version 10.2 (Molecular Devices, USA).

The percentage of abnormal Ca^{2+} transients, such as multiple peaks comprising of two peaks, irregular phases, oscillations, and varying amplitude manifested as low peaks, were calculated from each studied cell line. Beating frequency and diastolic Ca^{2+} levels of CMs were analyzed during spontaneous baseline beating, and during adrenaline perfusion. These parameters were compared between mutated and control cell lines and also between each mutated cell line. Some results of Ca^{2+} cycling of CPVT-*P2328S* and control cell lines have been published before [21] and parts of these results have been included here.

For dantrolene studies, the changes in Ca^{2+} were recorded during spontaneous baseline beating, spontaneous beating during 1 μM adrenaline perfusion and spontaneous beating during 1 μM adrenaline together with 10 μM dantrolene (Sigma) perfusion. If a CM displayed Ca^{2+} transient abnormalities during adrenaline perfusion, it was exposed to dantrolene. Drug

effects of dantrolene were categorized into three groups. In "responder" group dantrolene abolished virtually all the Ca^{2+} handling abnormalities, in "semi-responder" group dantrolene reduced them by more than 50%, in "non-responder" group dantrolene reduced them by less than 50%. Diastolic Ca^{2+} levels and beating frequency were compared between adrenaline and dantrolene responses of responder CMs (*exon 3 del*, *P2328S*, *T2538R*, *L4115F*) and non-responder CMs (*Q4201R*, *V4653F*, controls). For this, adrenaline response values were divided by dantrolene response values, separately for each cell.

Statistical Analysis

Statistical analysis of *in vivo* studies was made with SPSS 21.0 statistical software package (SPSS, Chicago, IL). Data are presented as average + 1 SD. Comparisons between phases were performed by the non-parametric Wilcoxon test. The significance of *in vitro* differences between two groups was evaluated with the unpaired Student's *t*-test. The significance of changes within a group was evaluated with the paired Student's *t*-test. Data are expressed as average ± S.E.M. and n refers to the number of cells. $P<0.05$ was considered statistically significant in both *in vivo* and *in vitro*.

Results

Antiarrhythmic Effects of Dantrolene in Cpvt1 Patients

In the baseline study, patients exercised on an average 8±2 minutes reaching a maximum heart rate of 134±17 min^{-1}. Exercise bicycle testing induced polymorphic PVCs in all patients and non-sustained ventricular tachycardia (NSVT, episodes of 3 to 4 consecutive PVCs) in three of them. The average threshold sinus rate for the appearance of PVCs was 105±9 min^{-1}. The total count of PVCs during the workload was 172±119 (range 43–391).

All six patients tolerated the target dose 1.5 mg/kg of intravenous infusion of dantrolene but reported considerable muscle weakness as a side-effect. Dantrolene did not affect atrioventricular conduction or the QT interval (Table 1). Dantrolene decreased the prevalence of PVCs in four patients, whereas in two patients the number of PVCs remained virtually the same (Fig 2). Dantrolene seemed to reduce arrhythmias in patients with the mutation in the N-terminal or central region of the *RyR2* protein (Figs 1 and 2). Thus, dantrolene abolished 97% of PVCs in the patient with exon 3 deletion (cluster 1), 88% of PVCs in the patient with *P2328S* mutation (cluster 2), 33% of PVSc in the patient with *T2538R* mutation (right after cluster 2) and 77% of PVCs in the patient with *L4115F* mutation (cluster 3). In contrast, dantrolene abolished only 1 to 2% of PVCs in patients carrying mutation closer to (*Q4201R*, end of cluster 3) or within the transmembrane region (*V4653F*, cluster 4).

Fig 3 illustrates an example of the PVCs and NSVT episodes during the baseline study and after dantrolene. Dantrolene increased significantly the threshold at which the arrhythmias appeared from 105±9 to 120±17 min-1. The duration of the exercise phase and the maximal heart rate achieved during the exercise were similar to those in the baseline study. On day 2, after wash-out of dantrolene, the prevalence of PVCs was approaching that in the first baseline test.

Characterization of iPSC Lines Confirms Pluripotent Stem Cell Characteristics

iPSC lines from six CPVT1 patients with above-mentioned *RyR2* mutations were generated. Results of the *P2328S* and control iPSC line characterizations have been published before. [21,27] All studied endogenous pluripotency genes (Nanog, Rex1, Oct 3/4 and Sox2) were

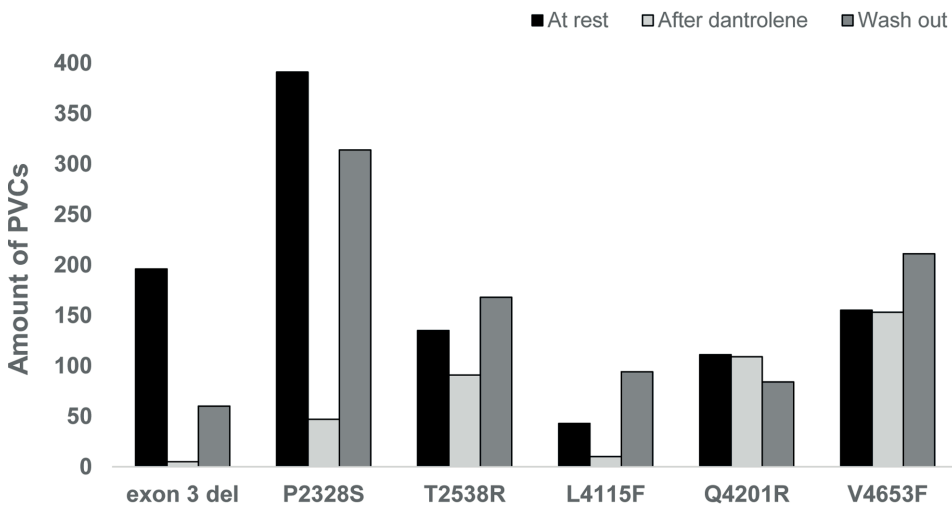

Fig 2. Features of the PVCs. Number of PVCs in exercise stress test before and after administration of intravenous dantrolene and 24 hours after dantrolene wash out.

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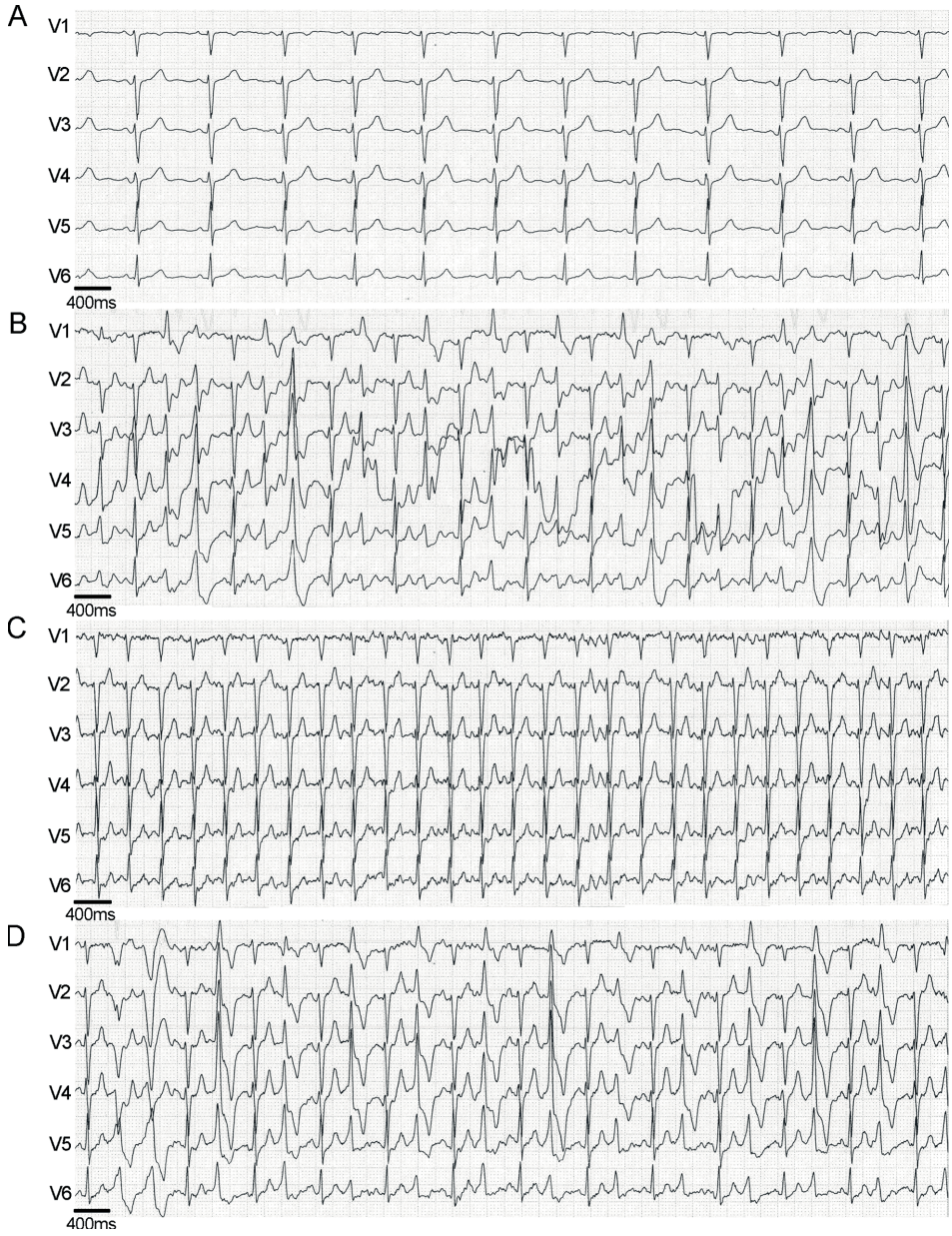
turned on and the expression of retrovirally encoded reprogramming factors c-Myc, Klf4, Sox2 and Oct 3/4 was silenced (Fig 4A and 4B). CPVT1 iPSC lines expressed endogenous pluripotent markers Nanog, Oct3/4, TRA 1–81 and SOX2 also at the protein level (Fig 4D). Pluripotency was further confirmed by teratoma formation and with *in vitro* embryoid body (EB) formation expressing all three germ layers (Figs 4C and 5G). The presence of the *RyR2* mutations was confirmed from all the CPVT1 iPSC lines with DNA sequence analysis (Fig 4E). All the iPSC lines had a normal karyotype (Fig 4F). In addition to sequencing, the presence of the *exon 3 deletion* was confirmed with PCR (S1 Fig).

iPSC Derived Cms Display Abnormal Ca^{2+} Cycling

iPSCs were differentiated into spontaneously beating CMs (Fig 5A). When compared to CMs derived from the healthy individual, CPVT1 CMs demonstrated marked Ca^{2+} transient abnormalities such as multiple peaks comprising of two peaks, irregular phases, oscillations, and varying amplitude manifested as low peaks (Fig 5B) both in baseline and in response to adrenaline. Although these abnormalities were common with all six mutations examined, some differences between *RyR2* mutations were also observed. Accordingly, Ca^{2+} transient abnormalities were somewhat more common in the cluster 4 mutation than in cluster 1, 2 and 3 mutations (Fig 5C).

Adrenaline increased the beating frequency of each cell line studied (Fig 5D). All *RyR2* mutated CMs had lower beating frequency both at baseline and during adrenaline perfusion than control CMs. Adrenaline produced significantly elevated diastolic Ca^{2+} levels only in *P2328S* CMs, while diastolic Ca^{2+} levels were lower or similar in other mutant CMs compared to control CMs (Fig 5E). *Exon 3 deletion* CMs had both lower beating frequency and diastolic Ca^{2+} level when compared to other mutations (Fig 5 and S2 Table).


A
V1
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V1
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V3
V4
V5
V6
400ms
D
V1
V2
V3
V4
V5
V6
400ms

Fig 3. ECG examples of a 38-year-old patient carrying the *RyR2 P2328S* mutation. (A) Resting ECG showing sinus rhythm and normal QRS morphology. (B) Exercise ECG at the highest work load of 105 W in the baseline study before dantrolene. PVCs include couplets and polymorphic NSVTs. (C) Disappearance of ventricular arrhythmias after administration of dantrolene (work load 105 W). (D) Exercise test on day two after 20-hours wash-out of dantrolene showing return of PVCs (work load 105 W).

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iPSC Derived Cpvt1 Cms Reproduced the Clinical Antiarrhythmic Responses to Dantrolene

Effects of dantrolene were divided into three groups based on their Ca^{2+} responses. In "responder" group dantrolene abolished all the Ca^{2+} handling abnormalities, in "semi-responder" group dantrolene reduced them by more than 50% and in "non-responder" group dantrolene reduced them by less than 50%. iPSC derived CMs were found to markedly reproduce the varying individual clinical responses of dantrolene (Fig 6). In cell lines with the mutation in the N terminal or central region of the *RyR2* protein dantrolene abolished or reduced the majority of Ca^{2+} transient abnormalities (Fig 6). These mutations were within or in close proximity of clusters 1, 2 or 3 (Fig 1). A detailed analysis indicated that in CMs with *exon 3 deletion*, *P2328S*, *T2538R or L4115F*, dantrolene abolished or reduced by more than 50% of Ca^{2+} abnormalities in 65–97% of cells (Fig 6).

In striking contrast, the effect of dantrolene was only minimal in CMs carrying a mutation at the end of cluster 3 (*Q4201R*) or in the transmembrane region (cluster 4, mutation *V4653F*) (Fig 6), in accordance with the *in vivo* dantrolene infusion data. Dantrolene had no effect on the Ca^{2+} transients in control CMs. Dantrolene did not significantly affect the diastolic Ca^{2+} levels of CMs in which Ca^{2+} transient abnormalities were abolished (S2 Fig). Dantrolene increased significantly the diastolic Ca^{2+} levels of control and *Q4201R* CMs where Ca^{2+} transients were unaltered by the drug. There was no correlation between the antiarrhythmic effect of dantrolene and its effect on beating frequency (S2 Fig).

Discussion

We have studied the antiarrhythmic potential of dantrolene in the treatment of CPVT1. To this end, we assessed the efficacy of intravenously administered dantrolene in patients carrying various *RyR2* mutations and compared these effects to *in vitro* studies using iPSC derived CMs generated from the same patients. Our findings demonstrate that intravenous dantrolene, a drug used to treat another ryanodine receptor disorder, malignant hyperthermia, abolished or markedly reduced arrhythmias in a subgroup of CPVT1 patients with specific *RyR2* mutations. By combining evidence from *in vivo* and *in vitro* studies, we propose that the location of the *RyR2* mutation affects the antiarrhythmic effect of dantrolene in CPVT1.

Previously dantrolene has been shown to have beneficial effects on cardiac function in experimental animal models of CPVT1 [13–15,29,30] and in iPSC derived CMs from a CPVT1 patient with an N-terminal *S406L* mutation [20], but no studies in patients have so far been reported. It is also important to take into consideration that even if a drug is found to be beneficial in the patient-derived iPSC-CMs, it cannot be automatically concluded that this will translate into a clinical benefit. Here we show that dantrolene given intravenously has an antiarrhythmic effect also in some but not in all patients with CPVT1. This antiarrhythmic effect was observed only in patients with *RyR2* mutations in the N-terminal or central regions of *RyR2* protein (clusters 1–3), whereas virtually no effect was seen in patients carrying mutations at the end of cluster 3 or in the transmembrane region (cluster 4). Although a dose-dependent effect cannot be excluded, similar observations on mutation-specific drug responses have been obtained in some other genetic disorders including long QT syndrome type 3 [31], cystic fibrosis [32], as well as in certain neoplastic diseases.[33] Recognition of potential mutation-specific

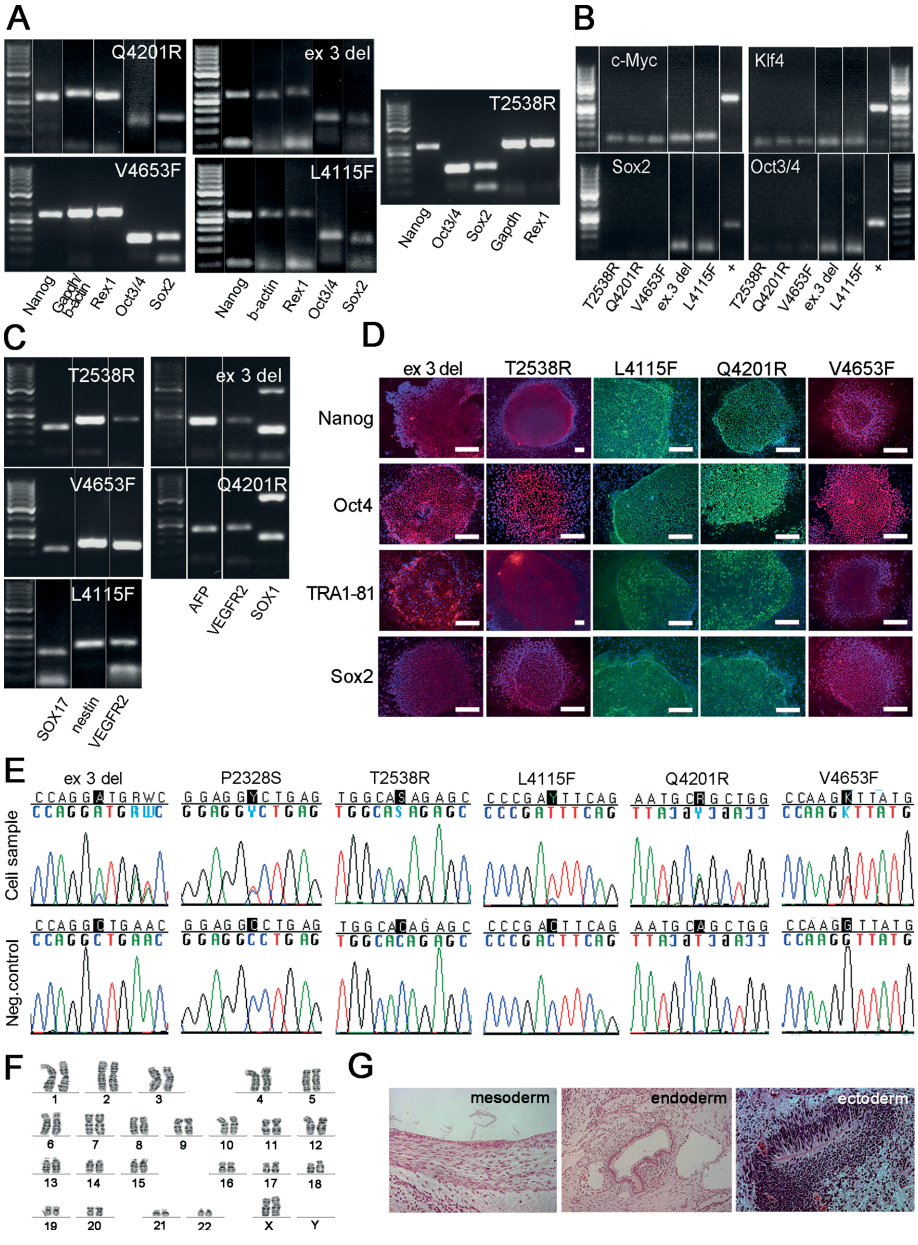
A
Q4201R
ex 3 del
T2538R
V4653F
L4115F
Nanog
Gapdh
b-actin
Rex1
Oct3/4
Sox2
B
c-Myc
Klf4
Sox2
Oct3/4
T2538R
Q4201R
V4653F
ex.3 del
L4115F
+
C
T2538R
ex 3 del
V4653F
Q4201R
L4115F
AFP
VEGFR2
SOX1
SOX17
nestin
VEGFR2
D
ex 3 del
T2538R
L4115F
Q4201R
V4653F
Nanog
Oct4
TRA1-81
Sox2
E
ex 3 del
P2328S
T2538R
L4115F
Q4201R
V4653F
Cell sample
Neg.control
F
G
mesoderm
endoderm
ectoderm

Fig 4. Characterization of CPVT1 iPSCs. (A) Expression of pluripotency markers shown by RT-PCR, β-actin or GAPDH serving as a housekeeping gene. (B) None of the exogenous genes are expressed in CPVT1 cell lines. (C) EBs express markers from all the three embryonic germ layers. (D) Immunocytochemical stainings and expression of pluripotency markers. Scale bar 200 μm. (E) Sequencing analysis confirmed the *RyR2* mutation in each cell line. (F) All the cell lines had normal karyotype, example picture from *Q4201R* cell line. (G) Teratomas made from a CPVT-iPSC line further confirms pluripotency, example pictures from *L4115F* cell line.

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responses will be important for future drug development: one drug may not work for all patients even if the phenotype is the same.

Currently, beta-antiadrenergic medication is the first line antiarrhythmic treatment for all CPVT patients. Flecainide has also shown beneficial effects.[34,35] ICDs are used if severe arrhythmic events occur despite optimal beta-blocking treatment. However, use of ICDs is not without risk since ICD-shocks may further aggravate catecholamine release and initiate an uncontrolled electric storm. Very recent data suggest that left cardiac sympatectomy may be highly effective in patients refractory to medical therapy.[7,36] Although dantrolene as such would not be suitable for long-term treatment of CPVT1 due to its side effects, and although only a subset of patients would benefit from it, our data shows its antiarrhythmic potential. It also suggest that it could be administered by intravenously to CPVT1 patients in emergencies such as incessant ventricular tachycardia.

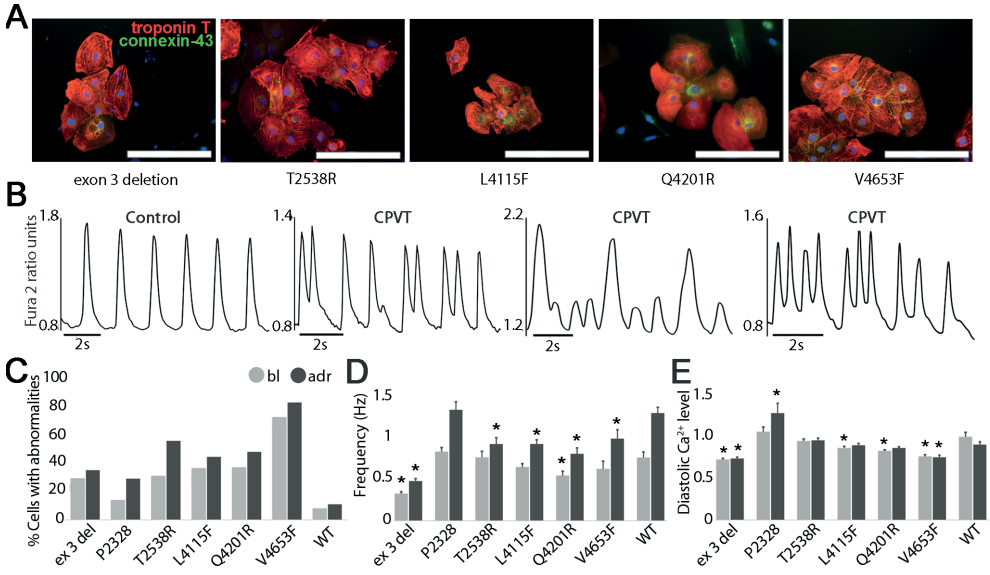

Fig 5. Characterization of CPVT-iPSCs derived CMs. (A) Immunocytochemical stainings of cardiac markers where red represents troponin T, green connexin-43 and blue DAPI-staining for nuclei. Scale bars 200 μm. (B) Representative traces of a control CM showing normal regular Ca^{2+} transients and CPVT1 CMs showing abnormalities like multiple peaks, low peaks, irregular phases and oscillations in Ca^{2+} handling. (C) Quantification of percentage of CPVT1 and control iPSC CMs exhibiting abnormal Ca^{2+} transients at baseline (bl) and during adrenaline perfusion (adr). (D) Frequency and (E) Diastolic level of intracellular Ca^{2+} of all CPVT1 and control CMs. Numbers of cells analyzed in C, D, and E, *exon 3 del* n = 48, *P2328S* n = 72, *T2538R* n = 52, *L4115F* n = 110, *Q4201R* n = 63, *V4653F* n = 29, Controls (WT) n = 28. As an exception, number of WT cells analyzed in D, and E, in bl n = 54 and adr n = 27 and number of *P2328S* cells in bl n = 90 and adr n = 47. Grey bars indicate cells at baseline and black bars during adrenaline perfusion. Error bars, SEM. *P<0.05 CPVT1 versus control, with Student's t-test. Significance's of mutation specific differences, see S2 Table.

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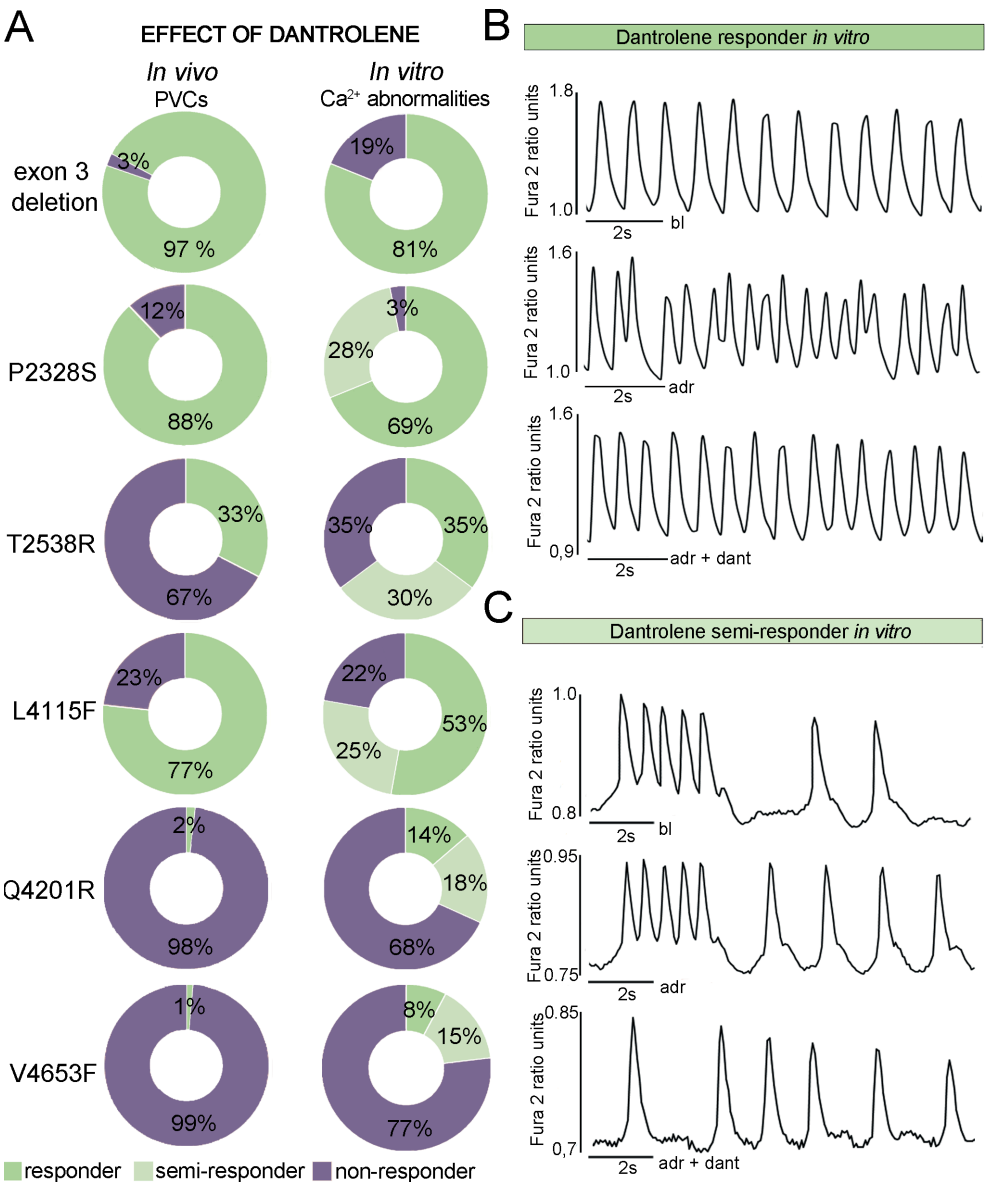

Fig 6. iPSC derived CMs reproduced the clinical responses of dantrolene. (A) *In vivo* and *in vitro* effects of dantrolene correspond within each *RyR2* mutation. *In vitro* drug effects were categorized into three groups (responders, semi-responders and non-responders) depending on how dantrolene affected to the amount of Ca^{2+} abnormalities when compared to adrenaline response. *In vivo* responder group show the percentage of the abolished PVCs when compared to the baseline. Numbers of cells analyzed in *exon 3 del* n = 16, *P2328S* n = 32, *T2538R* n = 17, *L4115F* n = 36, *Q4201R* n = 22, *V4653F* n = 13. (B)

Representative traces of dantrolene responder *in vitro* in an *L4115F* mutated CM. Adrenaline causes Ca^{2+} cycling abnormalities and dantrolene abolishes all the abnormalities. (C) Representative traces of semi-responder *in vitro* in a *P2328S* mutated CM. The cell has abnormal Ca^{2+} cycling at baseline and during adrenaline perfusion and dantrolene reduces the abnormalities by abolishing the oscillation but leaving some low peak Ca^{2+} spiking.

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More than 150 mutations in *RyR2* gene have been reported so far and they are clustered in four hotspots.[11] One third of the reported mutations are in clusters 1 and 2 and the rest are equally distributed between clusters 3 and 4. Only 10% of *RyR2* mutations have been found outside these clusters.[11] The location of the *RyR2* mutation appears to be critical for a favorable effect of dantrolene. The binding site for dantrolene is localized in the N-terminus of *RyR2* between amino acid 601 and 620.[13,37] The dantrolene-binding sequence is considered to constitute part of the domain switch region, suggesting that dantrolene is involved in the correction of defective unzipping and allosteric stabilization of interdomain interactions between the N- terminal and central regions of *RyR2*, resulting in inhibition of Ca^{2+} leak [16,30,38] and in fact, this has been demonstrated in previous studies.[13,16,37] Also our data demonstrate that dantrolene abolished arrhythmias in CPVT1 patients with mutations of N terminal or central domain, suggesting that a defective inter-domain interaction within the *RyR2* could be the underlying arrhythmogenic mechanism in the *exon 3 deletion*, *P2328S*, *T2538R* and *L4115F*. However, dantrolene did not suppress *T2538R*-related arrhythmias to the same extent as arrhythmias caused by other central region mutations. It has been speculated that differences in the mode of interdomain interaction in dantrolene binding regions may result in differences in its antiarrhythmic efficacy.[30] Furthermore, other drug-binding regions in the carboxyl-terminal half of the *RyR2* or additional low affinity drug binding sites in the N-terminal area could exist.[13] No previous studies on the effects of dantrolene on *RyR2* mutations in or close to the transmembrane are available; here we show that dantrolene has no or only minimal effect on arrhythmias if mutations are located in these areas. It is interesting that the patient with *Q4201R* mutation did not respond to dantrolene even though this mutation is located in cytosolic portion of *RyR2* and in cluster 3 although in its terminal part. This finding indicates that the location of the mutation in certain mutation cluster does not necessarily determine the antiarrhythmic response, and highlights the utility of the iPSC model for individual functional analysis.

Our data showing similar patient-to-patient variation in dantrolene effects in the clinical setting and corresponding iPSC-CM models suggest that, at least in theory, it may be possible to tailor an individual's medication in cell culture without predisposing the individual to the potentially serious side-effects of a drug. Dantrolene did not affect normally beating CMs. This is consistent with previous reports showing that dantrolene inhibits only abnormal Ca^{2+} release and has no effect on the normal Ca^{2+} transients, suggesting that the native conformation of *RyR2* may restrict binding of the drug and that dantrolene binding to *RyR2* might be dependent on a specific conformational state present only in mutated cells.[13,37] Defective calmodulin binding caused by *RyR2* domain unzipping has also been shown to be restored by dantrolene [29,39] which may as well explain why dantrolene exerts effects on diseased but not healthy hearts.

Besides differences in drug responses, we also saw both similarities and differences in the CPVT1 *in vitro* phenotypes depending on the nature of the mutation. The beating frequency of CPVT1 CMs was lower than that in control CMs. This is in line what has been reported also with CPVT1 patients.[5,40] All the CPVT1 CMs showed similar disturbances in intracellular Ca^{2+} cycling. Ca^{2+} transient abnormalities were somewhat more common in the cluster 4 mutation than in cluster 1, 2 and 3 mutations. *Exon 3 deletion* differed from all the other mutations by having lower diastolic Ca^{2+} levels and beating frequency both at baseline and during

adrenaline perfusion. Exon 3 encodes secondary structure elements that are crucial for folding of the N-terminal domain. It has been proposed that *RyR2* with *exon 3 deletion* has evolved additional means to regulate Ca^{2+} release, by altering the conformation of the domain [41], which may result in the observed differences in Ca^{2+} transients.

There are certain limitations in our study. First, only six CPVT1 patients and their iPSC cell lines were studied; however, we emphasize that despite the very rare nature of this disease we were able to examine in detail six different disease-causing mutations. Second, under the conditions of the study design we were permitted to study only acute effects of intravenously administered dantrolene. Third, although we titrated the dose of dantrolene according to the weights of the patients, serum levels of the drug were not measured and could have varied from patient to patient, resulting in concentration-dependent variation in clinical responses. Fourth, we used only a fixed concentration of dantrolene, selected on the basis of the work by Jung et al. [20], in our iPSC studies. Fifth, immature phenotype of the iPSC-CMs may produce variation in arrhythmias. However, in our previous study [21] the electrophysiology of CPVT1 iPSC-CMs appeared fairly mature. We have also shown here and in our previous study [21] that arrhythmias are substantially more consistent in CPVT1 CMs than in control CMs.

In conclusion, we have shown here the proof of principle that intravenously administered dantrolene suppresses ventricular arrhythmias in the congenital *RyR2* defect and that the location of the *RyR2* mutation may affect the antiarrhythmic effect of this drug. We also demonstrate that iPSC derived patient-specific CMs correctly predict the clinical response to dantrolene in CPVT1 patients with varying *RyR2* mutations. Our data support the notion that iPSC-derived CMs could serve as a platform for drug development and for design of personalized medication.

Supporting Information

S1 Fig. Confirmation of *exon 3 deletion*. *Exon 3 deletion* of 05605. CPVT cell line was also confirmed with PCR and agarose gel electrophoresis.
(TIF)

S2 Fig. Ca^{2+} transient parameters as a response to dantrolene. (A) Diastolic Ca^{2+} level and (B) beating frequency in responder and non-responder CMs. Values during adrenaline perfusion were divided by values during dantrolene perfusion, separately for each cell. Green bars indicate responder CMs and purple bars non-responder CMs. Error bars, SEM. * indicates significant difference between adrenaline versus dantrolene within a group, $^{*}P<0.05$. Numbers of cells analyzed in *exon 3 del* n = 13, *P2328S* n = 29, *T2538R* n = 11, *L4115F* n = 28, *Q4201R* n = 15, *V4653F* n = 10, Control (WT) n = 20.
(TIF)

S1 Protocol. English translated trial study protocol.
(DOCX)

S2 Protocol. Trial study protocol in the original language (Finnish).
(DOC)

S1 Table. Primer sequences for EB RT-PCR.
(DOCX)

S2 Table. Differences between RyR2 mutations in their Ca^{2+} transient properties during baseline and adrenaline perfusion. CL indicates cluster numbers. Upward pointing arrow indicates significantly ($p<0.05$) higher and downward pointing arrow significantly lower diastolic Ca^{2+} level or beating frequency of the first mentioned mutation when compared to the

second mentioned mutation. NS indicates that there was no statistical significance between mutations. As parallel pointing arrows between comparison groups indicate, the average of the beating frequency and diastolic Ca^{2+} level inside one mutation group corresponded and the average of these parameters decrease when moving from *P2328S* towards transmembrane area mutations.
(DOCX)

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Author Contributions

Conceived and designed the experiments: KP HS KK KAS. Performed the experiments: KP HS SV AML KAS. Analyzed the data: KP HS JP KAS. Contributed reagents/materials/analysis tools: KAS. Wrote the paper: KP HS SV JP AML KK KAS.

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PLOS ONE

CORRECTION

Correction: Antiarrhythmic Effects of Dantrolene in Patients with Catecholaminergic Polymorphic Ventricular Tachycardia and Replication of the Responses Using iPSC Models

Kirsi Penttinen, Heikki Swan, Sari Vanninen, Jere Paavola, Annukka M. Lahtinen, Kimmo Kontula, Katriina Aalto-Setälä

There are errors in Fig 2, "Features of the PVCs," and its caption. Please see the corrected Fig 2 and its caption here.

Citation: Penttinen K, Swan H, Vanninen S, Paavola J, Lahtinen AM, Kontula K, et al. (2015) Correction: Antiarrhythmic Effects of Dantrolene in Patients with Catecholaminergic Polymorphic Ventricular Tachycardia and Replication of the Responses Using iPSC Models. PLoS ONE 10(7): e0134746. doi:10.1371/journal.pone.0134746

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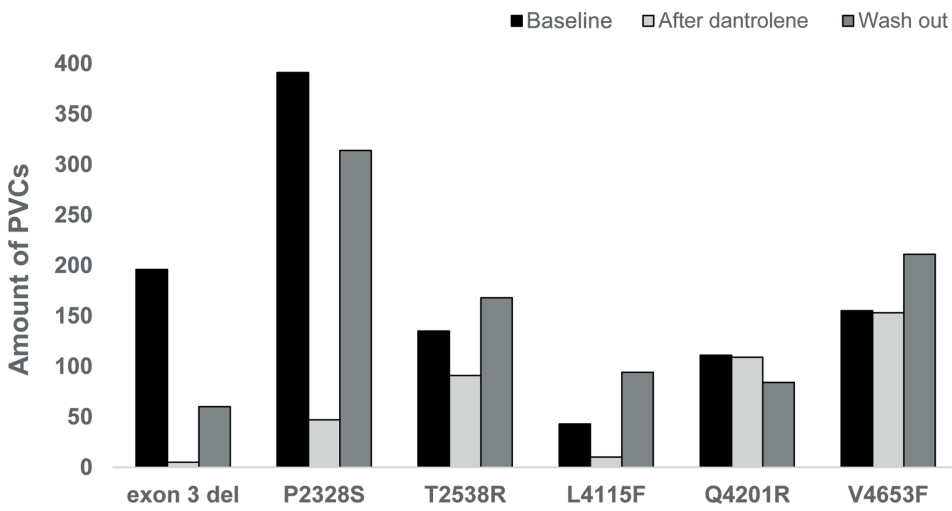

Fig 2. Features of the PVCs. Number of PVCs in exercise stress test before (baseline) and after administration of intravenous dantrolene and 24 hours after dantrolene wash out.

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PUBLICATION

IV

Quantitative comparison of 12-lead electrocardiograms and in vitro cardiac field potentials with different beating frequencies.

Sari U. M. Vanninen*, Ville J. Kujala*, Ilkka Pörsti, Katriina Aalto-Setälä.

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A Quantitative Comparison of 12-Lead Electrocardiograms and *In Vitro* Field Potentials of Stem Cell-Derived Cardiomyocytes

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Abstract

Objective: The human pluripotent stem cell -derived cardiomyocyte (hPSC-CMs) represent a novel biomedical technology that hold great promise for investigating cardiac differentiation and characteristics. The electrophysiological aspects of hPSC-CM can be studied using microelectrode arrays (MEAs). However, there are no reports about the relationship of electrophysiological characteristics between the human heart and hPSC-CMs.

Methods: We analyzed 12-lead electrocardiograms (ECG) of healthy individuals with low basic heart rate and they all performed stress exercise test to increase heart rate and to match the beating rate of hPSC-CMs. All participants were on sinus rhythm. The field potential (FP) recordings of hPSC-CMs were obtained with MEAs. We compared FP duration (FPD) to QT time of ECG and the effect of beating frequency on repolarization time was analyzed.

Results: The heart rate in ECG ranged from 39 to 120 beats per minute (bpm) and the beating hPSC-CMs clusters were selected to match this range. The uncorrected mean FPD value was 375 ms with a mean beating rate of 73 bpm, while the uncorrected mean QT was 397 ms with a mean heart rate 76 bpm. When FPD was corrected with Bazett's formula, cFPD was 400 ms with standard deviation (SD) 64 ms. Volunteers had a mean QTc 432 ms with SD 39 ms. The duration of uncorrected and corrected QT and FPD were similar in ECG and MEA recordings in this beating range.

Conclusions: Our results further validate hPSC-CM clusters as proper *in vitro* models of the native human heart and demonstrate the quantitative relationship of corresponding components in ECG and cardiac MEA recordings.

Keywords: Electrocardiogram; Microelectrode array; Cardiac field potential; Cardiac parameters; Dynamics, QTc, cFPD

Running title: Cardiac Dynamics;

Introduction

The first stable human embryonic stem cell (hESC) lines were derived in 1998 [1] and first human induced pluripotent stem cell (iPSC) lines in 2007 [2]. Since then, these human pluripotent stem cells (hPSCs) have been used in a variety of applications. They are able to differentiate into cell types of all the three germ layers: ectoderm, mesoderm, and endoderm. Hence, one of their advantages is that they enable to study of the functionality of human cells and pathophysiology of genetic diseases *in vitro* without the need of biopsies of the diseased organ. Biopsy is potentially a risky procedure and the fully differentiated cells in the biopsy usually de-differentiate fast in cell culture conditions, as is the case with human cardiomyocytes [3, 4].

The hPSC-derived cardiomyocytes (hPSC-CMs) have been proposed as a source of scalable and comparable origin of human cardiomyocytes [5]. Using microelectrode arrays (MEAs) it is possible to record electrical activity of cultured cells. Field potentials (FPs) of beating cells provide detailed information about the origin and spread of excitation in the heart [6]. FP represents spread of excitation and the conduction velocity in MEA, and FP corresponds intrinsic action potential (AP). In addition to AP duration is same than FP duration (FPD) [7]. The MEA with hPSC-CMs represents a platform that enables medium-throughput analysis of human cardiac tissue. This is of interest especially for basic research, but also for pharmaceutical industry because novel human models for preclinical safety testing are urgently needed. In order to be used in preclinical tests, however, more detailed knowledge on how hPSC-CMs correspond to the clinical ECG measurements is needed.

The hPSC-CMs represent a powerful platform to study human cardiac tissue and they hold a great promise for investigating cardiac differentiation and characteristics. These cardiomyocytes were for the first time reported just three years after the derivation of the hESC lines [8] and since that they have been extensively used *in vitro* for electrophysiological recordings [8 -10]. The FPD is analogous with the QT interval on the ECG [11].

The QT interval represents the duration of ventricular electrical systole, especially from the beginning of contraction to the end of relaxation [12]. In epidemiologic studies the abnormal duration of the QT interval has been found to identify individuals at increased risk of sudden cardiac death [13], therefore measurement of QT interval is important.

QT time is the interval of the beginning of ventricle activation (beginning of the Q wave) to the end of repolarization (end of the T wave), and RR is the time from onset of one QRS complex to another in seconds [12]. The QT interval is known to be rate-dependent [14]. In order to be able to compare QT intervals of different beating rates, the Bazett's formula ($QTc = QT/\sqrt{RR}$ and $cFPD = FPD/\sqrt{PPI}$ (equation 1)) has been created. This formula has also been widely used to correct FPD for beating rate of cardiomyocytes in MEA recordings [15,16]. However, we found no studies directly comparing the cardiac parameters between ECG and MEA recordings. Therefore, we compared sinus rhythm 12-lead ECG from healthy adult individuals to cardiac FP recordings of hPSC-CMs. Our aim was to investigate how well the ECG and cardiac FP recordings correspond to each other. This provided further information how well hPSC-CMs recapitulate the electrophysiological characteristics of the adult human heart. These results indicate similar electrophysiological field potential characteristics, which further validate the hPSC-CM clusters as reliable *in vitro* models of the human myocardium.

In this study we compared with FPD to QT time and PPI to RR, in this context, also the comparability of the cFPD and QTc formulas.

Materials and Methods

Ethical Approval

The local Ethics Committee gave their approval for the study (R07110M and R08070).

Stem cell culture

H7H7 hESCs (WiCell) [1] were cultured on mitomycin C inactivated mouse embryonic fibroblasts (MEFs) in hES medium, which consisted of DMEM/F-12 (Invitrogen) supplemented with 20% KnockOut Serum Replacement (KO-SR), serum replacement (Invitrogen), 1% nonessential amino acids (Lonza), 2 mM L-glutamine (Glutamax, Invitrogen), 50 U/ml penicillin/streptomycin (Lonza), 0.1 mM beta mercaptoethanol (Invitrogen), and 7.8 or 4 ng/ml basic fibroblast growth factor (R&D Systems). The hES medium was refreshed daily, and the stem cell colonies were passaged onto a new MEF layer once a week using 1 mg/ml collagenase IV (Invitrogen). The stem cell derived cardiomyocytes are called hPSC-CMs in the results.

Cardiomyocyte differentiation and plating on microelectrode arrays

The hPSCs were differentiated into cardiomyocyte clusters by co-culturing them with mouse visceral endoderm –like cells as described before [17]. Spontaneously beating hPSC-CM clusters were excised and plated onto FBS (Invitrogen) and 0.1% gelatine (Sigma-Aldrich) coated 6wellMEA200/30iR-Ti-mr MEAs (Multi Channel Systems MCS GmbH) in EB medium consisting of Knockout DMEM (KO DMEM) (Gibco Invitrogen, USA) supplemented with 20% foetal bovine serum (FBS) (Gibco Invitrogen, USA), 1% non-essential amino acids (Cambrex BioSciences, Verviers, Belgium), 1% L-glutamine (Invitrogen, USA), and 50 U/ml penicillin/streptomycin (Cambrex BioSciences, Verviers, Belgium). Cells were fed three times per week.

Electrocardiogram recordings

ECGs were obtained from five normal body weight healthy and no smoking individuals (mean age 38.4 + 20 years, 3 males and two females). Nobody has left ventricular hypertrophy using ECG Sokolow-Lyon-criterions. Other clinical characteristics of the healthy volunteers are described in Table I. Individuals with low baseline beating rate were selected to have similar rage of beating rate in clinical and *in vitro* situations. Electrodes were positioned using standardized protocol at a paper speed of 50 mm/s and 10 mm/mV standardization. All participants were in sinus rhythm and with normal conduction, depolarization and repolarization times. The volunteers performed a stress exercise test on a cycle ergometer (GEMS IT CardioSoft V4.2).

Table I. Clinical characteristics of the healthy volunteers.

Volunteers	Gender	Age (years)	Slowest BR	QRS duration (ms)	QT time(ms) 60 bpm	Previous diseases	Medication
1	M	38	44	90	400	no	no
2	F	52	40	100	410	hypothyroidism after treatment with radio-Iodine*	levothyroxine*
3	M	49	39	105	455	no	no
4	F	18	42	80	435	no	no
5	M	35	42	90	440	no	no

Abbreviations: Gender (M, male; F, female); beating rate, BR; millisecond, ms; * with levothyroxin euthyreotic

Before the stress test, 12-lead electrocardiography was recorded at rest until heart rate decreased to less than 50 bpm. Starting at 20 W the work rate was then increased 5 W manually by degrees. The workload was adjusted for each volunteer so that heart rate increased as smoothly as possible. Each test person pedaled continuously at a cadence of 60 rpm (revolutions per minute), and the exercise stress test was interrupted when the target heart rate (130 bpm) was reached. None of the participants had symptoms limiting their exercise.

RR and QT interval were measured on a single selected lead V2 or V3 because of the clearest T wave during exercise. Heart rate was determined by RR cycle lengths (ms) before measured QT interval. QT interval was manually measured from the beginning of the QRS complex to the tangent to the end of the T wave. Measurements were taken to the nearest 5 ms. The QT interval and heart rate measured by the analysis programs was used in the heart rate adjustment formulae from the Framingham Study, adjusting the measured QT intervals for heart rate using Bazett's formula [18].

Field potential recordings with microelectrode arrays

FPs were recorded in 5% FBS containing EB medium from 9 hESC-CM clusters in room air with a USB-MEA amplifier (Multi Channel Systems MCS GmbH) using a 20 kHz sampling rate. MC_rack 4.0.0 software (Multi Channel Systems MCS GmbH) was used for data acqusition. MEAs were covered with a gas-permeable membrane (ALA Scientific) during recordings to keep cultures sterile. Recordings were made at +37 °C using a TC02 heater controller.

Data analysis

The different parameters were measured manually from the ECG recordings and using Clampfit 10 software (Molecular Devices, Inc.) from the FP recordings. Heart rate (HR, beats per minute [bpm]), beating rate (BR, bpm), heart-rate corrected QT-interval (QTc, Bazett's formula, equation 1), beating-rate corrected field potential duration (cFPD, Bazett's formula, equation 1), RR interval (ECG) and peak-to-peak interval (PPI, FPs) were determined from the recordings.

$$QTc = QT/\sqrt{RR} \text{ and } cFPD = FPD/\sqrt{PPI} \text{ (equation 1) [19]}$$

Where, QTc is the heart rate corrected QT interval, QT is the QT-interval in milliseconds, and RR is the time from onset of one QRS complex to another in seconds (often 60 bpm). The same formula was used for FP recordings as well, only then QTc became cFPD, QT was FPD and RR was PPI (determined as time between two depolarizing sodium [Na^+] peaks). FPD (Figure 1) was determined as the time between the onset of initial deflection and return to baseline as described before [15]. FPD and PPI were measured in triplicate from each FP trace and the mean value was used for further analysis. The Poincaré maps were generated using Neuroexplorer 4 software (Nex Technologies).

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analyses were performed between the groups with two-tailed t-test using SPSS software (IBM).

Results

The QT and RR as well as FPD and PPI values were measured from the ECG and MEA recordings, respectively (Table II). The healthy volunteers were chosen so that the baseline beating rate was low to match the slowest beating rate in hPSC-CMs and stress exercise test was performed to match the highest beating of the cells.

A characteristic hPSC-CM FP trace is presented in figure 1 with the different cardiac FPD and PPI along with the sodium (Na+), calcium (Ca2+), and potassium (K+) components depicted. Millivolt scale recording of hPSC-CMs is possible with the MEA platform and these cardiomyocytes exhibited stable beating rhythms (Figures 1 and 4). The corresponding QT and RR intervals are also presented in Figure 1.

The relationship of the dynamics of native QT interval/FPD and RR/PPI is presented in Figure 2. These parameters have a clear positive correlation. The coefficient of determination of approximately 0.71 for hPSC-CMs (linear fitting) in figure 1 shows that 71% of the FPD prolongation is explained by concurrent prolongation of the PPI. The coefficient of determination was about 0.68 for ECG.

Figure 3A demonstrated the relationship between QTc and cFPD measurements. The hPSC-CMs had a mean cFPD of 400 ms with s SD 64 ms. Volunteers had a mean QTc 432 ms, SD 39 ms. Uncorrected mean FPD value was 375 ms with a mean BR of 73 bpm. Uncorrected mean QT was 397 ms with a mean heart rate 76 bpm.

We investigated the relationship of electrical activation and beating rate clinically in the adult myocardium with ECG and *in vitro* with MEA recordings of hESC-derived cardiomyocytes. Figure 3B depicts the mean values of measured QT-to-RR (QT/RR) and FPD-to-PPI (FPD/PPI) relationships in ECG and MEA recordings, respectively. Their FPD/PPI mean value was 0.44, ranging between 0.358-0.545. Volunteers´ QT/RR mean value was 0.485, ranging between 0.312-0.634.

Figure 4 shows the PPI dynamics of cardiac MEA recordings with Poincaré plots where each the duration of each PPI interval is plotted against the preceding PPI interval. The plots show stable beating dynamics just above or below 1 hertz (Hz), which is evident from the small scatter and linear relationship.

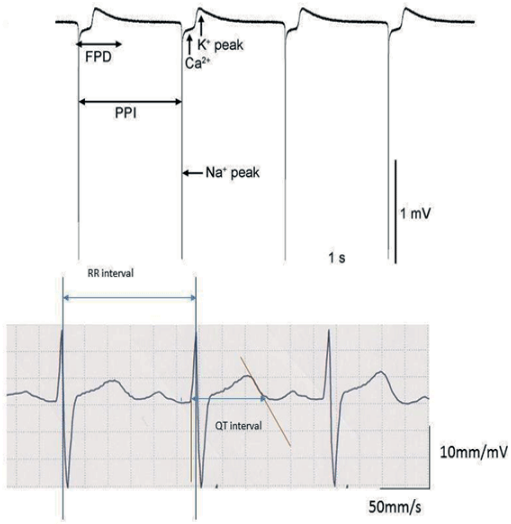

Figure 1: Characteristics of field potential (FP) (upper panel) and ECG (lower panel) recordings. A FP trace from one of the recording electrodes on the microelectrode array generated by the spontaneously beating human embryonic stem cell –derived cardiomyocytes. The field potential duration (FPD) and peak-to-peak interval (PPI) is also depicted in the first cardiac FP cycle along with the sodium (Na+), calcium (Ca2+), and potassium (K+) currents in the cardiac FP components.

We compared FPD with QT time and PPI with RR. An ECG is shown how heart rate is determined by RR cycle lengths (ms) before measured QT interval. QT interval is measured from the beginning of the QRS complex to the tangent to the end of the T wave.

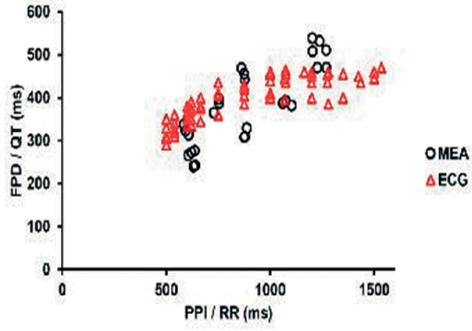

Figure 2: Correlation of beating rate and QT and FPD duration. RR-interval (ms) of electrocardiograms is plotted against QT- intervals (ms) and peak-to-peak interval (PPI, ms) of pluripotent stem cell –derived cardiomyocyte (hPSC-CM) recordings against field potential durations (FPD, ms). For hPSC-CMs linear fitting shows a value of approximately 0.71 for the coefficient of determination and for ECGs about 0.68.

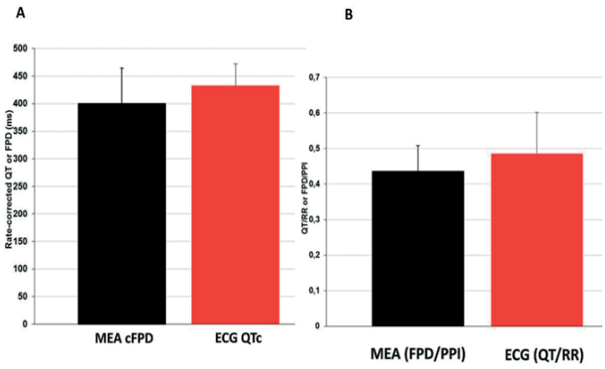

Figure 3: Comparison on electrocardiogram (ECG) and field potential (FP) parameters. A) Rate-corrected QT interval (QTc) and field potential duration (cFPD) times (ms). B) The ratio of QT interval (ECG) or FPD microelectrode arrays (MEA) to duration of one beat (RR with ECG and PPI with MEA recordings). The ratio was calculated with all the intervals measured in milliseconds.

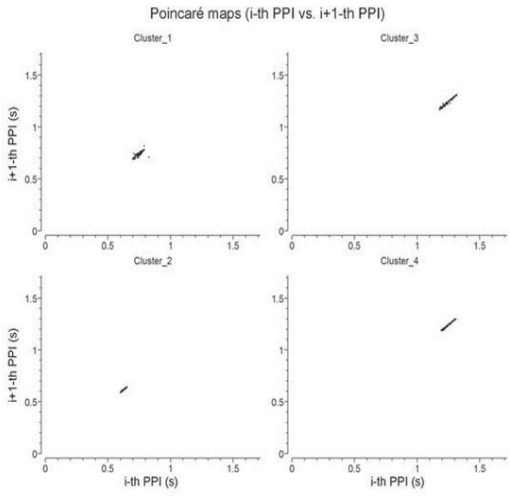

Figure 4: Beat rate dynamics within microelectrode arrays (MEA) recordings of human embryonic stem cell -derived cardiomyocytes (hESC-CM) clusters. Representative Poincaré maps of peak-to-peak (PPI) intervals. Poincaré maps show the relation of a given PPI (x-axis) to its preceding PPI (y-axis) in seconds. Tight grouping and linear relationship of the data points suggest stable beating dynamics in the field potential (FP) recordings.

Discussion

Electrical properties of the heart are due to ion transfer across the cell membrane. *in vivo*, the transfer rate of potassium ions is dependent on the beating rate [20]. The repolarization time presented as the QT interval in the ECG is, thus, dependent on how fast the heart beats [14]. Because heart rate is the principal determinant of repolarization length, there are several formulas how to correct the measured QT interval so that QT intervals with different beating frequencies can be compared [12]. The simplest and most widespread approach to correct the QT interval is to divide its value by the square root of the preceding RR interval expressed in seconds ($QTc = QT/\sqrt{RR}$), i.e., by using Bazett's formula [21].

The definition of normal QT time has been difficult because abnormal QT interval can be either "too long" or "too short". Large

population studies suggest that, for the adult population, normal QTc values for males are 350 to 450 ms and for females 360 to 460 ms [22]. Therefore, the upper limit for the normal QTc for both genders is 0.44 seconds [12]. Nevertheless, the Bazett's correction overestimates the number of patients with a prolonged QT [23]. However, marked abnormalities of the QT interval may be caused by many different reasons and situations: genetic disorders (e.g., long/short QT syndrome), pharmacologic agents (e.g., antiarrhythmics, antipsychotics, antibiotics), electrolyte abnormalities (e.g., hypokalemia and hypomagnesemia), and their interactions [13]. Especially when pharmacologic agents cause QT changes, we can choose individual treatments for patients by combining evidence from *In vivo* and *in vitro* studies using hPSC-CM- techniques [5]. Disease-specific likewise patient-specific hPSC-CMs also function in the study of heart diseases such as arrhythmias and electrophysiologcal abnormalities in patients [24, 25].

It is important that we have methods to examine cardiomyocyte function that correlates with *in vitro* models of the native human myocardium. At the moment there are no direct reports about the relationship of intricate electrophysiological characteristics between the human heart and hPSC-CMs. Here we investigated the relationship of electrical activation and beating rate clinically using ECG and MEA recordings *in vitro* of hPSC-CMs, and we found that the results correlated well with each other.

With hPSC-CMs these same correction formulas have been used without any evidence whether the same formulas are effective during *in vitro* situations [11]. In this paper we chose control individuals with low baseline beating rate and performed a stress exercise test to obtain QT variation over the same beating frequency spectrum as obtained with spontaneously beating hPSC-CMs. The cells were cultured on MEA platform and the FPD, corresponding to QT in ECG, was measured. A strong correlation was observed between QT intervals and FPDs, and also the correction of these parameters using Bazett's formulation gave similar results thus confirming that the ion fluxes in cell culture present with corresponding performance as they do *in vivo* in the heart.

The hPSC-CMs beating rates in our cell cultures varied from 55 (minimum) to 100 (maximum) bpm with the average of 73 bpm, which is in the same range as typically observed in human heart. Normally the QT interval of the heart decreases with increasing heart rate due to increase [14] in potassium flux across the cell membrane [20]. We compared the cardiac parameters between ECG and MEA recordings and could show that QT and RR parameters correlate excellently with the FPD and PPI results. In the volunteers who were used for comparison the heart rate varied 39-120 bpm with an average of 76 bpm (Table II and Figure 2).

Table 2: The cardiac parameters measured from the electrocardiogram (ECG) and microelectrode array (MEA) recordings. Parameters were QT and RR interval in ECG measurements and field potential duration (FPD) and peak-to-peak interval (PPI) in MEA measurements. The first measurement is heart rate 40 beats per minute (bpm), second measurement 80 and third measurement 120 bpm in the ECG.

ECG			
Parameter (Volunteer/hiPSC-CM Cluster #)	first measurement	second measurement	third measurement
QT (Volunteer #1)	405	365	290
RR (Volunteer #1)	1350	750	500
QT (Volunteer #2)	450	410	330
RR (Volunteer #2)	1500	750	500
QT (Volunteer #3)	455	410	350
RR (Volunteer #3)	1500	750	500
QT (Volunteer #4)	435	385	310
RR (Volunteer #5)	1435	750	500
QT (Volunteer #5)	460	390	305
RR (Volunteer #5)	1425	750	500
MEA			
FPD (Cluster #1)	382	393	387
PPI (Cluster #1)	1103	1076	1060
FPD (Cluster #2)	240	240	242
PPI (Cluster #2)	632	632	640
FPD (Cluster #3)	308	309	329
PPI (Cluster #3)	875	879	888
FPD (Cluster #4)	442	469	456

PPI (Cluster #4)	877	862	875
FPD (Cluster #5)	365	387	397
PPI (Cluster #5)	729	756	753
FPD (Cluster #6)	313	323	338
PPI (Cluster #6)	608	593	585
FPD (Cluster #7)	277	272	266
PPI (Cluster #7)	637	621	606
FPD (Cluster #8)	470	470	539
PPI (Cluster #8)	1271	1228	1203
FPD (Cluster #9)	511	532	508
PPI (Cluster #9)	1269	1238	1204

QT/RR value means how quickly and how much QT-interval changes when heart rate changes [26], corresponding to FPD/PPI values in MEA. The over- or under correction of QTc may lead to significant and systematic bias with both false positive and false negative findings, whereas QT/RR patterns in all different subjects will be characterized by the same mathematical form. Population risk stratification studies investigating QT/RR patterns would also benefit from this mathematical description avoiding the heart rate influence [27]. QT/RR mean value of our volunteers was 0.433, ranging 0.40-0.46. The mean FPD/PPI value of 0.44 indicates that the basal FPD in relation to the time between individual beats corresponds with QT/RR values.

The coefficient of determination value of 0.71 is good for biological samples. Our results indicate that 71% of the FPD prolongation is explained by concurrent prolongation of the PPI. However, the FPD generally increased when PPI was prolonged. Taken together with the observation that hPSC-CMs demonstrated fairly stable PPI dynamics within recordings, we conclude that they are rather reliable models in terms of their electrophysiological aspects, especially when corrected for beating rate –dependent FPD modulation. Of note, in different individuals the predicted QT times may normally vary up to 90 ms even if the RR-cycle lengths are the same [12]. QT time variation between our volunteers was less than 60 ms at various RR cycle lengths. The coefficient of determination of our volunteers was about 0.68 for ECG.

The cFPD value of 400 ms for hPSC-CMs is within the physiological normal range for QTc values. Because uncorrected FPD was 375 ms at 73 bpm, the Bazett's formula is adequately compensating for the rate-induced FPD shortening also in hESC-CMs. Analogously, the volunteers had a mean QTc 432 ms and their uncorrected mean QT was 397 ms with a mean heart rate 76 bpm. However, heart rate of 60 bpm is the most optimal when using Bazett's formula [21]. Usually the relationship between QT/RR adaptation and mean QTc values means that those subjects who show longer QTc intervals have steeper QT/RR patterns [19]. In our study we demonstrated that the relationship FPD/PPI adaptation and cFPD values functioned similarly to the relationship between QT/RR adaptation and QTc values.

In this study beat rate was a little slower than heart rate, therefore cFPD was a slightly shorter than QTc. It is also known that Bazett's formula overcorrects the QT interval at high heart rates [12] and under-corrects QT time at low heart rates [18]. This probably is the reason for this small difference between our cFPD and QTc values.

In this research we did not compare the different correction formulas. However, there is no consensus, which would be clinically the optimal formula (Fridericia's or Bazett's formula) [19]. Although Bazett's formula is the most widely used for QT-rate correction, AHA/ACCF/HRS has also recommended that linear regression functions rather than the Bazett's formula be used for QT-rate correction [28]. However, the Bazett's formula is easy to use and widely applied and thus it was chosen in this study to compare *in vitro* and *In vivo* electrical parameters in different beating rates.

Conclusions

QT time is beating rate dependent and QTc is the rate corrected QT interval adjusted for the changes due to beating rate and corresponding ion flux across the cell membrane. The same correction formula was equally applicable to the corresponding repolarization parameter (FPD) in hPSC-CMs and thus the same rate correction formula can be applied to electrical recordings in cell culture situation.

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Declarations

Conflict of Interest

There are no conflicts of interest by any of the authors of this manuscript

Competing interests

The authors declare that they have no competing interests.

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