The role of molecular genetics in the diagnoses of sudden arrhythmic death syndromes in New Zealand

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2010
Abstract

Inherited cardiac arrhythmias are leading causes of death and disability. The cardiac channelopathies, of which long QT syndrome (LQTS) is most common, are a heterogeneous group of disorders of cardiac ion channels that can cause sudden unexplained death in up to 80, otherwise healthy, young New Zealanders each year. LQTS is characterised by recurrent syncope (fainting), seizure and sudden death. Clinical diagnosis can be challenging and therapeutic intervention often depends on the gene responsible. Therefore, it is important to obtain a molecular diagnosis wherever possible to establish preventative clinical best-practice. The Cardiac Inherited Disease Group (CIDG) is a multidisciplinary group that aims to save the lives and support the families of children and young adults who have inherited cardiac arrhythmia syndromes. This study forms part of the research arm of the CIDG. To identify and assess the frequency and spectrum of LQTS associated mutations, both clinically diagnosed LQTS families and a sudden unexpected death cohort were screened for mutations in the five LQTS genes using denaturing high performance liquid chromatography (dHPLC) and direct sequencing. The mutation detection rate was 52%. To investigate the molecular genetic cause of LQTS symptoms in the remaining mutation-negative cohort, multiplex ligation-dependent probe amplification and comparative genomic hybridization arrays were used to investigate copy number variation in LQTS-associated genes. Altered exon copy number was detected in 3 (11.5%) patients: (1) a KCNQ1 exon 13-14 deletion (2) a KCNH2 exon 6-14 deletion and (3) a KCNH2 exon 9-14 duplication, suggesting that sequencing-based mutation detection strategies should be followed by deletion/duplication screening in all LQTS mutation-negative patients. As catecholaminergic polymorphic ventricular tachycardia (CPVT) presents similarly to LQTS, the CPVT-associated cardiac ryanodine receptor gene (RYR2) was screened for mutations using direct sequencing. Five RYR2 variants were detected among the LQTS mutation-negative cases. Lastly, a selection of unclassified variants or single nucleotide polymorphisms (SNPs) were typed using Taqman genotyping assays as a pilot study to investigate their role as potential susceptibility loci or genetic modifiers of established mutations. No significant associations were identified. However, certain trends worth following up in a larger cohort were noted. Although the genetic cause of arrhythmia symptoms has been established in nine mutation-negative index cases, further studies using different strategies are required to establish the genetic cause in a greater proportion of mutation-negative cases.
Acknowledgements

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This work is dedicated to New Zealand patients and families

with life-threatening, inherited cardiac arrhythmias
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Abbreviations

α  Alpha
β  Beta
AD  autosomal dominant
ADHB  Auckland District Health Board
AF  atrial fibrillation
AP  action potential
AR  autosomal recessive
ARVD/C  arrhythmogenic right ventricular dysplasia or cardiomyopathy
AV  atrioventricular
bp  Base pair
BrS  Brugada syndrome
BVT  bidirectional ventricular tachycardia
CHD  coronary heart disease
CIDG  Cardiac Inherited Diseases Group
CPVT  catecholaminergic polymorphic ventricular tachycardia
C_t  Threshold cycle
dNTPs  Deoxyribonucleic acids
ECG  electrocardiogram
EDTA  Ethylenediaminetetraacetic acid
GMS  Grantham matrix score
HCM  hypertrophic cardiomyopathy
HIPC  Health Information Privacy Code
Hrs  Hours
ICD  implantable cardiac defibrillator
JLNS  Jervell and Lange Nielsen syndrome
Kb  Kilobase
KDa  Kilodalton
LQTS  long QT syndrome
MAF  minor allele frequency
mRNA  messenger RNA
NCBI  National Centre for Biotechnology Information
NTC  no template control
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PK  Proteinase K
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>PolyPhen</td>
<td>Polymorphism phenotyping</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>Rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RWS</td>
<td>Romano-Ward syndrome</td>
</tr>
<tr>
<td>SAD</td>
<td>sudden arrhythmic death</td>
</tr>
<tr>
<td>SCA</td>
<td>sudden cardiac arrhythmia</td>
</tr>
<tr>
<td>SCD</td>
<td>sudden cardiac death</td>
</tr>
<tr>
<td>SD</td>
<td>sudden death</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SIFT</td>
<td>Sorts intolerant from tolerant</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SQTS</td>
<td>short QT syndrome</td>
</tr>
<tr>
<td>SSS</td>
<td>sick sinus syndrome</td>
</tr>
<tr>
<td>SUD</td>
<td>sudden unexplained death</td>
</tr>
<tr>
<td>SUDY</td>
<td>sudden unexplained death in the young</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetic acid EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VF</td>
<td>ventricular fibrillation</td>
</tr>
<tr>
<td>VT</td>
<td>ventricular tachycardia</td>
</tr>
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</table>
CHAPTER 1:

INTRODUCTION
1 Introduction

1.1 Sudden death in the young

Sudden death (SD) in the young is a relatively common problem which is recently receiving increasing recognition (Chugh 2004; Albert 2010). Depending on the underlying cause, SD can be divided into sudden cardiac death (SCD), defined as SD from a cardiac cause, and SD due to noncardiac causes, for example, intracranial hemorrhage, epilepsy, pulmonary embolism, or asthma (Tough et al. 1996; Wren et al. 2000). This subdivision is clinically relevant because cardiac causes are usually inherited, whereas noncardiac causes are not. Death in absence of a diagnosis despite autopsy is generally termed sudden unexplained death (SUD) and if it occurs in individuals aged less than 40 years, sudden unexplained death in the young (SUDY).

The incidence of SD in the general population ages 20 to 75 years is 1 in 1000 individuals, which accounts for 18.5% of all deaths (De Vreede-Swagemakers et al. 1997). The vast majority of cases are considered to be SCD and, of these, most are attributed to coronary artery disease in older individuals, mainly men (Zipes et al. 1992). The overall number of cases of SD in those at younger ages (less than 40 years) is still largely unknown, although estimates from the mid-nineties range from 1.3 to 8.5 per 100 000 person-years (Shen et al. 1995; Libethson 1996).

In the USA, an incidence of sudden cardiac death in children higher than leukaemia, has led to a bill before the senate to raise funds to address the issue. The Teague Ryan Sudden Child Cardiac Arrhythmia Syndromes Screening and Education Act of 2007 (H.R. 2887) was introduced to "amend the Public Health Service Act to provide for a program of screenings and education regarding children with sudden cardiac arrhythmia syndromes."
In the United Kingdom, there are about 500 “unsolved” cases of young sudden death per annum. In response to a campaign led by surviving relatives of these victims and sudden cardiac death charities, there was parliamentary legislation (Dari’s bill) to add sudden death syndromes to the Cardiac National Service Frameworks with appropriate funding. In Australia, recent post mortem studies estimate the annual incidence of sudden death in children and young adults to be approximately 20 per million (Doolan 2004). The incidence of SD in the 1-40 year age group in New Zealand is unknown. However, if it is similar to that in the USA, UK and Australia, we may expect about 80 such deaths per year in otherwise healthy, young New Zealanders.

It is difficult to extract more accurate estimates, mostly because there is no unifying diagnosis that allows the incidence of SUDY to be tracked. Worldwide, such unexplained deaths are often certified by general practitioners as myocardial infarction, epilepsy, or by the mechanism of death which might have been drowning, road traffic accident or other apparently accidental cause of death. Cases fortunate enough to be selected to undergo a post mortem examination are often categorised as death by “natural causes” or possible cardiac arrhythmia if no diagnosis can be made.

To make it even more difficult to assess the incidence of SUDY, these deaths tend to fall into three age groups (0-1, 1-16 and 16-40 years) with overlapping major causes of sudden death, some of which may be identified at autopsy. Within the 16-40 year age group, 35-59% of deaths have been attributed to structural causes that include coronary heart disease (CHD) (Doolan 2004). Hypertrophic cardiomyopathy (HCM), arrhythmogenic right ventricular cardiomyopathy (ARVC) and dilated cardiomyopathy were identified as the major non-CHD structural causes (Doolan 2004; Eckart 2006; Chugh 2004). Sudden death in young athletes is most often attributed to one of these inherited heart diseases that have
a structural component, particularly HCM. However, population-based autopsy series have revealed that it is more common for SUDY to occur in individuals with a structurally normal heart (Doolan et al. 2004; Puranik et al. 2005).

Genetic screening of sudden death victims and/or cardiological investigation of family members, have revealed that inherited disorders of cardiac ion channels, such as long QT syndrome (LQTS), Brugada syndrome (BrS) and catecholaminergic polymorphic ventricular tachycardia (CPVT), contribute up to 35% of such deaths in 1–40-year-olds (Tan et al. 2005; Tester and Ackerman 2007) and at least 10% in infants (Arnestad et al. 2007). These “cardiac channelopathies” result in disturbance of the cardiac action potential, and generally these hearts appear completely normal under the microscope.

Multiple genes and allelic variants of channelopathies have been discovered in the last decade. The growing understanding of the pathophysiology of arrhythmic syndromes has highlighted an extremely high degree of genetic heterogeneity; more than 30 genes and hundreds of variants within these genes have been identified in association with these disorders, and revealed the complex nature of the relation between genotype and phenotype. Incomplete penetrance of mutations and extreme variability in clinical manifestations that range from distinguishing electrocardiographic patterns to life-threatening ventricular arrhythmias, have been observed. Even within a family, the same genetic mutation may result in striking phenotypic variation, from sudden death of an infant or young child to asymptomatic carriage in the elderly. However, results of genotype-phenotype correlation studies have also revealed distinguishing features of individual mutations or variants. Genetics is now emerging as an important tool contributing not only to a better diagnosis but also to risk stratification, treatment and management of arrhythmic syndrome patients at risk of sudden death.
1.2 Cardiac conduction

To understand the pathogenic effects of disrupted ion channels in congenital arrhythmia syndromes, it is helpful to be reminded of the physiology behind “normal” cardiac conduction and the resultant electrocardiographic waveforms and intervals.

1.2.1 Sequence of excitation

Contraction of cardiac muscle, like that of other muscle types is triggered by depolarisation of the plasma membrane of the cells making up the muscle. In myocardial cells, action potentials (AP) spread from one cell to another via gap junctions, allowing the excitation of one cell to result in the excitation of all cells.

Two types of cardiac tissue allow conduction of electrical impulses, ordinary myocardium (atrial and ventricular) and the specialised cardiac conduction system which includes the sinoatrial, or sinus node; anterior, middle and posterior internodal tracts; atrioventricular (AV) node; His bundle; right and left bundle branches; anterior-superior and posterior-inferior divisions of the left bundle and the Purkinje network. The normal electrical impulse is generated by the sinus node (located in the right atrium), resulting in a wave of depolarisation that quickly travels through the atria (Fisch 1980) (Figure 1.1). After reaching and stimulating the atrioventricular junction, which consists of the atrioventricular node and the His bundle, the impulse slows slightly as it moves through the specialised atrioventricular tissue, allowing blood to transfer from the atria to the ventricles (Goldberger 1994). The impulse then quickly travels through the His bundle as it bifurcates into left and right bundle branches and the terminal filaments of the Purkinje fibres, resulting in a wave of ventricular depolarisation and contraction. After the surge of
depolarisation moves through the heart, the final step of each cardiac conduction cycle is tissue repolarisation, which is necessary before another cycle can occur.

**Figure 1.1 Electrical Conduction System of the Heart.** Image adapted from Beth Israel Deaconess Medical Centre (http://www.bidmc.org/CentersandDepartments/Departments/Medicine/Divisions/CardiovascularMedicine/DiseasesandConditions/Arrhythmias/)

### 1.2.2 The electrocardiogram

The electrocardiogram (ECG) is primarily a tool for evaluating the electrical events within the heart. As an impulse progresses through the cardiac tissue, an upward movement on the ECG represents the wave of depolarisation moving toward a positive electrode. Based on the complex cycle of depolarisation and repolarisation, typical electrocardiographic waveforms are generated (Figure 1.2). These waveforms include the P wave (atrial depolarisation), QRS complex (ventricular depolarisation), and T wave (ventricular repolarisation). The U wave (not shown on Figure 1.2) follows the T wave and represents repolarisation of the Purkinje fibres and ventricular relaxation. Electrocardiographic
intervals are also important; the PR interval is the time for atrial depolarisation and impulse propagation through the atrioventricular junction, and the QT interval is the time for ventricular depolarisation and subsequent repolarisation (Fisch 1980).

Figure 1.2 A typical one-cycle ECG tracing.

A direct relationship exists between the action potential in a single myocardial fibre and the recorded ECG (Figure 1.3). The shape of the action potential is based on the balance between the inflow of positive ions (sodium and calcium) and outflow of positive ions (potassium) (Viskin 1998). When an electrical impulse reaches a myocardial fibre, it initially results in the brisk movement of sodium ions inward, resulting in a sharp, rapid upstroke of the action potential (phase 0). Phase 1 repolarisation is mediated by transient potassium efflux. The plateau (phase 2) is due to the balance created by calcium ions entering slowly while potassium ions exit the cell. Phase 3 repolarisation is caused predominantly by outward potassium current. At the end of the action potential there is electrolyte equilibration (phase 4).
An ECG is not a direct record of the changes in membrane potential across cardiac muscle cells but rather a measure of the currents generated in extracellular fluid by these changes. Because arrhythmia disorders alter normal ion transfer across myocardial cells, and thereby the shapes and timing of the waves, an ECG is a powerful tool in the diagnosis of the cardiac “channelopathies.”

![Diagram of cardiac action potential and ECG](image)

**Figure 1.3** Temporal relationship of a representative cardiac action potential (a) with a surface ECG (b). Diagram (a) shows ion movement during the action potential. Phase 0 = sodium enters the cell through fast sodium channels; Phase 1 = fast sodium channels close; 2 = calcium and additional sodium enter the cell through slow channels; 3 = potassium exits the cell, and resting membrane potential is reestablished; 4 = equilibration of sodium and potassium occurs. The 400ms indicates the typical time length of the ST interval.
1.3 Inherited cardiac arrhythmia syndromes associated with sudden death

Arrhythmic disorders can be secondary to congenital, acquired, or sporadic disorders. Yet, recent research demonstrates that even acquired arrhythmias may be associated with genetic variants in specific cardiac channels (Lehnart et al. 2007; Roden 2008). More than 30 genes have been identified in association with arrhythmic syndromes. The relation between genotype and phenotype is not straightforward in most genetic disorders, due to incomplete penetrance of mutations and the variability in clinical manifestations. This is especially true in the inherited cardiac arrhythmia syndromes, where one gene can be responsible for different disorders, and even in the same family a single disorder can be due to mutations in any one of several genes.

The following is an introduction to the main inherited arrhythmia disorders that fall within the scope of this thesis: long QT syndrome, Brugada syndrome and catecholaminergic polymorphic ventricular tachycardia. Other inherited arrhythmias that are less commonly responsible for SUDY (such as short QT syndrome (SQTS), sick sinus syndrome (SSS) and atrial fibrillation (AF)) or are classified more as structural cardiomyopathies (such as hypertrophic cardiomyopathy (HCM) and arrhythmogenic right ventricular dysplasia or cardiomyopathy (ARVD/C)) are introduced briefly, thereafter.

1.3.1 Long QT syndrome
Long QT syndrome (LQTS), described initially by Jervell and Lange-Nielsen in 1957 and named such due to the lengthened QT interval observed in patients’ ECG tracings, is the most common inherited arrhythmia syndrome.
1.3.1.1 Epidemiology

LQTS was originally thought to be an extremely rare disorder, however, the early speculation that the disorder was “undoubtedly more unrecognised than rare” is in fact more true (Roden 2008). The prevalence of LQTS mutation carriers in the USA is estimated at 1 in 1000 – 3000, resulting in as many as 3000-4000 sudden deaths in children and young adults each year (Priori et al. 1999; Yang et al. 2002). The data is still insecure for the incidence of LQTS in New Zealand. However, the Cardiac Inherited Disease Group (CIDG) is aware of at least more than 100 probands with LQTS, and the number is growing. If one assumes an average of 10 gene carriers within the extended family per proband, then the incidence would be at least 1000 per 4 million, or 1 in 4000, which makes LQTS nearly as common as cystic fibrosis. During the preparation of this thesis, Schwartz and colleagues found an incidence of 1 in 2500 from an ECG screening programme of infants in Italy, backed up by genetic diagnosis (Schwartz et al. 2009).

1.3.1.2 Symptoms

The typical characterisation of LQTS is prolonged ventricular repolarisation which may manifest as syncope, apneic seizures or sudden death via rapid ventricular tachycardia (torsades de pointes), which can deteriorate to ventricular fibrillation, in otherwise fit and healthy young people (Moss et al. 1991; Ackerman et al. 1998; Keating and Sanguinetti 2001). Symptoms usually occur after a precipitating event, such as physical activity, emotional stress or auditory stimuli but may also occur during periods of sleep or rest in some individuals (Schwartz et al. 2001). This is particularly true in infancy, where the cardiac sodium channel encoded by the gene SCN5A accounts for about half of infant deaths related to LQTS, whereas it occurs in about 8% of the older population presenting with LQTS (Splawski et al. 2000; Schwartz et al. 2001; Towbin 2001). LQTS has also been confused with epilepsy (Towbin and Vatta 2001).
LQTS can occur as an inherited or sporadic disorder, or it may be acquired (Towbin and Vatta 2001). Although the clinical presentation is similar in all forms, there are some minor variations. Two inherited forms have been characterised based on their patterns of transmission: Romano-Ward Syndrome (RWS) (Romano et al. 1963) and Jervell and Lange-Nielsen Syndrome (JLNS) (Jervell and Lange-Nielsen 1957). RWS is the most common form of inherited LQTS and is transmitted as an autosomal dominant trait with a pure cardiac phenotype (prolonged QT interval with associated symptom complex of syncope, seizures and possibly sudden death) (Dumaine and Antzelevitch 2002). In contrast, JLNS is rare, and inherited in an autosomal recessive manner with a more malignant course and an association with congenital deafness (Dumaine and Antzelevitch 2002). LQTS can be acquired under conditions of exposure to certain drugs (antiarrhythmics, some antihistamines and antibiotics), electrolyte abnormalities, dietary deficiencies and some cardiac conditions such as myocardial infarction and dilated cardiomyopathy (Roden et al. 1996). Recent studies have indicated that acquired LQTS, the most common form of LQTS, also has a predisposing genetic basis in genes associated with congenital LQTS. Specific examples have been identified in individuals with drug-induced LQTS (Sesti et al. 2000; Makita et al. 2002; Yang et al. 2002; Harrison-Woolrych et al. 2006). Sporadic or seemingly not inherited LQTS is also recognised when diagnostic features of LQTS occur in a patient, but with no family history and test negative for mutations in the typical LQTS candidate genes (Towbin and Vatta 2001).

**1.3.1.4 The genetics of LQTS**

The extreme heterogeneity of LQTS’s clinical natural history, ranging from asymptomatic longevity to sudden death in infancy, and ECG phenotype, ranging from a completely normal resting ECG to extreme QT prolongation with varying T-wave alternans, is carried
through to the disorder’s genetic underpinnings (Ackerman 1998). At the outset of this project, in 2006, over 300 mutations involving eight genes had been reported in LQTS. Over the past four years, the numbers have grown to well over 700 mutations in 12 genes, almost all of which encode critical components of cardiac ion channels that coordinate the cardiac action potential (Table 1.1, references therein). Dominant mutations in five of these genes are typically associated with RWS, whereas homozygous or heterozygous compound mutations of either $KCNQ1$ or $KCNE1$ are linked to JLNS (Neyroud et al. 1997; Schulze-Bahr et al. 1997; Splawski et al. 1997). There are also examples where two mutations in two different LQTS genes coexist and often leading to severe and complex arrhythmia (Kobori et al. 2004; Lupoglazoff et al. 2004).
<table>
<thead>
<tr>
<th>Disorder</th>
<th>Locus</th>
<th>Gene</th>
<th>Protein</th>
<th>Ion channel</th>
<th>Frequency</th>
<th>Inheritance</th>
<th>ECG characteristics</th>
<th>Trigger</th>
<th>Functional Consequences</th>
<th>Ref*</th>
</tr>
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<tbody>
<tr>
<td>LQTS1</td>
<td>11p15</td>
<td>KCNQ1</td>
<td>KvLQT1</td>
<td>I\textsubscript{Ks}</td>
<td>~50%</td>
<td>AD</td>
<td>broad base 'early onset' T wave</td>
<td>exercise, especially swimming</td>
<td>Loss of function I\textsubscript{Ks} ↓</td>
<td>a)</td>
</tr>
<tr>
<td>LQTS2</td>
<td>7q35</td>
<td>KCNH2</td>
<td>hERG</td>
<td>I\textsubscript{Kr}</td>
<td>30-40%</td>
<td>AD</td>
<td>small late T wave</td>
<td>adrenergic triggers, nightly noise</td>
<td>Loss of function I\textsubscript{Kr} ↓</td>
<td>b)</td>
</tr>
<tr>
<td>LQTS3</td>
<td>3p21</td>
<td>SCN5A</td>
<td>Na.1.5</td>
<td>I\textsubscript{Na}</td>
<td>5-10%</td>
<td>AD</td>
<td>'Late onset' T wave with normal configuration</td>
<td>rest or sleep</td>
<td>Gain of function I\textsubscript{Na} ↑</td>
<td>c)</td>
</tr>
<tr>
<td>LQTS4</td>
<td>4q25</td>
<td>ANK2</td>
<td>AnkyrinB</td>
<td>I\textsubscript{Na}, K</td>
<td>&lt;1%</td>
<td>AD</td>
<td>Abnormal targeting of Ca\textsuperscript{2+} regulatory proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LQTS5</td>
<td>21q22.1</td>
<td>KCNE1</td>
<td>minK</td>
<td>I\textsubscript{Ks}</td>
<td>&lt;1%</td>
<td>AD/AR</td>
<td></td>
<td></td>
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<tr>
<td>LQTS6</td>
<td>21q22.1</td>
<td>KCNE2</td>
<td>MiRP1</td>
<td>I\textsubscript{Ks}</td>
<td>&lt;1%</td>
<td>AD</td>
<td>Loss of function I\textsubscript{Ks} ↓</td>
<td>e)</td>
<td></td>
<td></td>
</tr>
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<td>LQTS7(^i)</td>
<td>17q23</td>
<td>KCNJ2</td>
<td>KIR2.1</td>
<td>I\textsubscript{Kr}</td>
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<td>AD</td>
<td>Loss of function I\textsubscript{Kr} ↓</td>
<td>f)</td>
<td></td>
<td></td>
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<td>12p13.3</td>
<td>CACNA1C</td>
<td>Ca.1.2</td>
<td>I\textsubscript{C\textsubscript{G\textsubscript{L}}}</td>
<td>&lt;1%</td>
<td>AD</td>
<td>Loss of function I\textsubscript{C\textsubscript{G\textsubscript{L}}}</td>
<td>g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LQTS9</td>
<td>3p25.3</td>
<td>CAV3</td>
<td>Caveolin 3</td>
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<td></td>
<td></td>
<td>alternating T waves</td>
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<td>11q23.3</td>
<td>SCN4B</td>
<td>Na.1.5 b4</td>
<td>I\textsubscript{Na}</td>
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<td></td>
<td></td>
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<td>7q21</td>
<td>AKAP9</td>
<td>Akap9</td>
<td>I\textsubscript{Kr}</td>
<td>1 family</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LQTS12</td>
<td>7q21</td>
<td>SYNTAI</td>
<td>Syntrophin1</td>
<td>I\textsubscript{S}</td>
<td>1 family</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


\(^i\) LQTS7 is also known as Andersen syndrome (AS), \(^2\) LQTS8 is also known as Timothy syndrome (TS)
1.3.1.5 The diagnosis of LQTS

A diagnosis of LQTS was originally made from a combination of clinical and family history, and by ECG assessment. One difficulty of QT interpretation is that the QT interval gets shorter as the heart rate increases. This problem can be solved by correcting the QT time for heart rate using the Bazett formula: $QTc = \frac{QT}{\sqrt{RR\text{ interval/sec}}}$.

LQTS patients have a QTc that ranges from about 410 ms to over 600 ms. The range of values in a normal population is about 350 ms to 460 ms with “cut off” normal values below 450 ms for men and below 460 ms for women (Rautaharju et al 2009). Consequently, there is overlap of QTc values between LQTS patients and control individuals in the 410 ms to 460 ms range. Approximately 30% of LQTS gene carriers have a normal to borderline QTc of 410 - 460 ms and about 60% of the normal population has a QTc of 410 - 460 ms, so when screening patients for possible LQTS, a large percentage will have QTc values which can neither make nor exclude the diagnosis of LQTS.

The diagnostic challenges of the QT interval are exacerbated by the T wave morphology. Moss and colleagues (1995) first reported a T wave pattern characteristic for each genotype. Zhang and colleagues (2000) further described patterns characteristic for each genotype, reporting four for LQT1, four for LQT2 and two for LQT3. These T patterns can be helpful for predicting the correct genotype in families, and can be of assistance in the diagnosis of LQTS in cases of borderline QT duration. Figure 1.4 shows representative ECG tracings of the three most common types of LQTS.
Figure 1.4 Typical ECG traces of the three most common forms of LQTS. LQTS1 - 'early onset' broad based T wave, LQTS2 - small late T wave and LQTS3 - prolonged QT interval with 'late onset' T wave with a normal configuration. Leads II, aVF and V5 are three of the leads from a standard 12 lead ECG. Please see section 8.3 in the Appendix for a more detailed explanation and Figure 8.2c for a 'normal' 12 lead ECG trace.

It has also become apparent that different genotypes tend to be associated with typical phenotypes and/or disease triggers; for example, symptoms of syncope and sudden death are most commonly triggered by exercise with \( KCNQ1 \) mutations, sudden noise with \( KCNH2 \) mutations, and occur mostly at rest with \( SCN5A \) mutations. The risk of cardiac events appears to be higher in males until teenage years, but then higher in females during adulthood (Locati et al. 1998; Hobbs et al. 2006; Sauer et al. 2007; Goldenberg et al. 2008). Boys and young men are at high risk of sudden death in \( KCNQ1 \) and \( SCN5A \) mutations, but young women are at highest risk with \( KCNH2 \) mutations (Hobbs et al. 2006; Sauer et al. 2007; Goldenberg et al. 2008). The position and type of mutation also affect phenotypic outcome. Mutations situated in the transmembrane and “pore” regions of the ion channels tend to be more malignant, and large deletions and duplications in LQTS genes are an emerging diagnostic consideration (Koopmann et al. 2006; Eddy et al. 2008 and Chapter 4).

Genetics, therefore, has an increasing role to play in the diagnosis of LQTS and, indeed, in the diagnosis of other inherited arrhythmic syndromes too, which are discussed next.
The mechanisms by which mutations in the genes responsible for arrhythmic disorders exert their pathogenic effect will be reviewed later (Chapter 1.4).

1.3.1.6 Treatment and management of LQTS

In the initial publication by Jervell and Lange-Nielsen the exercise/emotion induction of events was clear and well described. Increased QT prolongation was evident during exercise and with epinephrine administration. Thus, the adverse influence of adrenergic tone was integral to the description of the disease and this has been confirmed in many subsequent publications. This led to the use of Propranolol around 1959 as an investigational agent. Subsequently, beta-blockers have been the mainstay of therapy for most patients with LQTS (Moss 1997, 1998; Priori et al. 1999 parts I, II and III). However, although beta-blocker treatment is effective for syncope prevention, sudden cardiac arrest (SCA) prevention with beta-blockers is not universal or complete throughout all LQTS genotypes or patient subpopulations (Moss et al. 2000; Hobbs et al. 2006). Beta-blocker treatment appears to be most successful in high risk adolescent LQTS patients, especially LQTS1, who have experienced recent syncope (Hobbs et al. 2006). They are effective, but less so, in patients with LQTS2 and possibly least effective in LQTS3. Beta-blocker medication can have significant side-effects which often results in non-compliance.

Implantable cardiac defibrillator (ICD) therapy is considered an effective primary and secondary therapy in high risk patients with a strong personal history of syncope and severe QT prolongation. There are, however, significant ICD device-specific risks too. ICD discharge causing patient emotional distress may aggravate catecholamine-dependent arrhythmias (Lenhart et al. 2008). Although ICD therapy in high-risk patients must be considered, many LQTS patients are at relatively low risk for sudden death and require careful risk stratification to justify ICD treatment (Moss et al. 2007). It is, therefore, highly
desirable to develop novel mechanism- or gene-based therapeutic strategies that do not interfere with adrenergic regulation of the heart and avoid the risks and limitations of ICD therapy.

Genetic information is continuously contributing to the understanding of phenotype expression and arrhythmia severity. Not only is it beneficial in assisting with lifestyle modification therapies like avoiding swimming and competitive sports for LQTS1 mutation carriers or avoiding sudden loud noises like alarm clocks for LQTS2 mutation carriers; but mutation characterisation may greatly aid therapy orientation for patients (Priori et al. 2003). For example, a particular mutation may result in a <50% reduction of ion channel function from a trafficking defect (haploinsufficiency) or in a >50% reduction because of a dominant negative mechanism affecting the function of a multimeric ion channel complex. Dominant negative defects cause approximately a doubling of arrhythmogenic risk, and mutations in transmembrane portions of KCNQ1 constitute an additional, independent risk factor (Moss et al. 2007).

It is important to identify the patient and family members at high risk for LQTS symptoms that may result in sudden death, tachyarrhythmias, particularly when they are young. Although treating asymptomatic mutation carriers is controversial, LQTS mutation carriers do require some prevention therapy as they have a 10% risk of suffering a major cardiac event by age 40 years (Priori et al. 2003). On the other hand, the variable penetrance of LQTS phenotype may result in false-negative diagnoses which results in up to 40% of LQTS mutation carriers remaining undetected after clinical testing. This provides strong argument for the need of more comprehensive approaches, beginning with genotyping, which can add the predictive information needed to implement specific SCA preventive therapeutic strategies.
1.3.2 Brugada syndrome (BrS)

The Brugada brothers were the first to describe this form of idiopathic ventricular fibrillation characterised by elevation of the ST segment and right bundle branch block (RBBB) in the right precordial (chest) ECG leads (V1-V3) and link them to sudden death (Brugada and Brugada 1992) (Figures 1.5). Before that, the characteristic ECG findings, were often mistaken for a right ventricle myocardial infarction and already in 1953, a publication mentions that the ECG findings were not associated with ischemia as people often expected (Osher and Wolff, 1953).

Figure 1.5 Typical ECG abnormalities in Brugada syndrome: a = broad P wave with some PQ prolongation, b = J point elevation, c = coved type ST segment elevation and d = inverted T wave.

1.3.2.1 Epidemiology

The arrhythmias in BS usually occur in patients between 30 and 40 years of age, although the age range covers 2-84 years. It is estimated that BrS is responsible for at least 4% of all sudden deaths and at least 20% of all sudden deaths occurring in patients without structural heart disease (Antzelevitch et al. 2005). BrS has also been linked unequivocally to a near-miss sudden infant death (Skinner et al. 2005). BrS seems to be most prevalent in Southeast Asia and Japan (Wong et al. 1992; Nademanee et al. 1997). Symptoms occur mostly at night, and the folklore of many of these countries is replete with stories of young men with 'Lai Tai' (Thailand), 'Bangungut' (Philippines), or 'Pokkuri' (Japan), thrashing, screaming,
and then dying suddenly in their beds. This disorder may be the leading cause of natural death among young men in the poverty-stricken northeast of Thailand. The annual mortality rate in this group is said to be as high as 26-38 per 100,000 (Nademane et al. 1997)

1.3.2.2 Symptoms

BrS is an inheritable arrhythmia syndrome with an autosomal dominant pattern of inheritance. BrS patients are susceptible to developing life threatening ventricular fibrillation, most commonly in young males during sleep (Brugada and Brugada, 1996, Chen et al. 1998). Males are more often symptomatic than females, probably due to the influence of sex hormones on cardiac arrhythmias and/or ion channels, and a different distribution of ion channels across the heart in males versus females (Matsuo et al. 2001).

1.3.2.3 Diagnosis of BrS

The ECG characteristics of BrS are dynamic and often concealed but can be unmasked by potent sodium channel blockers such as ajmaline, flecainide, procainamide, disopyramide, propafenone and pilsicainide (Brugada et al. 2000, Priori et al. 2000). Three ECG patterns in the right precordial leads are recognised as being associated with BrS (Wilde et al. 2002) (Figures 1.6 and 1.7). Type 1 is diagnostic of Brugada pattern and is characterised by a coved ST-segment elevation ≥2 mm followed by a negative T wave. A definitive diagnosis of BrS can be made when a type 1 ST-segment elevation pattern is observed in >1 right precordial lead (V1 to V3) along with one of the following: 1) documented polymorphic VT or VF; 2) a family history of sudden cardiac death at <45 years of age; 3) similar type ECGs in family members; 4) inducibility of VT/VF during an electrophysiology study; 5) unexplained syncope, or 6) history of nocturnal agonal respiration (Antzelevitch et al. 2005) (Figure 1.6).
The type 2 ST-segment elevation pattern has a saddleback appearance with a ST-segment elevation of ≥2 mm, a trough displaying ≥1 mm ST elevation, and then either a positive or biphasic T wave. The type 3 pattern has either a saddleback or coved appearance with an ST-segment elevation of <1 mm. Unlike the type 1 pattern, type 2 and 3 pattern ECGs are not diagnostic of the Brugada pattern. In order for a diagnosis of BS to be made in patients with either a type 2 or 3 pattern, conversion to a more diagnostic type 1 pattern must be observed, either spontaneously or after administration of a sodium channel blocker (e.g. ajmaline), in conjunction with one or more of the clinical criteria described above (Antzelevitch et al. 2005) (Figure 1.7).
Figure 1.7 Examples of the various types of ECG patterns seen in patients with Brugada syndrome. Image adapted from ECG traces displayed at http://www.genedx.com/site/brugada-syndrome

1.3.2.4 The genetics of BrS

Since BrS is an autosomal dominant disorder, offspring of people with the mutant gene have a 50% chance of inheriting it from their affected parent. A sporadic form is also seen due to spontaneous mutations in the parent’s germ cells (ova or sperm) affecting the segments of DNA that code for the specific Brugada proteins. Loss-of-function mutations in the cardiac sodium channel subunit, SCN5A have been identified in 25%–30% of Brugada patients (Lehnart et al. 2007), the majority of which are dominant missense mutations. This is the same gene that causes LQTS3 (gain-of-function mutations) and isolated cardiac conduction defect (ICCD; Lenegre disease) (Wang et al. 1995b; Schott et al. 1999; Tan et al. 2001). The position of the mutation in the gene and the resulting differences in the electrophysiological abnormalities of expressed proteins determine the outcome of SCN5A mutations. BrS due to mutations in SCN5A is now termed BrS1 as an
additional seven genes have been associated with BrS2-8, respectively (Table 1.2, references therein).

**Table 1.2 Genes associated with BrS**

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Locus</th>
<th>Gene</th>
<th>Protein</th>
<th>Ion channel</th>
<th>Ref*</th>
</tr>
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<tbody>
<tr>
<td>BrS1</td>
<td>3p21</td>
<td>SCN5A</td>
<td>Na_1.5</td>
<td>I_Na</td>
<td>Chen 1998</td>
</tr>
<tr>
<td>BrS2</td>
<td>3p24</td>
<td>GPD1L</td>
<td>Glycerol-3-phosphate dehydrogenase 1-like</td>
<td>I_Na</td>
<td>Weiss 2002</td>
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<td>BrS3</td>
<td>12p13.3</td>
<td>CACNA1C</td>
<td>Ca_1.2</td>
<td>I_Ca-L</td>
<td>Antzelevitch 2007</td>
</tr>
<tr>
<td>BrS4</td>
<td>10p12.33</td>
<td>CACNB2b</td>
<td>Ca_β2b</td>
<td>I_Ca-L</td>
<td>Antzelevitch 2007</td>
</tr>
<tr>
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<td>MIRP2</td>
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<td>Sodium channel type 3 β</td>
<td>I_Na</td>
<td>Hu et al. 2009</td>
</tr>
<tr>
<td>BrS8</td>
<td>15q24</td>
<td>HCN4</td>
<td>hyperpolarization activated cyclic nucleotide-gated cation channel 4</td>
<td>I_K</td>
<td>Ueda et al. 2009</td>
</tr>
</tbody>
</table>

* First reference of association with BrS
# Mutations in SCN5A are the dominant cause of BrS, identified in 20-25% of cases.

**1.3.2.5 Treatment of BrS**

Currently, ICDs are the only proven treatment for BrS (Brugada, Brugada and Brugada 1998, 1999 and 2000). Patients who are symptomatic (unexplained syncopes, ventricular tachycardias or aborted sudden cardiac death) may have a symptom recurrence risk of 2 to 10% per year and since SCA may be the initial manifestation of the disease, it is critically important to identify patients who may benefit from ICD implantation. These patients include those with the type 1 ECG pattern (either spontaneous or induced by administration of a sodium channel blocker) who have been successfully resuscitated from SCA or have had unexplained syncope (loss of consciousness), seizures, or nocturnal agonal respirations (Zipes et al. 2006). The indications for ICD become less clear in asymptomatic patients with the Brugada pattern on ECG and, unfortunately, there is no consensus among physicians. ICD therapy in young patients, where the risk of SCA may accumulate over time must be balanced against the risk of the ICD complications,
themselves, which also increase with time (Sherrid and Daubert 2008). It appears that inappropriate ICD shocks far outweigh appropriate shocks in patients with BrS in three and four year follow-up studies (Sacher et al. 2006 and Sarkozy et al. 2007, respectively).

Asymptomatic patients, in whom the Brugada ECG characteristics are present (either spontaneously or provoked by fever or sodium channel blockers like ajmaline, procainimde or flecainide) are often given life style advice. This includes avoiding a number of medications (including sodium channel blockers and certain anti-depressants and anti-arrhythmics) and rigorously treating fever with paracetamol, for example, as fever may elicit a Brugada ECG and arrhythmias in some patients.

The pharmacological approach to therapy is based on rebalancing of currents during the action potential. Anti-arrhythmic drugs, that affect the transient outward potassium current (Ito), have shown promise because they re-establish the action potential dome. Quinidine is such a drug and has been used in some BS patients to re-establish a normal contour to the action potential and normalise the ECG pattern (Antzelevitch et al. 1999). However, clinical trials demonstrating the long-term efficacy of quinidine use are limited and, therefore, it is not recommended as sole first-line therapy for BrS patients (Alings et al. 2001; Belhassen et al. 2002). Other agents which augment calcium current via different mechanisms have been considered as potentially useful in patients with BrS (Antzelevitch 2001, Tsuchiya et al. 2002). Appropriate clinical trials are yet to be carried out to establish the effectiveness of pharmacological agents in BrS and currently, most medications are reserved for controlling incessant episodes of VT and/or VF in BS (Antzelevitch et al. 2005).
### 1.3.3 Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT)

Stress induced bidirectional ventricular tachycardia in children was described in 1962 (Horan and Venebles) and 1975 (Reid et al.). The term "catecholaminergic polymorphic ventricular tachycardia" occurring in the structurally intact heart was used to describe the condition by Coumel et al. (1978) and by Leenhardt et al. (1995). CPVT is a potentially lethal, heritable arrhythmia syndrome often manifesting as exercise-induced ventricular arrhythmias, syncope or sudden death (Leenhardt et al. 1995). CPVT is a rare disorder with uncertain prevalence, although estimates reach 1 in 10 000 (Liu et al. 2008).

#### 1.3.3.1 Symptoms of CPVT

Patients with structurally normal hearts and normal QT intervals present with recurrent syncope, seizures, and sudden death (Leenhardt et al. 1995; Tester et al. 2004; Liu et al. 2007). Similar to LQTS, ventricular tachycardia can degenerate into ventricular fibrillation and sudden death may be the first manifestation of the disease (Kontula et al. 1995). However, CPVT tends to have a higher malignancy than LQTS, and though much rarer, presents in similar numbers in the molecular autopsy of young sudden death victims, particularly teenagers (Tester and Ackerman 2007). Another phenotypic similarity to LQTS is swimming–triggered arrhythmia events (Choi et al. 2004). It is believed that the combination of physical activity, voluntary apnoea and possibly cold water causes co-activation of sympathetic and parasympathetic pathways and an increased release of catecholamines which trigger the arrhythmogenic symptoms (Gooden 1994; Marsh et al. 1995).

The mean age of onset of symptoms is 8 years, but the first syncope may also occur in adulthood in some instances. Approximately 30% of affected individuals have symptoms
before age 10, and nearly 60% of patients have at least one syncopal episode before the age of 40 years (Priori et al. 2002; Cerrone et al. 2005).

1.3.3.2 The diagnosis of CPVT

The original description of the disease includes a peculiar arrhythmia, bidirectional ventricular tachycardia (BVT), characterised by a beat-to-beat 180° rotation of the QRS axis, where the QRS complexes in the electrocardiogram are alternately positive and negative (Figure 1.8). Notably, in the first study carried out in a relatively large group of patients, only 35% showed BVT, whereas polymorphic ventricular tachycardia was also observed (Priori et al. 2002). Cases in which VF occurs as the first manifestation are often classified as idiopathic VF, given the absence of structural cardiac abnormalities and a normal resting ECG (Priori et al. 2002). The induction of BVT or PVT with exercise is usually reproducible and, therefore, represents the single most important test for diagnosis.
Figure 1.8 a) A normal ECG of a CPVT patient at rest, b) the ECG of the same patient with CPVT during exercise. Asterices mark polymorphic ventricular beats. Image adapted from ECG traces displayed at http://en.ecgpedia.org/wiki/Catecholaminergic_Polymorphic_Ventricular_Tachycardiamage.

For patients who are more susceptible to emotional stress triggers compared to physical exercise, holter monitoring may be useful. The use of epinephrine infusion to induce arrhythmias is indicated in patients with a negative exercise test result, normal resting ECG and in whom syncope or cardiac arrest occurred during exercise or emotion.
The complexity of arrhythmias tends to worsen gradually with increasing work load, starting with isolated premature ventricular beats, and ending with bidirectional ventricular tachycardia to polymorphic ventricular tachycardia (Priori et al. 2002).

1.3.3.3 The genetic basis of CPVT

Mutations in two genes have been identified in CPVT cases (Table 1.3). RYR2, the first gene associated with the autosomal dominant form of CPVT (CPVT1), encodes the cardiac ryanodine receptor (RyR2) (Priori et al. 2001). RyR2 controls Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) to the cytosol in response to the Ca\(^{2+}\) entry mediated by L-type channels during the plateau phase of the action potential. RYR2 mutations are identified in 55-60% of clinically affected patients and more than 70 different RYR2 mutations have been reported (Medeiros-Domingo et al. 2009).

A missense mutation in the CASQ2 gene, which encodes calsequestrin, was identified in a large consanguineous family of Bedouin origin affected by a recessive form of CPVT (CPVT2) (Lahat et al. 2001). Calsequestrin is an important Ca\(^{2+}\) buffering protein of the SR and it participates, together with junction and triadin, in modulating the responsiveness of the RyR2 to luminal Ca\(^{2+}\). CASQ2 mutations are found in only 1-2% of CPVT patients, with less than 10 mutations reported to date (Lahat et al. 2001, 2004; di Barletta et al. 2006; http://www.fsm.it/cardmoc/). A second autosomal recessive form of CPVT has been mapped to chromosome 7p14-22, although the gene involved has not yet been identified (Bhuiyan et al. 2007).
Table 1.3 Genes associated with CPVT

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Locus</th>
<th>Gene</th>
<th>Protein</th>
<th>Frequency</th>
<th>Inheritance</th>
<th>Ref*</th>
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<td>RYR2</td>
<td>cardiac ryanodine receptor</td>
<td>55-60%</td>
<td>Dominant</td>
<td>Priori et al 2001</td>
</tr>
<tr>
<td>CPVT2</td>
<td>3p24</td>
<td>CASQ2</td>
<td>calsequestrin</td>
<td>1-2%</td>
<td>Recessive</td>
<td>Lahat et al 2001</td>
</tr>
</tbody>
</table>

* First reference of association with CPVT

1.3.4 Short QT syndrome (SQTS)

The first report of the so-called "short QT syndrome" was provided by Gussak et al. (2000) who described a familial distribution of a persistently short QT interval associated with paroxysmal atrial fibrillation (AF) in one patient. They also reported similar ECG changes in an unrelated case associated with sudden cardiac death. More recently, these observation have been refined by Gaita et al. (2003) who described two families with severe history of sudden cardiac death, QTc interval constantly below 290ms, ventricular premature beats and documented VF.

SQTS is characterised by syncope, paroxysmal AF, a remarkable familial history of sudden cardiac death and a typical, hyperkalemic-like T wave pattern (tall and peak T wave) on the resting ECG. SQTS is presented either as familial or sporadic cases usually in a young population without structural heart disease (Lehnart et al. 2007). Evidence is emerging that SQTS is more common than previously expected, but it still remains rare; less than 50 families have been reported worldwide. SQTS has been implicated in ventricular fibrillation and SCD cases that previously were considered idiopathic (Bjerregaard and Gussak 2005).

Mutations have been identified in three LQTS associated genes, KCNQ1, KCNH2, and KCNJ2 (Bellocq et al. 2004; Brugada et al. 2004; Hong et al. 2005; Priori et al. 2005).
However, unlike LQTS which arises due to loss of function mutations in these genes, the SQTS phenotype is caused by gain of function mutations.

ICD implantation is currently the treatment of choice for symptomatic patients with SQTS and a family history of sudden cardiac death (Schimpf et al. 2003). Quinidine has been shown to normalise ventricular repolarisation (at variance with other antiarrhythmic drugs including sotalol and amiodarone) in some patients but, at present, its role in preventing recurrences of cardiac events is not known, and it is not yet considered an alternative therapy to ICD.

1.3.5 Sick sinus syndrome (SSS)

The term 'sick sinus syndrome' encompasses a variety of conditions caused by sinus node dysfunction. The most common clinical manifestations are syncope, presyncope, dizziness, and fatigue. ECG tracings typically show sinus bradycardia, sinus arrest, and/or sinoatrial block (Benson et al. 2003). Episodes of atrial tachycardias coexisting with sinus bradycardia ('tachycardia-bradycardia syndrome') are also common in this disorder. SSS occurs most often in the elderly associated with underlying heart disease or previous cardiac surgery, but can also occur in the foetus, infant, or child without heart disease or other contributing factors, in which case it is considered to be a congenital disorder (Benson et al. 2003).

Mutations in the cardiac sodium channel, SCN5A, can result in autosomal recessive congenital SSS (SSS1) (Benson et al. 2003). Autosomal dominant sinus bradycardia syndrome (SSS2) can be caused by mutation in the cardiac pacemaker channel gene HCN4 (Schulze-Bahr et al. 2003; Ueda et al. 2004; Milanesi et al. 2006; Nof et al. 2007).
1.3.6 Atrial Fibrillation (AF)

Although AF is the most common arrhythmia and accounts for 30% of all strokes in adults over the age of 60 years (Brugada et al. 1997; Roberts 2006), it is not a common cause of SUDY and is most commonly an acquired disorder associated with other cardiac pathology rather than an inherited cardiac arrhythmia syndrome (Beyer et al. 1993). It has an overall prevalence of 0.89% which increases rapidly with age, to 2.3% between the ages of 40 and 60 years, and to 5.9% over the age of 65.

Several chromosomal loci and four genes have been associated with AF, KCNQ1, KCNE2, KCNJ2, and CJA5 (Chen et al. 2003; Yang et al. 2004; Xia et al. 2005; Gollob et al. 2006). In contrast to commonly occurring germline variations in cardiac conditions, a rare somatic mutation has been revealed in CJA5 in atrial cells but is absent from germline cells (Gollob et al. 2006). Cardiac sodium channels encoded by SCN5A are expressed in atria and ventricles, and in BrS both ventricular and atrial fibrillation may occur. (Kusano et al. 2008).

1.3.7 Hypertrophic cardiomyopathy (HCM)

HCM is a disorder affecting cardiac myocytes, with a population prevalence of 1 in 500 (Maron 2002). It is generally characterised by thickening of the left ventricular wall that is not attributable to a known secondary cause, such as systemic hypertension or valvular aortic stenosis. It can lead to heart failure and ventricular arrhythmias (Hughes and McKenna 2005) and causes 40%–50% of cases of SCD in young athletes in the United States (Maron et al. 1996).

HCM exhibits remarkable genetic and clinical heterogeneity. The genetics of HCM have recently been reviewed by Konno et al. (2010). More than 450 different mutations within
13 myofilament-related genes have been identified. The majority of HCM associated mutations occur in the genes \( MYH7 \) and \( MYBPC3 \), which encode the \( \beta \)-myosin heavy chain (\( \beta \)-MyHC) and myosin binding protein C (MyBP-C), respectively. Mutations in cardiac troponin T (\( TNNT2 \)), cardiac troponin I (\( TNNI3 \)), essential myosin light chain (\( MYL3 \)), regulatory myosin light chain (\( MYL2 \)), \( \alpha \)-tropomyosin (\( TPM1 \)), and cardiac actin (\( ACTC \)) genes are rarer causes of HCM. About 50% of patients have no mutation in a sarcomeric or sarcomere-related gene.

**1.3.8 Arrhythmogenic right ventricular dysplasia or cardiomyopathy (ARVD/C)**

ARVC is a heart muscle disease, often familial, that is characterised pathologically by atrophy and fibro-fatty replacement of myocytes in the right ventricle, fibrosis and ultimately thinning of the ventricular wall with chamber dilation. Although not a channelopathy, it can lead to syncope and young sudden death, often with only subtle histological changes identified, or to progressive cardiac failure. ARVC has been reported to account for 17% of sudden death in the young in the United States (Towbin 2001) and is the commonest cause of sudden death in young athletes in Italy (Corrado et al. 2001).

Congenital ARVC is predominantly an autosomal dominant disorder. To date, eight chromosomal loci and four definitive genes encoding desmosomal proteins have been identified, plakoglobin, desmoplakin, desmoglein 2, and plakophilin-2 (McKoy et al. 2000; Hodgkinson et al. 2005).

**1.4 Biology of channelopathy arrhythmias**

Channelopathy disorders arise from cardiac potassium-, sodium- or calcium-ion channels that are faulty, present in reduced or increased numbers or entirely absent. The biological
mechanisms by which mutations in the genes encoding these ion channel subunits exert their pathogenic effect are reviewed below.

1.4.1 Cardiac potassium channelopathies

K⁺ channels conduct different potassium currents. The delayed rectifier current (Iₖ) is the major repolarising current during phase 3 of the cardiac action potential and has slow (Iₖ,ₙ) and rapid (Iₖ,ᵣ) components. The inward rectifier current (Iₖ,ᵢ) participates in the control of the resting membrane potential, phase 4 of the cardiac action potential (Figure 1.9). Mutations in the genes encoding these ion channels have been associated with different cardiac channelopathies. The genes implicated are KCNQ1 and KCNE1 encoding for the α-subunit (KvLQT1) and β-subunit (minK), respectively, of the Iₖ,ₙ channel (Figure 1.10), KCNH2 and KCNE2 encoding for the α-subunit (HERG) and β-subunit (MiRP1), respectively, of the Iₖ,ᵣ channel (Figure 1.11), and KCNJ2 encoding for the Iₖ,ᵢ channel.
Figure 1.9 The key ion channels in cardiac cells. K⁺ channels (green) mediate K⁺ efflux from the cell; Na⁺ channels (purple) and Ca²⁺ channels (orange) mediate Na⁺ and Ca²⁺ influx, respectively. Ionic currents and genes underlying the cardiac action potential: top – depolarising currents as functions of time, and their corresponding genes; centre – a ventricular action potential; bottom – repolarising currents and their corresponding genes.
Mutations in these genes can give rise to at least four different clinical disorders: LQTS (Priori et al. 2004), SQTS (Gussak et al. 2000), Anderson syndrome (AS) or LQTS7 (Anderson et al. 1971), and familial atrial fibrillation (FAF) (Chen et al. 2003) (Table 1.4). This is not as surprising as it may seem initially, as the clinical manifestations reflect the electrophysiological abnormalities caused by the specific genetic defect. Two types of mutation can be identified: those in which the activity of the channel is increased (“gain of function” mutations) and those in which the function of the channel is substantially diminished or even lost (“loss of function” mutations). Therefore, there are disorders caused by loss of function mutations of potassium channels, such as LQTS1, LQTS2, LQTS4, LQTS5 and LQTS7 (AS), and there are disorders caused by gain of function mutations like SQTS and FAF (Table 1.4).
Table 1.4 Genes and functional abnormalities of ion channels in inherited arrhythmias

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Functional alteration</th>
<th>Phenotype(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNQ1</td>
<td>I\textsubscript{Ks} potassium channel α subunit (KvLQT1)</td>
<td>Loss of function</td>
<td>LQTS1, JLN1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gain of function</td>
<td>SQTS2, FAF</td>
</tr>
<tr>
<td>KCNH2</td>
<td>I\textsubscript{Kr} potassium channel α subunit (hERG)</td>
<td>Loss of function</td>
<td>LQTS2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gain of function</td>
<td>SQTS1</td>
</tr>
<tr>
<td>KCNE1</td>
<td>I\textsubscript{Ks} potassium channel β subunit (minK)</td>
<td>Loss of function</td>
<td>LQTS5, JLN2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gain of function</td>
<td>-</td>
</tr>
<tr>
<td>KCNE2</td>
<td>I\textsubscript{Kr} potassium channel β subunit (MiRP)</td>
<td>Loss of function</td>
<td>LQTS6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gain of function</td>
<td>FAF</td>
</tr>
<tr>
<td>KCNJ2</td>
<td>I\textsubscript{K1} potassium channel (Kir2.1)</td>
<td>Loss of function</td>
<td>LQTS7/AS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gain of function</td>
<td>SQTS3</td>
</tr>
<tr>
<td>SCN5A</td>
<td>Cardiac sodium channel α subunit (Na\textsubscript{v}1.5)</td>
<td>Loss of function</td>
<td>BrS, CCD, SSS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gain of function</td>
<td>LQTS3</td>
</tr>
<tr>
<td>CACNA1C</td>
<td>Cardiac channel α subunit (Ca\textsubscript{v}1.2)</td>
<td>Loss of function</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gain of function</td>
<td>LQTS8/TS</td>
</tr>
<tr>
<td>RYR2</td>
<td>Cardiac ryanodine receptor (RyR2)</td>
<td>Loss of function</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gain of function</td>
<td>CPVT</td>
</tr>
</tbody>
</table>

Figure 1.11 a) Schematic diagram of KCNH2 genomic structure b): Two dimensional schematic representation of predicted KCNH2 polypeptide (HERG) with MiRP1
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1.4.1.1 Potassium ion channel assembly

Ion channels typically consist of a homomeric or heteromeric assembly of four voltage-gated, pore-forming $\alpha$-subunits along with accessory $\beta$-subunits plus regulatory proteins. A typical cardiac $K^+$ $\alpha$-subunit is a plasmalemmal protein with six transmembrane segments (S1–S6) linked by intracellular and extracellular loops, a voltage sensor (S4), and a pore loop containing a conserved $K^+$-selective signature sequence between S5 and S6. Both amino- and carboxy-terminus are located in the cytoplasm. In order to make a functional channel, $\alpha$-subunits combine to form homotetramers and they co-assemble with $\beta$-subunits (small proteins with a single transmembrane segment (Figure 1.12).

Taking a closer look, at the $I_{Ks}$ channel complex for example, the channel is composed of three different proteins in a specific ratio of $KCNQ1$ isoform 1 (the channel pore), $KCNQ1$ isoform 2 (an endogeneous N-terminal truncated $KCNQ1$ splice variant), and $KCNE1$ (Barhanin et al. 1996; Sanguinetti et al. 1996). The heterogeneity of $I_{Ks}$ amplitude across the heart depends on the balance of expression of $KCNQ1$ isoform 1 and 2 (Pereon et al. 2000). $KCNE1$ appears to regulate the $KCNQ1$ channel activity in a concentration-dependent manner (Romey et al. 1997). Hormones such as oestrogens can alter the level of $KCNE1$ expression and this may account for some of the gender differences observed in LQTS and vulnerability to torsades de pointes (Makkar et al. 1993; Kawasaki et al. 1995; Drici et al. 1996; Rodriguez et al. 2001; Moller and Netzer 2006).
Figure 1.12 K⁺ channel assembly. The α-subunits (encoded by KCNQ1 and KCNH2) contain six transmembrane spanning segments. Positively charged amino acids occur at every third position in each of the S4 segments and probably comprise the voltage sensor. Four identical α subunits combine to form a tetramer with the S5 and S6 transmembrane segments of each domain making up the channel pore and associated ion selectivity filter. The β-subunit proteins may be cytoplasmic proteins or transmembrane spanning proteins, like minK (encoded by KCNE1) or MiRP1 (encoded by KCNE2).

1.4.1.2 Functional consequences associated with loss of function mutations

The LQTS variants linked to potassium channel mutations are due to loss of function mutations (Table 1.4). In vitro expression studies of mutant channels have been instrumental in the characterisation of phenotypic consequences of LQTS-associated mutations and in identifying the physiological mechanisms by which the clinical phenotype is produced (Dumaine et al. 1996; Bianchi et al. 1999; Priori et al. 1999; Sanguinetti 1999; Lees-Miller et al. 2000; Huang et al. 2001; Roden 2001; Isbrandt et al. 2002; Tristani-Firouzi et al. 2002; Fodstad et al. 2004; Thomas et al. 2005). They have clearly shown the impairment of repolarising currents. This abnormal slowing down of the repolarisation process prolongs the action potential, and this prolongation is reflected in the lengthened QT interval on ECG.
Expression studies have demonstrated that LQTS mutations of genes related to K⁺ currents produce loss of channel function by three mechanisms (Keating and Sanguinetti 2001; Aizawa et al. 2004; Priori et al. 2004, Wilson et al. 2005): (1) a net current reduction by altering the channel gating and kinetic properties (dominant negative effect), (2) prevention of assembly of functional channel protein (haploinsufficiency) and (3) an abnormal intracellular protein trafficking (dominant negative effect if wild-type subunits are affected and haploinsufficiency if only “mutant” subunits are affected).

Most mutations are heterozygous, meaning that 50% of the proteins, those encoded by the wild-type allele are functionally normal, and this leads to the formation of ion channels that are made of varying proportions of “normal” and “mutant” proteins. The integration of mutant proteins into an ion channel can be deleterious and even alter the function of the wild-type protein. This causes a dominant negative interaction which results in >50% reduction in current. If the defective or “mutant” proteins lose their ability to co-assemble with wild-type subunits, they are unable to participate in channel structuring resulting in a ≤50% current reduction (haploinsufficiency). Often abnormal proteins are retained in the Golgi apparatus (trafficking defect) which can result in dominant negative interactions if wild-type subunits are “held back” or haploinsufficiency if the functional defect is simply due to a “lack” of proteins reaching the cell membrane.

Coexpression of \( KCNQ1 \) missense mutations with wildtype \( KCNQ1 \) can result in various degrees of \( I_{Ks} \) reduction (Chouabe et al. 1997; Donger et al. 1997). Mutations in transmembrane segments S1-S3 modify coassembly with \( KCNE1 \) (Li et al. 1998) and mutations in the voltage sensor segment (S4) and the pore regions (S5 and S6) are usually associated with the dominant negative effects due to the disruption of the K⁺ ion transport (Vatta et al. 2002). Mutations in the C- or N-termini and nonsense mutations seem to
affect the subunits assembly and tetramerisation of the channel (Wollnik et al. 1997; Dahimene et al. 2006; Wiener et al. 2008).

A particular C-terminal KCNE1 mutant, D76N, suppresses the I_{Ks} current in a dominant-negative manner (Splawski et al. 1997). This functional defect was rescued by the binding of activators of the I_{Ks} channel complex, such as 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) or mefenamic acid, to the extracellular N-terminal boundary of the KCNE1 transmembrane segment, indicating that the C-terminal mutant produces loss of function by locking the cytoplasmic domain into inactive conformations (Abitbol et al. 1999). Trapping of KCNE1 channels in the endoplasmic reticulum (ER) has also been reported, resulting in the prevention of KCNQ1/KCNE1 association (Schulze-Bahr et al. 1997; Duggal et al. 1998). Studies have suggested that clinical manifestations of LQT5 mutations may be more complicated than expected because KCNE1 mutations exert differing effects on KCNQ1 and KCNH2 encoded polypeptides (Bianchi et al. 1999; Ohyama et al. 2001; Priori et al. 2001). KCNH2 interacts with KCNE1, when its usual interactor KCNE2 is absent (Bianchi et al. 1999; Ohyama et al. 2001; Priori et al. 2001).

Expression of some KCNH2 mutant channels generate I_{Kr} current with altered gating or reduced amplitude, but no simple correlation was found in these cases between the position of the mutation and the functional consequences (Roden and Spooner 1999; Roden 2001). Where KCNH2 mutants produced no I_{Kr} current, two functional mechanisms have been reported. First, when the channel protein is not detected at the cell surface level, there may be defects in trafficking or protein stability (Zhou et al. 1998; Furutani et al. 1999; Petrecca et al. 1999). Mutated proteins are unglycosylated and retained in the ER, resulting in the reduction in the number of subunits for tetramerisation. Secondly, when channel protein is detected at the cell surface, a primary defect in gating is suggested
(Furutani et al. 1999). The trafficking deficient subunits have also shown to tag tetrameric channel complexes for retention in the ER on co-assembly (an acquired trafficking defect) (Ficker et al. 2000).

Unlike with KCNQ1, co-expression of KCNH2 mutants and wild-type KCNH2 showed no current suppression. However, when KCNE2 was co-expressed, K⁺ current was partially reduced. KCNE2 mutants reduce current due to the change in the gating of the channel, or faster deactivation kinetics (Abbott et al. 1999). KCNE2 also transformed the voltage dependant KCNQ1 to a voltage-independent channel (Tinel et al. 2000).

KCNJ2 mutations associated with LQT7 or AS cause loss of function mainly through a dominant-negative suppression of wild-type Kir2.1 subunits (Tristani-Firouzi et al. 2002). Using computer simulation studies, Tristani-Firouzi and colleagues (2002) demonstrated that an impaired Kir2.1 protein prolongs the terminal phase of the cardiac action potential and, in the setting of a reduced extracellular potassium concentration, induces delayed after-depolarisations (DADS) and spontaneous arrhythmias.

As mentioned earlier in this chapter (section 1.3.1.3), Jervell and Lange-Nielsen Syndrome (JLNS) is the rarer form of inherited LQTS with an autosomal recessive pattern of inheritance. JLNS has a severe clinical phenotype and is often associated with congenital deafness (Jervell and Lange-Nielsen 1957; Dumaine and Antzelevitch 2002). The coexistence of deafness with JLNS is largely accounted for by the roles played by KCNQ1/KCNE1 in the recycling circuit of K⁺ ions in the cochlea and the affect on the sensitivity to auditory stimuli (Robbins 2001). However, patients with homozygous JLNS mutations of KCNQ1 do not always present with coassociated deafness, reflecting the complexity of the mechanisms involved in K⁺ channel-induced deafness (Priori et al. 1998).
Heterozygous carriers of JLNS mutations generally have mild clinical effects, and two
mutant alleles are required to cause the severe phenotype (Keating and Sanguinetti 2001). Expression studies of JLNS mutations reduced $K^+$ current by 50%, most likely due to haploinsufficiency (Chouabe et al. 1997; Wollnik et al. 1997; Mohammad-Panah et al. 1999). JLNS mutations appear to interfere with either the C-terminal or N-terminal assembly domains (Schmitt et al. 2000; Tyson et al. 2000), or may reduce protein expression due to nonsense-mediated mRNA decay (NMD) (Frischmeyer and Dietz 1999). It remains difficult, however, to predict phenotype and different effects of the mutation between individuals, as the functional effect of mutations differ by the relative amounts of mutant and wild-type protein (Huang et al. 2001).

1.4.1.3 Functional consequences associated with gain of function mutations

A gain of function mutation in one of the genes encoding potassium ion channels generally causes SQTS. The $KCNH2$ mutation induces remarkable alteration of $I_{Kr}$ kinetic with a loss of channel rectification at depolarised potentials. This abnormality results in a larger outward potassium current during the initial phases of the cardiac action potential (Brugada et al. 2004). Current kinetic is also affected by $KCNQ1$ gain of function mutations, where significantly faster activation and negative shift of activation voltage result in a larger $I_Ks$ current (Bellocq et al. 2004). Finally, the $KCNJ2$ gain of function mutation causes a net increase of outward current and a positive shift of peak $I_{K1}$ (Priori et al. 2005).

1.4.2 Cardiac sodium channelopathies

The $\alpha$-subunit of the cardiac sodium channel (Nav1.5), encoded by the $SCN5A$ gene, is a transmembrane protein composed of four homologous domains (DI-DIV), each containing six transmembrane segments (S1-S6) (Figure 1.13). The SCN5A polypeptide with two auxiliary $\beta$-subunits forms the cardiac sodium channel and conducts the $I_{Na}$
current recorded in cardiac myocytes, the major determinant of the phase 0 (upstroke) of the cardiac action potential (Figure 1.9). It has been suggested that the beta-subunits modulate the ionic flux through the channel. A defect in the interaction between the SCN5A polypeptide and the β-subunits can, therefore, influence channel inactivation (An et al. 1998; Kupershmidt et al. 1998).

**Figure 1.13** a) Schematic diagram of SCN5A genomic structure b): Two dimensional schematic representation of predicted SCN5A polypeptide.

SCN5A is expressed in human myocardium but not in skeletal muscle, liver, or uterus (Wang et al. 1995). Recently, an alternative SCN5A isoform was identified in the human central nervous system (Wang et al. 2008) and SCN5A is also expressed in limbic circuitry of rat brain (Hartmann et al. 1999). This may provide an explanation for LQTS patients presenting with suspected seizures, or phenocopies of seizures (Bezzina et al. 2001) although heart tissue remains the predominant site of expression.

SCN5A mutations that cause even subtle defects in fast or slow gating in SCN5A receptors have profound effects on cardiac electrical activity, leading to significantly
different cardiac phenotypes that have a high incidence of sudden death, usually at night or rest. As described for potassium channels, $\text{SCN5A}$-related phenotypes are also secondary to either gain (LQT3) or loss (BrS, CCD and SSS) of function mutations (Table 1.4). $\text{SCN5A}$-associated phenotypes include the following genetic arrhythmogenic disorders:

1. The LQT3 variant of LQTS (Wang et al. 1995),
2. SIDS and near SIDS cases (Schwartz et al. 2000; Arnestad et al. 2007). Interestingly, Schwartz et al. (1998) found a prolonged QT interval in 50% of the 24 SIDS cases who died within the first year of life (SID cases), indicating the potential role of LQTS in SIDS. (ECG traces taken before the time of death were made available for the study). This suspicion has been confirmed in postmortem studies in young unexpected death syndrome cohorts and isolated retrospective diagnostic testing (Ackerman et al. 2001; Skinner et al. 2004). More recently Arnestad and colleagues (2007) demonstrated that 9.5% of cases diagnosed as SIDS carry functionally significant genetic variants in LQTS genes. Most variants were found in $\text{SCN5A}$ (50%) followed by $\text{KCNQ1}$ and $\text{KCNH2}$ (19% each).
3. BrS (Chen et al. 1998),
4. Sudden unexplained nocturnal death syndrome (SUNDS), a disease allelic to BrS, is common in southeast Asia, and causes SCD (usually in males) during sleep (Vatta et al. 2002),
5. The progressive cardiac conduction defect (PCCD) which is defined by isolated prolongation of the conduction parameter in the His-Purkinje conduction system but no ST segment elevation or QT prolongation (Schott et al. 1999; Tan et al. 2001),
6. The Sick Sinus syndrome (SSS), also known as sinus node dysfunction. $\text{SCN5A}$ mutations are associated with dominant form of SSS, whereas mutations in $\text{HCN4}$
occur in recessive cases of SSS (Benson et al. 2003; Milanesi et al. 2006; Nof et al. 2007).

It is, therefore, apparent that single \( SCN5A \) mutations may cause “overlap syndromes,” that is, phenotypes that combine features of LQT3, BrS, and conduction disease (Tan et al. 2003). Model systems investigations confirm that the interchangeable phenotypes can resonate from the same missense mutations (Abriel 2007). This raises the possibility that all \( SCN5A \)-associated diseases are allelic disorders or they may present the same syndrome, \( SCN5A \) syndrome, with different penetrance and severity.

Physiologically, \( SCN5A \) mutations result in defective \( I_{Na} \) and prolonged depolarisation due to a delayed activation or incomplete inactivation of the \( Na^+ \) channel (Wehrens et al. 2000). Mutations in DIII-DIV linker, the D1-DII linker, and the C-terminal demonstrate a prolonged \( Na^+ \) activity by altering the inactivation gating (Bennett et al. 1995; Dumaine et al. 1996; Wehrens et al. 2003). Heterologous expression of \( SCN5A \) mutations in DIII-DIV linker exhibit a small, persistent \( Na^+ \) current during the AP plateau by allowing channel reopening, while a missense mutation in the S4 segment of the DIV region reduces channel activation by prolonging the channel opening with bursting behaviour (Makita et al. 1998). Interestingly, a \( SCN5A \) mutation, 1795insD in the C-terminus region produces opposite effects on the fast and slow components of inactivation (Bezzina et al. 1999; Veldkamp et al. 2000). At a slow heart rate, the sustained inward current was produced during the AP plateau, whereas at a fast heart rate, slow inactivation was enhanced by delaying recovery of channel availability between stimuli. This illustrates how one mutation can be associated with both LQTS and BrS, by exerting different phenotypic effects.
1.4.3 Cardiac calcium channelopathies

During the plateau phase of the cardiac action potential, extracellular Ca$^{2+}$ that enters through L-type calcium channels (ICa) elicits Ca$^{2+}$ release from the sarcoplasmic reticulum (SR), mainly through the cardiac ryanodine receptor (RyR2). RyR2 is the cardiac counterpart of RyR1, the skeletal muscle ryanodine receptor that is involved in malignant hypothermia (Tiso et al. 2001). RyR2 is responsible for calcium-induced calcium release (CICR) from the SR (Fabiato 1983) (Figure 1.14). Therefore, Ca$^{2+}$ fluxes in cardiac cells altered due to mutations in genes encoding these major calcium channels may cause susceptibility to life threatening cardiac arrhythmias.

1.4.3.1 Mechanism of arrhythmia due to mutation in CACNA1c

Timothy syndrome (TS), also known as LQT8 (Splawski et al 2004), is caused by mutations in CACNA1c, which encodes the pore-forming α-subunit (Ca,v1.2) of the cardiac L-type Ca$^{2+}$ channel, a key protein in excitation-contraction coupling in the heart that is also expressed in the brain, smooth muscle, immune system, teeth, and testes (Marks et al. 1995a). TS is a rare and malignant variation of LQTS presenting with QT interval prolongation, syndactyly, and a spectrum of additional cardiac and extracardiac manifestations (Marks et al. 1995a, 1995b; Levin 1996; Joseph-Reynolds 1997).

The molecular basis of TS was characterised in a collaborative study in which Splawski and colleagues (2004) identified a missense mutation, G408R, in 13 patients. In vitro functional characterisation showed that the molecular defects induce a gain of function by delaying the inactivation of the channels due to loss of the voltage-dependent component of the activation process. This abnormality leads to a net increase of I$_{Ca}$ and a prolongation of action potential duration (Splawski et al. 2004).
Figure 1.14 Calcium induced intracellular calcium release via the ryanodine receptor. Depolarisation of myocardial cells opens L-type calcium channels which allow small amounts of calcium ions to enter the cell. These, in turn, stimulate the release of large amounts of calcium ions from the SR via RyR2. RyR2 is also affected by stimulus via adrenergic beta receptors on the cell surface via cyclic AMP and phosphokinase A (PKA).

1.4.3.2 Mechanism of arrhythmia due to mutation in RYR2

Functional studies using genetically engineered mouse models have shown that CPVT arrhythmias result from intracellular Ca\textsuperscript{2+} overload which causes delayed after-depolarisations (DADS)-induced triggered activity (Cerrone et al. 2005; Kannankeril et al.
The presence of DADs as the origin of CPVT arrhythmias is also supported by the clinical evidence of direct correlation between the coupling interval of ventricular beats and the preceding RR interval (a typical feature of triggered arrhythmias) (Monteforte et al. 2007). Additionally, Paavola et al. (2007) showed the occurrence of DADs with monophasic action potential recordings from the right ventricular septum in RyR2-CPVT patients.

Although there is general agreement that the induction of DADs and triggered activity caused by uncontrolled calcium release (leakage) is the final common effect of CPVT mutations, three major biophysical/molecular mechanisms causing this effect have been proposed.

1. Enhanced store overload induced Ca\(^{2+}\) release.

The probability of an open RyR2 channel increases in the presence of an increase of sarcoplasmic reticulum (SR) Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{SR}}\)). When [Ca\(^{2+}\)]\(_{\text{SR}}\) reaches a critical threshold, spontaneous Ca\(^{2+}\) release (spill over) can occur even in the presence of normal channels (store overload induced Ca\(^{2+}\) release, or SOICR). [Ca\(^{2+}\)]\(_{\text{SR}}\) is physiologically increased as an effect of adrenergic stimulation. Jiang et al. (2002) showed that CPVT RYR2 mutations have a reduced threshold for SOICR. An increased Ca\(^{2+}\) sensitivity of RyR2 channels (that is, a lower threshold for diastolic release) was also observed more recently in functional experiments carried out by Fernandez-Velasco and colleagues (2009).

2. Defective domain-domain interactions

As proper folding and domain-domain interactions are crucial for RyR2 channel function, it was proposed that a loose interaction or unzipping between the N-terminal and central regions could hyperactivate RyR2, causing diastolic Ca\(^{2+}\)
leakage (Ikemoto et al. 2002) Studies of several synthetic peptides that mimicked CPVT mutants all caused spontaneous Ca\(^{2+}\) release by destabilising interdomain interactions (Oda et al. 2005). The domain-domain interaction hypothesis gained further support by George and colleagues (2006). Their experiments, using high-resolution confocal microscopy and Fluorescence Resonance Energy Transfer (FRET), suggested that RyR2 mutants cause an unstable channel structure through different mechanisms depending on their location.

3. Disruption of FKBP12.6 binding

FKBP12.6 (or calstabin2) is a RyR2 binding protein that tends to stabilise the channel in the closed state during diastole, thus preventing Ca\(^{2+}\) release. Marks et al. (2000) suggested that in the setting of heart failure, increased dissociation of FKBP12.6 from RyR2 may cause triggered activity. Further studies suggested that CPVT mutations disrupted FKBP12.6–RyR2 interaction and increased susceptibility to complex ventricular arrhythmias (Wehrens et al. 2004). Interestingly, the treatment with a benzothiazepine derivative able to restore FKBP12.6-RyR2 binding, exerted an anti-arrhythmic effect (Wehrens et al. 2004, Lehnart et al. 2008). Although the FKBP12.6-RyR2 interaction hypothesis is appealing for its therapeutic implications, there has been some conflicting evidence of mutant RyR2 that interacts normally with FKBP12.6 both in control and during adrenergic stimulation (George et al. 2003; Jiang et al. 2005; Liu et al. 2006). Thus, it seems possible that the molecular pathophysiology of CPVT is mutation-specific.

The local anesthetic tetracaine has been used to inhibit RyR2 and suppress spontaneous sarcoplasmic reticulum Ca\(^{2+}\) release in isolated myocytes (Venetucci et al. 2006). However, tetracaine causes a rebound increase in sarcoplasmic reticulum
Ca\textsuperscript{2+} release events during prolonged exposure (Gyorke et al. 1997), effective inhibitory concentrations (Shoshan-Barmatz and Zchut 1993) are too high for clinical use, and systemic administration is contraindicated in humans. Watanabe and colleagues have recently shown that that flecainide, an approved antiarrhythmic drug known to block sodium channels, showed remarkable efficacy in suppressing spontaneous sarcoplasmic reticulum Ca\textsuperscript{2+} release by inhibiting RyR2. Flecainide treatment completely prevented adrenergic stress–induced arrhythmias in a mouse model of CPVT and in humans with CASQ2 or RYR2 mutations that were refractory to standard drug treatment (Watanabe et al. 2009). DADs of sufficient amplitude activate voltage-gated Na\textsuperscript{+} channels and trigger full action potentials. Flecainide reduces triggered beats by a higher percent than its reduction of spontaneous Ca\textsuperscript{2+} release events, data consistent with flecainide’s known inhibition of Na\textsuperscript{+} channels to prevent triggered beats (Rosen and Danilo 1980). Taken together, their results indicate a dual mode of flecainide action in CPVT: suppression of spontaneous sarcoplasmic reticulum Ca\textsuperscript{2+} release events via RyR2 inhibition and suppression of triggered beats via Na\textsuperscript{+} channel block (Watanabe et al. 2009).

1.5 Interpretations from the literature

There are several challenges facing the molecular diagnostics of inherited cardiac disorders. One of the most important and all-encompassing, perhaps, is correlating a specific genetic variant to the clinical phenotype. One of the major conclusions that can be drawn from the literature, reviewed here, is that the clinical manifestations of arrhythmia syndromes are highly variable. Understanding the pathogenetic link between genotype and clinical phenotype is vital to the diagnosis and treatment of inherited arrhythmias. This includes the
biophysical phenotype of abnormal protein function (for example, effects on currents), the cellular phenotype caused by this abnormal function (for example, effects on action potential and Ca\(^{2+}\) loading), and the tissue and organ phenotype (for example, ECG change and type of arrhythmia) that characterises the clinical phenotype (seizures, syncope, sudden death). At each phenotype level, environmental factors, such as acidosis, autonomic nerve activity or ion concentrations, and other genetic factors, like genetic background and modifier genes, may affect the phenotypes (Makielski 2006). Better understanding of these complex interrelations is especially important for inherited arrhythmia syndromes and has immediate implications for the discovery of new mutations and for cost-effective screening. Most importantly, insights into these relationships will ultimately result in better therapy strategies and management of patients and their families.

### 1.6 The Cardiac Inherited Disease Group (CIDG)

Given the range and complexity of the challenges facing molecular diagnostics of arrhythmia disorders, it is important to have a cohesive programme, a solid infrastructure and input from multidisciplinary specialists. The Cardiac Inherited Disease Group (CIDG) is a New Zealand National multidisciplinary group made up of specialist cardiologists, molecular and clinical geneticists, pathologists, and genetic counsellors and associated professional staff whose main aim is to prevent sudden cardiac death due to inherited heart conditions in the young. The CIDG was formed in 2001 with the gradual development of a molecular genetic screening program for LQTS in New Zealand. Clinical diagnosis in family members may be uncertain in as many as 50%. In families where a mutation has been identified, 30% of mutation carriers have a normal electrocardiogram. They are nevertheless at risk for adverse cardiac events. Furthermore identification of the individuals at highest risk, and possibly in need of a defibrillator pacemaker, and appropriate lifestyle
Chapter 1: Introduction

counselling and medication depends on knowledge of the genotype which allows risk stratification (Priori et al. 2003). For example, a female carrying a KCNH2 mutation has an average annual risk of sudden death of 0.8%, compared to 0.3% for a female with a mutation in KCNQ1. Examples of particular advice (summarised on www.cidg.org) are that swimming is contraindicated with KCNQ1 mutation carriers, and loud noises during the night (alarm clocks, radios) need to be avoided as far as possible for KCNH2 mutation carriers (Schwarz et al. 2001).

The initial pilot study, funded by the Child Health Research Foundation (Cure Kids), consisted of 10 probands, demonstrating a clinical LQTS phenotype, who were tested for the three LQTS genes, KCNQ1, KCNH2, and SCN5A. Over the years this grew to include the screening of both clinically diagnosed LQTS families and a sudden unexpected death cohort for mutations in these five genes using denaturing high performance liquid chromatography (dHPLC) and direct sequencing. At the start of this project in 2005, over 100 probands had been analysed and approximately 30 mutations had been identified. A number of single nucleotide polymorphisms (SNPs) in these genes were also identified by the mutation-screening strategy. Family members of patients with positive genotypes were subsequently screened. These individuals, through careful counselling and medical management were empowered to make appropriate changes in their life styles, including, for most, taking beta blocker medication, which alone reduces the risk of sudden death by up to ten fold (Moss et al. 2000). The work described above is covered in more detail in Chapter 3 as part of the work towards this project included additional molecular genetic screening. Over the years, the CIDG has also established a clinical service and an ethically approved national cardiac inherited disease registry, which helps to streamline the screening of relatives of affected individuals who are spread across New Zealand and overseas.
1.6.1 The Cardiac Inherited Disease Group Registry

An ethically approved CIDG Registry has been operational in the New Zealand Northern region since 2002. National ethical approval was obtained in January 2008. Each individual enters the Registry following informed, written consent via CIDG clinicians and associated members in Auckland, Hamilton, Tauranga, New Plymouth, Palmerston North, Wellington, Christchurch and Dunedin. Currently this report is maintained by a coordinator and team support funded by Cure Kids for a limited period. The CIDG Registry database is managed within the Auckland District Health Board (ADHB). Maintenance of the registry is the responsibility of the ADHB, represented by the multidisciplinary CIDG. Clinicians and others, who work with the CIDG outside the ADHB are bound by the Registry’s privacy framework. The privacy framework for the management of the health information about identifiable individuals contained within the CIDG Registry falls within the provisions of the Privacy Act 1993 and the Health Information Privacy Code 1994 (HIPC). The HIPC, in particular, provides a broad framework of controls for the management of information about identifiable individuals. The framework applies to all information collected in the CIDG Registry and all individuals and organisations that collect, control and access information in the CIDG Registry. Personal data regarding any individual who could be identified would not be released to a third party without the individual’s prior knowledge and informed consent.

The key objectives of the registry are to:

1. Facilitate effective clinical and genetic screening of families who have an inherited heart disease.
2. Facilitate effective clinical and genetic screening of families who have suffered a sudden unexplained death in a young person.
3. Accumulate incidence and prevalence data for health resource planning.
4. Provide a resource for clinicians and patient groups to advise of new developments of potential benefit to patient care.

5. Provide a continued resource for consent based research into inherited heart disease and young sudden death.

1.7 Molecular autopsy from new-born screening Guthrie cards

The importance of obtaining molecular diagnoses in cardiac arrhythmia syndromes has been emphasised in the introduction to this work. The value of a molecular diagnosis, as described above, extends to post-mortem negative sudden unexplained death victims. A molecular autopsy can deliver a diagnosis of LQTS, Brugada syndrome, and CPVT in 40% of cases (Tester and Ackerman 2007). Such diagnoses enable effective screening and management of the deceased's family, with the aim of preventing other sudden deaths in the same family. However, obtaining DNA posthumously is problematic if appropriate samples are not collected at the time of the postmortem. Stored formalin-fixed, paraffin-embedded tissue blocks may not yield DNA of sufficient quality for testing as DNA can be degraded by the process and will also undergo degradation over time (Carturan et al. 2008; Doolan et al. 2008). In some instances, tissue blocks are not archived adequately for retrieval. Pathologists and coroners may also be under pressure to avoid unnecessary tissue storage after public concern over long-term organ or tissue retention. New best-practice recommendations for the investigation of young natural sudden death were recently developed by a clinical and scientific collaboration of Australia and New Zealand known as TRAGADY (the Trans-Tasman Response Against Sudden Death in the Young). These stipulate that tissue or blood suitable for DNA extraction should be stored (Skinner et al. 2008, TRAGADY, Post-mortem in Sudden Unexpected Death in the Young: Guidelines on Autopsy Practise, 2007)

However, not all practitioners adhere to these guidelines, and many families still seek a diagnosis for deaths occurring many years ago. Neonatal screening (Guthrie) cards provide a potential source of DNA in countries where the cards are stored long term and are available for retrieval.

In New Zealand, blood spots on Guthrie cards have been collected from newborns since 1969. Standardised cards of untreated Whatman no. 903 paper are used to collect four heel-prick blood spots (Figure 1.15). These are used to screen for diseases of inborn errors of metabolism by the National Testing Centre (www.nsu.govt.nz/Current-NSU-Programmes/566.asp). The cards are then stored indefinitely at ambient temperature and are available on request from the individual to whom the sample belongs or from other individuals who have the authority to access according to a strict protocol (such as the coroner or next of kin). They may also be requested for the purposes of research after approval by the program advisory committee and an Ethics Committee.

Genetic testing of blood spots from Guthrie cards occurring years or decades after collection has not been well described. With the increased awareness of cardiac channelopathies as a cause of young sudden death, and in particular LQTS, families and their clinicians may seek a molecular genetic diagnosis months or years after the death has occurred. LQTS was diagnosed from the neonatal screening card in a 12-year-old boy who was misdiagnosed as epileptic during life and in whom the cause of death was ascribed to sudden unexplained death in epilepsy (Skinner et al. 2004). Since most pathologists have not, until recently, stored blood or tissue suitable for DNA extraction, it would be valuable to know whether adequate DNA for testing could be obtained from Guthrie cards, which may be decades old.
The aim of this study was to investigate the role and implications of molecular genetics in the diagnosis and subsequent management of sudden arrhythmic death syndromes in New Zealand (NZ). At the outset of the project, molecular screening to determine the spectrum of missense mutations and small insertion- and deletion-mutations in NZ LQTS patients and sudden death victims was incomplete. The first aim of the thesis was to complete the screening in both patients and posthumous samples to establish a platform from which to proceed. Following these findings, which were consistent with global reports of approximately 25 -30% of clinically affected LQTS patients remaining mutation negative, the more specific hypotheses of the thesis were developed. In an attempt to identify the molecular genetic causes that contribute to the clinical phenotype in LQTS gene-negative patients, the following hypotheses were explored:
1. Given that large single- or multi-exon deletions or duplications comprise a large proportion of the mutation spectrum for several disorders, these mutations occur commonly in LQTS too.

2. LQTS is an extremely heterogenous disorder. Several genes are associated with LQTS, with new genes being identified almost every year and clinical phenotype can closely mimic other arrhythmic disorders. LQTS gene-negative individuals carry mutations in the RYR2 gene, associated with CPVT.

3. SNPs in LQTS-associated genes play a role as susceptibility loci or genetic modifiers of established mutations and, therefore, are occur at different frequencies in mutation-positive and mutation-negative samples.

The ultimate aim is to work towards establishing a molecular diagnosis for each patient and SCD victim, to enable the best possible patient and family care and prevent further sudden cardiac deaths in New Zealand families. A more detailed outline of the aims and objectives of the project is given below.

1.9 Aims and objectives

The work towards this degree forms part of the large, ongoing research endeavour of the CIDG that ultimately aims to prevent sudden death in young New Zealanders by identifying and understanding the genetic causes of LQTS and other cardiac arrhythmia syndromes in affected New Zealand families. Obtaining molecular diagnoses in cardiac arrhythmia syndromes is clearly crucial in establishing preventative clinical best-practice. It is clear, however, that in up to a third of families with obvious disease, or in those who have experienced sudden death in their families, no obvious gene defects have been identified (Splawski et al. 2000; Tester et al. 2005; Chung et al. 2007). We aim to study the
complex genetics of inherited arrhythmic disease, and develop new approaches and strategies to detect newly identified genetic causes. This will largely focus on those individuals whose ECG and clinical diagnosis indicate arrhythmic disease but in whom we haven’t identified any LQTS gene mutations using current screening methodology. The health outcomes will reduce the likelihood of sudden death in young people and provide genetic information that will result in preventative clinical best-practice. The more specific objectives of this thesis are outlined below.

1.9.1 Completing molecular genetic screening

The first objective of this project was to complete the molecular genetic screening of LQTS genes \textit{KCNQ1, KCNH2, SCN5A, KCNE1} and \textit{KCNE2} in our cohort of clinically diagnosed LQTS patients in order to accumulate sufficient mutation spectrum data to enable the molecular diagnostic screening programme to move from a research setting to a clinically accredited laboratory (Lab Plus, the laboratory diagnostic arm of the ADHB). This would enable the proportion of clinically affected LQTS patients who do not carry mutations in any of the above five LQTS-related genes to be determined and a phenotypically described, LQTS “gene negative” cohort from all samples that have passed through the CIDG registry to be established.

In addition to the molecular genetic screening described above, a series of young sudden unexplained deaths was investigated, up to 13 years after the death occurred, where no tissue suitable for DNA extraction had been retained at autopsy. The only source of DNA available for these samples was that extracted from their new-born screening Guthrie cards. Current methods for mutation screening of the LQTS genes are denaturing high-performance liquid chromatography (dHPLC) analyses of all exons and then direct sequence analyses of all those samples/exons with abnormal dHPLC profiles. Although
this approach yields a high detection rate and to date has been the most cost and time efficient, it does have some limitations. dHPLC may fail to detect variations in exons/PCR amplicons with sequences that do not allow appropriate dHPLC melting temperatures to span the entire length. The gold standard methodology in this case would be complete sequencing of the genes of interest. This has become much more feasible recently, with the reduction in sequencing costs and increase in high throughput technology, therefore, sequencing is now considered to be the standard approach in this type of study.

1.9.2 Screening for large duplications and deletions

As mentioned previously, mutations in the genes mentioned above are found only in approximately two thirds of cases. Point mutations and small deletions/insertions in the LQT genes can be detected by sequencing, dHPLC and other methods. These methods do not detect large gene rearrangements, such as large duplications or deletions (not infrequent mutations in other disorders), because of the presence of the remaining normal allele. Although yet unknown genes could be involved in the gene-negative patients, a number of cases might be attributable to large genomic rearrangements in these genes. We used a quantitative fluorescent approach, multiplex ligation-dependent probe amplification (MLPA), to detect deletions and/or duplications of one or more exons of the genes. There are a range of disorders where such deletions and/or duplications of one or more exons of a gene account for a significant proportion of detected mutations, such as Duchenne muscular dystrophy (Den Dunnen et al. 1989), breast cancer (Hogervorst et al. 2003) and Fanconi anaemia (Morgan et al. 1999). There is no reason why LQTS should be an exception. During the course of this study, a duplication of 3.7 kb in KCNH2 was identified in a Dutch family with LQTS (Koopman et al. 2006)
DNA microarrays are a well-established technology for measuring gene expression levels. More recently, however, high-density oligonucleotide-based whole genome microarrays have emerged as a preferred platform for genomic analysis beyond simple gene expression profiling. These ‘tiling arrays’ have a diverse range of applications in decoding information contained within the genome. We trialled the use of tiling arrays to confirm the existence, and delineate the boundaries of the deletions/duplications that identified by MLPA. If this were successful, we would be in a position to implement this as a routine diagnostic tool.

### 1.9.3 Cardiac ryanodine receptor gene (*RYR2*) screening

As LQTS and CPVT present very similarly, it is possible that some of our LQT gene-negative samples carry mutations in CPVT-associated genes. The contribution of *RYR2* mutations to the cardiac arrhythmias in New Zealand is completely unknown. *RYR2* is a large gene, made up of 105 exons and screening for mutations within the entire gene is a challenge. However, mutations tend to occur in three critical regions of the gene, that amount to 30-40 exons. We hope to screen those exons that include these regions as well as all previously reported *RYR2* mutations and assess the mutation detection rate in New Zealand. It seems likely that this will prove to be an important cause of inherited tendency to sudden death, and since the risk of death is reduced by beta blockers and pacemaker defibrillators, and it is often familial, screening for this condition is certainly desirable in young sudden death victims.

### 1.9.4 The role of SNPs and ‘unclassified’ variants

The cardiac channelopathies exhibit extreme variation in phenotype expression. The mechanisms underlying such phenotypic diversity remain unknown. Through the clinical screening program, several unclassified variants or single nucleotide polymorphisms (SNPs) have been identified that have uncertain clinical significance. For many complex diseases,
SNPs are known to predispose to the development of the disease. We would like to determine whether any of a selection of SNPs in LQTS-related genes play a role as susceptibility loci or genetic modifiers of established mutations. We intend to type these variants using realtime PCR and Taqman probes in our LQTS cohort and investigate their significance/pathogenicity through case association with various phenotypic parameters including QT interval.

The cardiac channelopathies are an extremely heterogenous family of disorders, through their clinical natural history that ranges from asymptomatic longevity to sudden death in infancy, ECG phenotype that ranges from completely normal resting ECG to extreme QT prolongation, to their complex genetic underpinnings. By pursuing the investigational areas outlined above, we hope to gain a better understanding of ion channel functioning and how these variable areas inter-relate. There are broader implications for the general public because of the suspected frequency of semi-penetrant cardiac channelopathies. This work will provide support for, or arguments against, the routine ECG screening of ‘at risk’ populations, or for the screening of SNPs in at risk groups. In general, the outcome of this project should enable clinicians to better manage affected families and improve the prevention of sudden death in disorders of cardiac arrhythmia in New Zealand families.
CHAPTER 2:

MATERIALS AND METHODS
2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

The chemicals used throughout the study were of analytical grade and were purchased from several suppliers: BDH (Pennsylvania, USA), BioLab (Clayton, Australia), Difco Laboratories (Detroit, USA), Invitrogen (Carlsbad, USA), Life Technologies (Gaithersburg, USA), Merck (Whitehouse Station, USA), Scharlau (Barcelona, Spain), Serva Electrophoresis GmbH (Heidelberg, Germany) and Sigma-Aldrich (St Louis, USA). The water used in this study was ultra pure pyrogen free water, and autoclaved where appropriate.

2.1.2 Enzymes

Taq DNA polymerases were purchased from Qiagen (Hilden, Germany), Qiagen Taq for standard polymerase chain reactions (PCR), Applied Biosystems (Foster City, USA) (Amplitaq Gold) and Roche (Basel, Switzerland), Expand Long Template PCR System for long range PCR. Proteinase K, used mainly in DNA extraction was purchased from Sigma-Aldrich. Restriction endonucleases were purchased from New England Biolabs (NEB) (Ipswich, USA). TaqMan Mastermix, SYBR Green PCR mastermix and BigDye Terminator v3.1 Cycle Sequencing Kits were purchased from Applied Biosystems.

2.1.3 Kits

Kits for purification of PCR products were purchased from Roche (Basel, Switzerland) and Qiagen Inc (Hilden, Germany). MasterPure™ Complete DNA and RNA Purification Kits were purchased from EPICENTRE Biotechnologies (Madison, USA). MLPA kits, SALSA
MLPA P108 SCN5A and SALSA MLPA kit P114 Long-QT, were purchased from MRC-Holland (Amsterdam, The Netherlands).

2.1.4 Primers

All primers designed for this research were purchased from Invitrogen and were of standard purity (that is, desalted). For PCR amplification, primers were resuspended in ultra pure pyrogen free water to a stock concentration of 100µM and a working concentration of 10µM. For quantitative real time PCR, primers were resuspended in 10mM Tris, 0.1mM EDTA, pH 8 to a stock concentration of 100µM and working concentrations of 3µM.

2.2 Methods

2.2.1 Patients

The New Zealand Cardiac Inherited Disease Registry, formed through the CIDG, has received ethical approval from the New Zealand multicentre ethics committee: (AKX/02/00/107)

Patients suspected of having an inherited cardiac arrhythmia syndrome or victims of sudden cardiac death (SCD) or sudden unexplained death (SUD) were referred to our cardiac service. The process of assessing clinical diagnosis was that first a referring clinician made the diagnosis based on his/her clinical evaluation, based on clinical and family history and the ECG findings. In each case the diagnosis was then reviewed by Dr Jon Skinner, with particular attention to accurate measurement of the QT interval. Borderline cases were reviewed by a small panel of expert cardiologists. The QT interval recorded was the heart-rate corrected QT interval (QTe), made by dividing the QT interval by the square root of
the preceding R-R interval (the Bazett formula). The longest QTc was taken, measuring in both lead V5 and lead II, (Monnig et al. 2006) and looking at serial ECGs when they were available (Goldenberg et al. 2006). In most cases QT intervals measured during and after exercise and 24 hour Holter recordings formed part of this assessment. QT intervals taken in the first few hours after a cardiac arrest were not taken as indicative, since transient QT prolongation is common in that scenario. Care was also taken to ensure that no other factors might have influenced the QT interval (metabolic derangement, cooling, QT prolonging medications etc). The Schwartz score is a clinical scoring system to assign probability of diagnosis of LQTS (Schwartz et al. 1993). This is based on multiple factors, including length of the QT interval, and presence of syncope or cardiac arrest, and presence of family history. The score, more particularly, is calculated by assigning different points to various criteria:

- QTc (Defined as QT interval / square root of RR interval)
  - >= 480 msec - 3 points
  - 460-470 msec - 2 points
  - 450 msec and male gender - 1 point
- Torsades de Pointes ventricular tachycardia - 2 points
- T wave alternans - 1 point
- Notched T wave in at least 3 leads - 1 point
- Low heart rate for age (children) - 0.5 points
- Syncope (one cannot receive points both for syncope and Torsades de pointes)
  - With stress - 2 points
  - Without stress - 1 point
- Congenital deafness - 0.5 points
- Family history (the same family member cannot be counted for LQTS and sudden death)
- Other family members with definite LQTS - 1 point
- Sudden death in immediate family (members before the age 30) - 0.5 points

With 4 or more points the probability is high for LQTS, and with 1 point or less the probability is low. Two or 3 points indicates intermediate probability.

This assessment score was used in particular to grade level of certainty in the diagnosis when the genetic test was uninformative, and is referred to later in the section on MLPA testing of the gene-negative cohort of LQTS subjects.

Evaluation of victims of young sudden unexpected death involved a team approach, with formal review of each case during the weekly cardiology/pathology/genetic team meetings.

Patients whose history and electrocardiogram findings supported a clinical diagnosis of LQTS or Brugada syndrome proceeded to molecular genetic analysis. Informed consent for genetic testing was obtained in all cases, following the protocols established in our multicenter ethical approval from the regional ethics committee.

Patient samples were received in the form of either 5-10ml whole blood or DNA previously extracted by the Diagnostic Genetics Laboratory at Lab Plus (ADHB). Sudden death samples were obtained from blood spots punched from neonatal screening (Guthrie) cards or from whole blood samples extracted at post-mortem examination. In New Zealand, blood spots on Guthrie cards have been collected from newborns since 1969. Standardised cards, containing untreated Whatman FTA paper, are used to collect four heel-prick blood spots. These are used to screen for diseases of inborn errors of metabolism by the National Testing Centre. The cards are then stored indefinitely at ambient temperature and are available upon request from the individual to whom the
sample belongs, the next of kin or by request from the coroner. Samples may also be requested for the purposes of research through a National Testing Review Panel and Ethics Committee.

2.2.2 Control samples

The control samples used in this study were recruited for a colleague’s study: SPRASA, a novel protein. Is it essential for fertility? (Prendergast 2009). Samples were obtained following written, informed consent and approved by the regional ethics committee. Blood samples were obtained by venipuncture from 104 fertile couples, who were recruited via popular media advertisements. Fifty of these couples (50 male and 50 female individuals) were used as control samples in the present study. Their ethnicities were all Caucasian and their ages ranged from 18-53 years.

2.2.3 DNA Extraction

2.2.3.1 DNA extraction from whole blood

Genomic DNA was extracted from 5-10mL of blood using a salting out method adapted from the protocol established by Miller and colleagues (1988). Whole blood collected in EDTA tubes (no older than two or three days) was transferred into a 50mL centrifuge tube and either frozen at -20°C for extraction at a later time or used as follows: the red blood cells were lysed with 40-45mL of cold Sucrose-Triton-X lysing buffer (Appendix) by inverting the tubes several times to mix. The white blood cells were pelleted by centrifugation at 3000rpm for 10 minutes. The supernatant was discarded and the cell pellet washed twice by the addition of 20mL Sucrose-Triton-X lysing buffer and placed on ice for 5 minutes. The cells were re-pelleted by centrifugation at 3000rpm for 5 minutes. The supernatant was discarded after each wash. After the final wash the cell pellet was
lysed with 3.2mL of Proteinase-K/Tris EDTA (PK/TE) mixture (AppendixI), inverted several times and lysed overnight at 42°C. The following day, 1mL of saturated NaCl (Appendix) was added to the lysate and mixed vigorously by inverting the tubes for 15 seconds. The cell lysate/NaCl mixture was allowed to stand for 10 minutes at -20°C followed by centrifugation at 3000rpm for 30 minutes. The supernatant containing the DNA was transferred into a new 50mL tube and two volumes of room temperature absolute ethanol added. DNA precipitated out of solution was gently spooled and washed in ice-cold 70% ethanol, transferred into a 1.5mL tube and the pellet allowed to air dry. The DNA pellet was resuspended in an appropriate (200µL – 1000µL) amount of 1xTris-EDTA buffer (Appendix). DNA stocks was diluted to a concentration of 50ng/µL and stored at -20°C.

2.2.3.2 DNA Extraction from blood spots obtained from newborn screening Guthrie cards

DNA from blood spots obtained from newborn screening Guthrie cards were extracted using the MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE Biotechnologies). DNA was stored at -20°C.

2.2.4 Polymerase Chain Reaction (PCR)

2.2.4.1 PCR Primers

PCR was used to amplify the all exons and flanking intron boundaries of LQTS genes (KCNQ1, KCNH2, SCN5A, KCNE1 and KCNE2) (section 3) and selected exons and flanking intron boundaries of the RYR2 gene (section 5). The oligonucleotide primers used to amplify the exons and flanking intron boundaries of genes KCNQ1, KCNH2, SCN5A, KCNE1, KCNE2 and RYR2 were either ordered ‘as is’ from previous reports (Splawski et al. 1998; Syrris et al. 2001; Tiso et al. 2001) or designed using Primer 3.0 program Whitehead Institute, Cambridge, MA (http://frodo.wi.mit.edu/cgi-bin/
primer3/primer3_www.cgi). The primer sequences are listed in the Appendix, Table 8.1-8.6. Previously reported primers were redesigned if they either contained reported single nucleotide polymorphisms (SNPs) or if the exon-flanking sequence was less than 50bp. This was required so that adequate DNA sequencing reads would be achieved post PCR.

SNPCheck (https://ngrl.manchester.ac.uk/SNPCheckV2/snpcheck.htm) was used to check for SNPs in predicted primer binding regions of all the primers used in this study. SNPCheck is a bioinformatic program launched by the National Genetics Reference Laboratory (NGRL), Manchester, for batch checking of oligonucleotide primers for SNPs. It uses the latest build of the human genome from NCBI (National Centre for Biotechnology Information - www.ncbi.nlm.nih.gov), and BLAST (Basic Local Alignment Search Tool) to identify the position in the sequence where the primers bind. The contents of the current release of dbSNP (NCBI) are used to identify if there are any known SNPs at the primer binding sites.

2.2.4.2 PCR amplification

All primer pairs were optimised using gradient PCR (on an iCycler thermal cycler, BioRad) to determine the best annealing temperature and whether or not Q solution (Qiagen) is required for the PCR reaction. Q solution is a proprietary agent from Qiagen for difficult templates, and aids DNA denaturation. PCR reactions were carried out in a total volume of 25μL, using 100ng of genomic DNA. Each reaction contained 2.5μL of 10X Qiagen Taq buffer (Qiagen), 100μM of each deoxynucleotide triphosphate (dNTP) (Roche), 10μM of forward and reverse primer, 5μL of Qiagen Q solution (for specified exons, Tables 8.1-8.6 in the Appendix), and 0.125μL of Qiagen Taq polymerase (5U/μL). PCR amplifications were performed in either an MJ-Research PTC-200 thermal cycler (MJ Research, Watertown, MA) or in an iCycler (BioRad).
Standard PCR conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, variable annealing temperature depending on primer pair for 1 minutes, and extension at 72°C for 1 minute. A final extension cycle was carried out at 72°C for 10 minutes. Certain primer pairs (Tables 8.1-8.6) required Touchdown PCR 70°C – 60°C for successful amplification. Touchdown PCR reduces the amplification of non-specific sequence (Dieffenbach and Dveksler, 1995). These cycling conditions consisted of an initial denaturation of 94°C for 5 minutes followed by 20 cycles of denaturation at 94°C for 45 seconds, annealing at 70°C for 45 seconds, and then to decrease the annealing temperature by increments of 0.5°C for every sequential cycle of the remaining ten cycles, and a primer extension at 72°C for one minute. This was followed by 15 additional cycles consisting of denaturation at 94°C, annealing at 60°C for 45 seconds and a primer extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 10 minutes.

2.2.4.3 DNA Electrophoresis on Agarose Gels

PCR products were visualised by electrophoresis on agarose gels to ensure that a single band of an expected size and sufficient concentration was amplified. Agarose (AppliChem) was dissolved in TAE buffer (Appendix) by heating in a microwave until all agarose particles had completely dissolved. The percentage of agarose used to make the gel was dependent on the fragment resolution required. DNA fragments were visualised by loading on to the gel 5µL of PCR products along with 1µL of 6 X loading dye of either bromophenol blue or Xylene cyanol loading buffer (Appendix), depending on the size of the amplicon and concentration of the gel. Samples were electrophoresed in TAE buffer for the required length of time and subsequently stained for 30 minutes with ethidium bromide (10mg/ml) (Appendix). Fluorescence of ethidium bromide-chelated DNA was
visualised with UV light and photographed with an EDAS 590mm filter, with the Kodak DC120 Zoom digital camera.

2.2.4.4 PCR Cleanup

PCR products to be used for sequencing were purified to remove excess primers and dNTPs with the High Pure PCR Product Purification Kit (Roche) according to manufacturer’s instructions. The purified PCR products were quantified by agarose electrophoresis, stained with ethidium bromide and visualised under UV light as described above.

2.2.5 Denaturing high pressure liquid chromatography (dHPLC)

The coding sequence and splice sites of LQTS genes (\textit{KCNQ1}, \textit{KCNH2}, \textit{SCN5A}, \textit{KCNE1} and \textit{KCNE2}) were screened for genomic variants by Transgenomic’s dHPLC system followed by DNA sequencing (described in Section 2.2.5). Before dHPLC, PCR products were denatured at 95°C for 5 minutes and slowly cooled to 4°C (0.1°C/sec) using an automated program on the PTC-200 PCR machine to generate either homoduplex or heteroduplex molecules if a mismatch of base pairs was present.

To identify single-nucleotide polymorphisms and mutations in the five LQTS genes described above, sequence variation was detected by dHPLC analysis in a mixture of DNA from index cases and normal control samples (Viskin et al. 2005). dHPLC was performed on the Transgenomic 2100 Wave DNA fragment analysis system (Transgenomic, Omaha, NE) using a DNASep HT cartridge and Navigator version 1.5.1 software (2003, Transgenomic, Omaha, NE). For each amplimer assay, the optimal partial denaturing temperatures were determined using interpretation of the DNA melting properties by the Navigator version 1.5.1 software (Table 8.7, Appendix).
PCR products of DNA samples with variant dHPLC profiles were purified with the Qiaquick PCR purification kit (Qiagen, Inc, Hilden, Germany) and sequenced (Section 2.2.5). The population frequency of SNPs and suspected functional variants were assessed in 100 control chromosomes using restriction fragment length polymorphisms (RFLP) analysis if a suitable restriction enzyme was available. Candidate mutations that failed to generate restriction fragment length polymorphism changes were assessed by dHPLC of controls vs mutation positive profiles to test the frequency of the abnormal profile within the control population. Variants were designated as mutations by excluding them from normal population controls and aligning them against other GeneBank databases to assess the degree of evolutionary/phylogenetic conservation. These results are presented in Chapter 3.

2.2.6 DNA sequencing

Bi-directional DNA sequencing for mutation screening of LQTS genes KCNQ1, KCNH2, SCN5A, KCNE1 and KCNE2 was carried out as described below. Unidirectional sequencing for mutation screening of RYR2 was performed by Macrogen DNA Sequencing Service (Korea). In all cases, sequence traces that varied from their respective reference sequences were repeated to confirm identified variants.

2.2.6.1 Sequencing reaction setup

Sequencing reactions were performed using the ABI prism Big Dye Terminator Sequencing Kit v3.1 (ABI) in a 20μl volume with approximately 10ng of PCR product per 100 bp of amplicon length. PCR products were quantified by agarose gel electrophoresis ethidium bromide staining. Sequencing reactions were carried out in 96-well plates, with each reaction consisting of 1.5μl of BigDye Terminator v3.1 premix, 10pmoles of primer, and the appropriate volume of sequencing buffer. In trouble shooting for amplicons that
proved difficult to sequence, sequencing reactions were supplemented with betaine (which helps in the denaturation of GC rich areas of DNA) at a final concentration of 1.4 M. Sequencing conditions comprised of an initial denaturation at 96°C for 1 minute, followed by 25 cycles of denaturation at 96°C 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes.

2.2.6.2 Sequencing reaction cleanup
Sequencing reaction extension products were precipitated by the MgSO₄ protocol recommended by the Australian Genome Research Facility (AGRF) and is described below. MgSO₄ precipitation solution consisting of 0.2mM MgSO₄ in 70% ethanol was prepared fresh for each experiment. To each sequencing reaction, 75µl of the 0.2mM MgSO₄ stock precipitation solution was added, mixed by inverting the plate several times, then left to precipitate in the dark at room temperature for either 15 minutes for fragments less than 200bp or 30 minutes for fragments greater than 200bp. The sequencing products were pelleted by centrifugation at 3000G for 30 minutes at room temperature. The plate was inverted and allowed to drain on a paper towel, and while in the inverted position the plate was placed onto a new paper towel and centrifuged at 700G for 1 minute. Samples were allowed to air dry for 10 minutes and then submitted to the Genome Research Facility at the School of Biological Sciences, University of Auckland, for capillary electrophoresis on a 3100/3130 Genetic Analyser (Applied Biosystems).

2.2.6.3 Sequence data analysis
The software programme Chromas Lite (http://www.technelysium.com.au/chromas.html) was used for the manual analyses of Applied Biosystems’ sequence chromatogram files for the LQTS genes KCNQ1, KCNH2, SCN5A, KCNE1 and KCNE2 (Chapter 3). The Applied Biosystems Variant Reporter Software was used for automated reviewing of RYR2
sequence data. The set up of the Variant Reporter® Software and the construction of an RYR2 “pseudo-reference sequence” for use in Variant Reporter® automated sequence analysis is described in Chapter 5 (Section 5.3).

2.2.7 Plasmids and cloning

The PCR products of exons in which small insertions or deletions were identified with DNA sequencing were cloned into the pGEM-T Easy plasmid (Promega) and transformed into bacterial cells. This provided increased amounts of DNA to allow isolation and amplification of individual alleles. All plasmids were transformed into Escherichia coli (E.coli) TOP10 competent cells (Invitrogen). All cells positive for plasmids were selected with ampicillin (50µg/ml) (Roche). Transformed E.coli cells were grown on Luria Bertani (LB) agar (Invitrogen) plates with the appropriate antibiotic for short term storage. For the isolation and purification of plasmid DNA, transformed E.coli cells were grown in LB broth with the appropriate antibiotic. For quantities less than 5ml, the plasmid DNA was isolated and purified with the plasmid DNA isolation kit from Promega, while larger quantities were isolated with the Qiagen plasmid DNA isolation kit, as per the manufacturer’s instructions.

2.2.8 Multiplex ligation-dependent probe amplification (MLPA)

2.2.8.1 MLPA method

MLPA is a quantitative fluorescent approach that was used to determine whether deletions and/or duplications of one or more exons of the main LQTS genes were present in an LQTS mutation negative cohort. Two MLPA kits, SALSA MLPA P114 LQT kit (lot 0508) and SALSA MLPA P108 SCN5A kit, (lot 0407) were purchased from MRC-Holland (Amsterdam, The Netherlands). MLPA analysis was performed according to the MRC Holland protocol (Schouten et al. 2002). In summary, 125ng of genomic DNA from each
sample was diluted in 5µl Tris EDTA buffer and denatured at 98°C for 5 minutes. MLPA buffer and probe mix (1.5µl of each) were added to allow the probes to anneal to their target sequences by heating at 95°C for 1 minute and incubating for 16 hours at 60°C. A buffer/ligase mixture (32µl) was added to each sample and incubated at 54°C for 15 minutes, followed by heating to 98°C for 5 minutes. 10µl of the ligation reaction was used for multiplex PCR amplification using a single universal primer pair suitable for all the probes in the kit. The SALSA polymerase was added at 60°C, followed by 36 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and a final extension step of 72°C for 20 minutes. 1µl of each PCR product was mixed with 0.5µl Genescan-Rox 500 size standard and 8.5µl of deionised formamide, and 1µl was injected into a 36-cm capillary (model 3130XL, Applied Biosystems Foster City, CA, USA) at 60°C. The MLPA process is illustrated in Figure 2.1.

2.2.8.2 MLPA data analysis

The electropherogram was analysed using either Genescan or Peakscanner software (Applied Biosystems). For each sample, the relative peak area was calculated and compared to five healthy controls using the Coffalyser version 2 software (MRC-Holland). This software calculates the relative peak area for each probe within the same test and compares each relative peak area to those obtained from the five controls to obtain the ratio peak area for each probe. All results with ratio peak areas suggesting aberrant copy numbers were independently repeated. Sequencing of apparent single exon deletions was performed as described in section 2.2.5.
Chapter 2: Materials and methods

Figure 2.1 The MLPA reaction. Each MLPA probe consists of two oligonucleotides which hybridise to a target sequence. Hybridisation: The MLPA probe mix is added to denatured genomic DNA and allowed to hybridise to their respective targets overnight. Ligation: Probes are ligated by a thermostable ligase. Amplification: A universal primer pair is used to amplify all ligated probes. The amplification product of each probe has a unique length (130 to 480 bp so that the amplification products of each probe can be separated electrophoretically).

2.2.9 Real-time quantitative PCR

Real-time quantitative PCR was used to validate the MLPA results. Primers were designed using the PrimerExpress software (Applied Biosystems) to correspond, in most cases, to the probe recognition sequences that appeared to be duplicated or deleted by MLPA analysis. Amplicons within exon 24 of the CFTR gene and exon 3 of the SPR-AVA gene were used as endogenous controls. Primer sequences are given in Table 8.8 (Appendix). PCR was carried out using an ABI Prism 7900HT Fast Real-Time PCR System and 384-well Clear Optical Reaction Plates (Applied Biosystems). The PCR was performed in a total volume of 10µl containing 100ng of genomic DNA, 5µl of SYBR GreenER qPCR supermix (Invitrogen, Carlsbad, CA, USA), and 2pmol each of forward and reverse primer, respectively. Cycling conditions were 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles
consisting of 15 seconds at 95°C, and 1 minute at 60°C. A dissociation curve analysis step of a rapid ramp to 95°C for 15 seconds, 15 seconds at 60°C, and then a slow ramp to 95°C for 15 seconds was added.

Real-time PCR reactions for each amplicon, including the CFTR and SPR-ASA controls for each individual, were performed in triplicate, and results were analyzed using the $2^{\Delta\Delta C_T}$ (comparative threshold cycle [$C_T$]) method (Livat and Schmittgen 2001)). The amplification efficiencies of the target and reference sequences were determined using the LinRegPCR software (Ramakers et al. 2003) and were approximately equal in most cases as shown in Table 8.8 (Appendix).

2.2.10 Oligonucleotide array–based comparative genomic hybridization (aCGH).

MLPA (Section 2.2.7) and real-time PCR (Section 2.2.8) are techniques than can be used to detect copy number variation. A pilot experiment was carried out to evaluate the use of aCGH as an alternative technique to detect such copy number variation. This work was carried out under the supervision of Associate Professor Don Love at LabPLUS, Diagnostic Genetics (ADHB) and at Roche Diagnostics (Mount Wellington, Auckland) with Anthony Thrush (Systems Specialist, Roche Diagnostics, New Zealand). A Roche Nimblegen CGH Custom Targeted Array was designed to target all exons of 67 genes that are tested in-house at LabPLUS, Diagnostic Genetics. Standard aCGH methods were used to test DNA samples for copy number aberrations, described briefly below. Analysis was tailored to the specific gene of interest using NimbleScan software.

While this work was carried out mainly by Anthony Thrush (Roche Diagnostics) and Elaine Doherty (Lab PLUS, Diagnostic Genetics), I was involved in some of the sample labelling and hybridisation of the array and subsequent data analysis.
2.2.10.1 Microarray construction and probe selection

A high-resolution oligonucleotide CGH tiling array was custom designed on a NimbleGen (Roche) 12x135K array format. The array contained probes covering exons of 67 genes tested at Diagnostic Genetics, LabPLUS.

However, only genes included in the array that are associated with inherited arrhythmia disorders will be discussed here. These genes, the disorder with which they are associated and their genomic coordinates are given in Table 2.1.

Table 2.1 The LQTS-associated genes included in the array, the disorder with which they are associated and their genomic coordinates.

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Gene</th>
<th>mRNA</th>
<th>Genomic (Range)</th>
<th>Chromosome</th>
<th>UCSC* genome coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQT1</td>
<td>KCNQ1</td>
<td>NM_000218.2</td>
<td>NC_000011.8 (2422797-2826916)</td>
<td>ch1p15.5-p15.4</td>
<td>chr11:2,422,797-2,826,915</td>
</tr>
<tr>
<td>LQT2</td>
<td>KCNH2</td>
<td>NM_000238.2</td>
<td>NC_000007.12 (15030947-150272982)</td>
<td>ch7q36.1</td>
<td>chr7:150,272,982-150,305,947</td>
</tr>
<tr>
<td>LQT3/BrS</td>
<td>SCN5A</td>
<td>NM_198056.1</td>
<td>NC_000003.10 (38666167-38654557)</td>
<td>ch3p22</td>
<td>chr3:38,564,557-38,666,167</td>
</tr>
<tr>
<td>LQT4</td>
<td>ANK2</td>
<td>NM_001148.3</td>
<td>NC_000004.10 (114190319-11424337)</td>
<td>ch4q26</td>
<td>chr4:114,190,319-114,243,334</td>
</tr>
<tr>
<td>LQT5</td>
<td>KCNCE1</td>
<td>NM_000219.2</td>
<td>NC_000021.7 (34806443-34740858)</td>
<td>ch21q22.12</td>
<td>chr21:34,740,859-34,806,443</td>
</tr>
<tr>
<td>LQT6</td>
<td>KCNCE2</td>
<td>NM_172201.1</td>
<td>NC_000017.9 (34658193-34665310)</td>
<td>ch21q22.11</td>
<td>chr21:34,658,193-34,665,310</td>
</tr>
<tr>
<td>LQT7/AS</td>
<td>KCNJ2</td>
<td>NM_000891.2</td>
<td>NC_000012.10 (65677271-65687780)</td>
<td>ch17q24.3</td>
<td>chr17:65,677,271-65,687,776</td>
</tr>
<tr>
<td>LQT8/TS</td>
<td>CACNA1c</td>
<td>NM_000719.5</td>
<td>NC_000003.10 (2032725-2072369)</td>
<td>ch12p13.33</td>
<td>chr12:2,032,725-2,077,376</td>
</tr>
<tr>
<td>LQT9</td>
<td>CAV3</td>
<td>NM_001234.3</td>
<td>NC_000018.8 (8750496-8763451)</td>
<td>ch3p25.3</td>
<td>chr3:8,750,496-8,763,450</td>
</tr>
<tr>
<td>LQT10</td>
<td>SCN4B</td>
<td>NM_174934.2</td>
<td>NC_000001.9 (11752874-117509302)</td>
<td>ch1q23.3</td>
<td>chr11:11,750,302-11,752,845</td>
</tr>
<tr>
<td>CPVT1</td>
<td>RYR2</td>
<td>NM_001035.2</td>
<td>NC_000001.9 (35272325-352603276)</td>
<td>ch1q43</td>
<td>chr1:235,272,325-236,063,911</td>
</tr>
<tr>
<td>CPVT2</td>
<td>CASQ2</td>
<td>NM_001232.2</td>
<td>NC_000001.9 (116112793-116044166)</td>
<td>ch1p13.1</td>
<td>chr1:116,044,149-116,112,929</td>
</tr>
</tbody>
</table>

*UCSC – University of California Genome Browser (http://genome.ucsc.edu/)
Oligonucleotide exonic probes of variable length (60 bp – 75 bp) were designed to overlap by 25 bp, therefore, using 8 probes covers a 200 bp exon. Each probe was spotted in duplicate. The results were normalised by using internal ‘backbone’ probes localised to regions of the genome outside of the genes of interest. These were spaced approximately every 175bp.

2.2.10.2 Sample labelling and hybridisation

High molecular weight genomic DNA samples were labelled using a NimbleGen Dual Colour DNA Labeling Kit (Roche) according to the manufacturer’s instructions. In brief, DNA samples (500ng) were denatured at 98°C in the presence of either Cy3-labeled random nonamer (for test samples) or Cy5-labeled random nonamer (for reference samples) in Random Primer Buffer (a kit component) and β-mercaptoethanol. The denatured samples were chilled on ice, and then incubated with 100 units Klenow fragment and 10mMdNTP mix for 2 hr at 37°C. Reactions were terminated by addition of 0.5 M EDTA; the end products were precipitated with isopropanol and resuspended in nuclease free water. For hybridisation 20µg each of test sample and reference sample were combined and dried in a DNA concentrator on low heat, protected from light. Each sample was resuspended in a unique Sample Tracking Control (STC) (a kit component), to which Hybridisation Solution Master Mix (a kit component) was added. The samples were denatured at 95°C for 5 min, and then cooled to 42°C. Hybridisations were carried out for 40-72 hours at 42°C. The arrays were washed using a NimbleGen Wash Buffer System (Roche) and immediately dried down by centrifugation.

Arrays were scanned at 2- and 5-micron pixel resolution using the GenePix 4000 scanner (Molecular Devices, Sunnyvale, CA). Data were extracted from scanned images using
NimbleScan 2.0 extraction software (NimbleGen Systems, Inc.), which allows for automated grid alignment, extraction, and generation of data files.

2.2.10.3 Data analysis

Normalised log₂ ratio data were analysed using analysis programs: (SegMNT or DNA copy) NimbleScan (Hupe et al. 2004). At least 2 affected probes were required to call a change. Log₂ above +0.2 indicated a duplication and Log₂ below -0.2 indicated a deletion.

2.2.11 TaqMan allelic discrimination assays

TaqMan allelic discrimination assays (Applied Biosystems) were used to screen for a selected cohort of SNPs in LQTS-associated genes. The SNPs genotyped and their selection criteria are given in Chapter 6. For SNPs without predesigned assays, custom assays were designed using the File Builder software (Applied Biosystems).

2.2.11.1 Designing the custom TaqMan SNP genotyping assay

BLAST-verified unique target sequences underwent a SNP BLAST search to confirm the absence of non-target SNPs within two bases of the SNP to be genotyped. Repeat Masker (http://www.repeatmasker.org) was used to ensure that the SNP of interest was not located within a masked repeat or within two bases of a masked repeat. Evaluated target sequences were used to create a submission file with File Builder for validation and assay order.

2.2.11.2 SNP genotyping

TaqMan PCR reactions contained a pair of oligonucleotide primers and two TaqMan allele-specific probes labeled with the fluorescent dyes VIC and FAM, respectively, and 10ng DNA. PCR was carried out in a total reaction volume of 5 μl in 384-well plates with the
following amplification protocol: denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds, finished with annealing and extension at 60°C for 1 minute. Post PCR, the genotype of each sample was attributed automatically by measuring the allele-specific fluorescence on the Applied Biosystems 7900 HT Fast Real Time PCR System using SDS 2.3 software for allelic discrimination (Applied Biosystems). Duplicate samples and negative controls were included to ensure accuracy of genotyping.

2.2.12 In silico analyses

The possible impact of unclassified, nonsynonymous variants on the structure, function and expression of their respective encoded proteins was assessed using several on-line prediction tools, SIFT (sorts intolerant from tolerant) (Ng and Henikoff 2003) predicts the functional importance of an amino acid substitution based on the alignment of highly similar orthologue and/or paralogue protein sequences (Rudd et al. 2005). SIFT scores were classified as intolerant (0.00-0.05), potentially intolerant (0.051-0.10), borderline (0.101-0.20), or tolerant (0.201-1.00) (Xi et al. 2004; Rudd et al. 2005). PolyPhen (polymorphism phenotyping (Ng and Henikoff 2001) predicts the functional effects of amino acid changes by assessing the level of sequence conservation between homologous genes over evolutionary time (Rudd et al. 2005). PolyPhen scores were designated as probably damaging (≥2.00), possibly damaging (1.50-1.99), potentially damaging (1.25-1.49), borderline (1.00-1.24), or benign (0.00-0.99) (Xi et al. 2004; Rudd et al. 2005). Grantham matrix scores (GMS) (Grantham 1974) predicts the effect of substitutions between amino acids based on chemical properties, including polarity and molecular volume (Rudd et al. 2005). GMS was designated as conservative (0-50), moderately conservative (51-100), moderately radical (101-150), or radical (≥151) (Rudd et al. 2005; Li et al. 1984).
2.2.13 Statistical analysis

The Hardy-Weinberg distribution of genotypes identified through the TaqMan genotyping assays was determined. Deviation from the Hardy-Weinberg equilibrium was tested using Pearson’s chi-squared test of the observed and expected genotype frequencies. Statistical analysis of the association between allele frequencies in different groups were performed using Fisher’s exact test (http://www.matforsk.no/ola/fisher.htm). All p-values were two-tailed and considered significant if \( p< 0.05 \).
CHAPTER 3:

MUTATION ANALYSIS IN LQTS GENES
3 Mutation analysis in LQTS genes

3.1 Molecular genetic screening study

Several LQTS genetic screening studies have been published, identifying over 600 mutations (Splawski et al. 2000; Tester et al. 2005; Napolitano et al. 2005). In response to the clear clinical importance of genetic screening for LQTS and Brugada syndrome, the CIDG established a university-based molecular genetic screening programme in 2002, to determine the local spectrum of mutations and ultimately assist in directing optimal patient and family treatment and management. An additional aim was to examine the factors relevant to establish a clinical genetic diagnostic service in New Zealand.

The genes associated with inherited arrhythmia disorders are reviewed in Chapter 1. The five genes most frequently affected in LQTS and analysed in the screening programme are KCNQ1, KCNH2, SCN5A, KCNE1 and KCNE2. As a brief reminder, KCNQ1 (LQT1) and KCNH2 (LQT2) encode α-subunits of the voltage-gated K⁺ channel and are responsible for I_{Ks} and I_{Kr}, respectively. Loss of function mutations result in prolongation of the QT interval. SCN5A (LQT3) encodes the α-subunit of a voltage-gated Na⁺ channel. Gain of function mutations in SCN5A cause LQT3, whereas those mutations creating a loss of function result in Brugada syndrome. KCNE1 (LQT5) and KCNE2 (LQT6) code for the β-subunit of the K⁺ channel, affecting I_{Ks} and I_{Kr}, respectively.

At the start of the work towards this thesis, molecular screening of the five LQTS genes mentioned above was incomplete. Screening between 2002 and 2005 was carried out by Seo-Kyung Chung, under the supervision of Professor Mark Rees. The first aim of the
project was to complete the screening in both patients and posthumous samples to establish a platform from which to proceed

### 3.2 Patients

Patients suspected of having LQTS or BrS were referred to the CIDG. The initial presentations to medical services included personal symptoms (syncope, seizures, or resuscitated sudden cardiac death (SCD)), SCD of a relative, and the incidental finding of a prolonged QTc interval. Section 2.2.1 outlines the process of assessing clinical diagnosis. During the course of this study, 20 index cases (or probands) whose history and electrocardiogram findings supported a clinical diagnosis of LQTS or Brugada syndrome and 24 sudden death victims were screened for mutations in five genes associated with LQTS: \( KCNQ \), \( KCNH2 \), \( SCN5A \), \( KCNE1 \) and \( KCNE2 \).

In eight of the cases of sudden unexplained death, a genetic screen was carried out using DNA samples extracted from the new-born screening Guthrie cards of the deceased, between two and 13 years after the death. Postmortem investigations had been negative in each case, and no tissue suitable for DNA extraction had been retained. Family cardiological investigations had either failed to make a diagnosis of inherited heart disease or had revealed some suspicion of LQTS.

Thirty mutation screening tests were carried out in relatives of patients (index cases) in whom mutations were detected.
3.3 Results

3.3.1 Molecular genetic screening of index cases

Genetic screening for mutations in all exons (65 in total) of five genes associated with LQTS: \textit{KCNQ1} (16 exons), \textit{KCNH2} (15 exons), \textit{SCN5A} (28 exons), \textit{KCNE1} (3 exons) and \textit{KCNE2} (3 exons, including 3’ untranslated amplicon) was carried out in 20 patients and 24 sudden death victims.

Thirteen mutations were detected in 11 cases (8 in patients and 3 in sudden deaths), reflecting a detection rate of 27.3%. These mutations are summarised in (Table 3.1). Four of the 13 mutations detected were novel (30.8%) and had not been previously reported in the literature or the Inherited Arrhythmia Database (www.fsm.it/cardmoc/). Novel mutations were distributed as follows: one in \textit{KCNH2}, and three in \textit{SCN5A} (Figure 3.1). The approximate genetic and protein locations of the identified mutations are illustrated in Figures 3.2 to 3.4. A case of Jervell and Lange-Neilsen syndrome, segregated with \textit{KCNQ1} compound heterozygosity (p.L266P/p.G269S). Another patient was heterozygous for two mutations, a frameshift in \textit{KCNQ1} p.L191fs281x and missense mutation in \textit{SCN5A} p.A425S.

Eleven of the mutations identified were missense mutations. Two frameshift mutations were detected, a 5 bp deletion in \textit{KCNQ1} and a 11 bp duplication in \textit{KCNH2}.

Two common non-synonymous SNPs were identified that have inconsistently reported functional effects, the \textit{KCNH2} p.K897T and p.R1047L. The p.K897T variant was detected in patients 1, 2, 8 (Table 3.1) and three other sudden death victims in whom no other mutations were identified. The modifying effect of p.K897T has been studied extensively,
but inconsistency exists among different studies (reviewed in Kaab and Schulze-Bahr, 2005, Zhang et al. 2008). The p.R1047L polymorphism was identified in two sudden death victims in whom no other mutations were identified, and is also reported to have inconsistent functional effects (reviewed in Kaab and Schulze-Bahr, 2005). The clinical relevance of both of these variants remains unclear. No mutations were identified in KCNE1 and KCNE2.

3.3.2 Cascade screening in relatives

With the identification of LQTS/BrS associated mutations, testing for at-risk family members of index cases was possible. The term ‘cascade screening’ is commonly used to describe the screening or mutation detection of a known mutation or variant among family members of the proband. Thirty cascade screening tests were performed by sequencing only the exon in which the mutation in the index case was identified. Ten family members tested positive for their particular family mutation. Mutation appropriate counselling and treatment was then initiated by CIDG cardiologists.

3.3.3 Gene-positive cases

3.3.3.1 Patient 1: G168R KCNQ1 mutation

A c.502G→A missense mutation in KCNQ1 (exon 3) was detected in a 32 year-old man with a prolonged QT interval and history of cardiac arrest (Patient 1, Table 3.1). This base pair substitution results in an amino acid change from glycine to arginine at codon 168 (p.G168R). The p.G168R mutation has been associated previously with LQTS and has been described in the literature (Donger et al. 1997; Jongbloed et al. 2002; Beery et al. 2003; Van Langen et al. 2003). The mutation is located in the S2 transmembrane domain of the KCNQ1 protein. This alteration presumably results in a dysfunctional K⁺ channel and prolonged ventricular repolarisation.
In addition to the detected mutation (described above), three polymorphisms were identified in this patient, \textit{KCNH2} (exon 8) c.1953C→T, p.Y652Y; \textit{KCNH2} (exon 11) c.2689A→C, p.K897T and \textit{SCN5A} (exon 24) IVS24+116 G→A. The numbering of these variants follows that in the footnote of Table 3.1.

### Table 3.1. LQTS genes mutations identified in this study.

<table>
<thead>
<tr>
<th>Sequence changes</th>
<th>Exon</th>
<th>Mutation type</th>
<th>Predicted consequence</th>
<th>Protein position</th>
<th>Patient number</th>
<th>Patient age/Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{KCNQ1}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.502G→A</td>
<td>3</td>
<td>missense</td>
<td>p.G168R</td>
<td>S2</td>
<td>Patient 1</td>
<td>32/male</td>
</tr>
<tr>
<td>c.del[572-576]</td>
<td>3</td>
<td>frameshift</td>
<td>p.L191fs281X</td>
<td>S2/S3 onwards</td>
<td>Patient 2*</td>
<td>13/male</td>
</tr>
<tr>
<td>c.797T→C</td>
<td>6</td>
<td>missense</td>
<td>p.L266P</td>
<td>S5</td>
<td>Patient 3*</td>
<td>7/female</td>
</tr>
<tr>
<td>c.805G→A</td>
<td>6</td>
<td>missense</td>
<td>p.G269S</td>
<td>S5</td>
<td>Patient 3*</td>
<td>7/female</td>
</tr>
<tr>
<td>c.944A→G</td>
<td>7</td>
<td>missense</td>
<td>p.Y315C</td>
<td>pore</td>
<td>Patient 4</td>
<td>15/female</td>
</tr>
<tr>
<td>\textit{KCNH2}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.248G→C</td>
<td>2</td>
<td>missense</td>
<td>p.Q81H</td>
<td>N-terminal</td>
<td>SD 1</td>
<td>27/male</td>
</tr>
<tr>
<td>c.1704G→C</td>
<td>7</td>
<td>missense</td>
<td>p.W568C</td>
<td>S5</td>
<td>Patient 5</td>
<td>56/female</td>
</tr>
<tr>
<td>c.1713AG→</td>
<td>7</td>
<td>missense</td>
<td>p.G572S</td>
<td>S5/pore</td>
<td>Patient 6</td>
<td>75/female</td>
</tr>
<tr>
<td>c.1861A→C</td>
<td>7</td>
<td>missense</td>
<td>p.S621R</td>
<td>S5/pore</td>
<td>SD 2</td>
<td>35/female</td>
</tr>
<tr>
<td>c.dup[1871-1881]</td>
<td>7</td>
<td>duplication</td>
<td>p.F627fs89X</td>
<td>S5/pore</td>
<td>Patient 7</td>
<td>43/female</td>
</tr>
<tr>
<td>\textit{SCN5A}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.725C→A</td>
<td>7</td>
<td>missense</td>
<td>p.A242D</td>
<td>DI S4/S5</td>
<td>Patient 8</td>
<td>14/male</td>
</tr>
<tr>
<td>c.1273G→T</td>
<td>10</td>
<td>missense</td>
<td>p.A425S</td>
<td>DI/DII</td>
<td>Patient 2*</td>
<td>13/male</td>
</tr>
<tr>
<td>c.4565T→A</td>
<td>27</td>
<td>missense</td>
<td>p.F1522Y</td>
<td>DIII/DIV</td>
<td>SD 3</td>
<td>&lt;1/female</td>
</tr>
</tbody>
</table>

Numbering of all mutations is based on reference sequences \textit{KCNQ1} NM_000218, \textit{KCNH2} NM_000238, \textit{SCN5A} NM_000335. Nucleotide numbering is from ATG (first position of cDNA).

*Two mutations were detected n these cases

Mutations in blue are novel to CIDG and had not been previously reported at time of detection

#### 3.3.3.2 Patient 2: \textit{p.L191fs281X KNCQ1} and \textit{p.A425S SCN5A} mutations

The patient in whom both of these mutations were detected presented with a long QT interval and episodes of palpitations (Patient 2, Table 3.1). A 5bp deletion in \textit{KCNQ1} (exon 3) was detected (del c.572-576; L191fs281X). This results in a frameshift from the leucine at codon 191 followed by 281 altered amino acids before the appearance of a stop codon.
resulting in premature termination of the protein. A c.1273G→T missense mutation in SCN5A (exon 10) was also detected in this patient. This results in an amino acid change from alanine to serine at codon 425 (p.A425S).

The p.L191fs281X mutation has been previously reported in association with LQTS (Tyson et al. 1997; Tranebjaerg et al. 1999; Choi et al. 2004). The 5bp deletion in the S2-S3 membrane-spanning segment of the KCNQ1 potassium channel causes a frameshift and is highly likely to be disease causing. The SCN5A alteration p.A425S is novel and is not described in the literature (Figure 3.1). It occurs between the first and second domain of the SCN5A encoded protein. Alignment of the protein sequence among species indicates that the alanine residue at codon 425 is conserved in mammals. This variant was absent in 100 control chromosomes. Although it may well be implicated in disease, the functional consequence of the p.A425S alteration needs to be established.

Several polymorphisms were also identified in this patient: KCNQ1 (exon13) c.1638G→A, p.S546S; (exon13) IVS13+36GA (homozygous); KCNH2 (exon 8) c.1953C→T, p.Y652Y; (exon 11) c.2689A→C, p.K897T; SCN5A (exon 10) IVS9-3C→A, and SCN5A (exon 17) c.3180A→G, p.E1060E.

3.3.3.3 Patient 3: p.L266P and p.G269S KCNQ1 mutations

The amino acid substitution p.L266P and p.G269S were detected in a 7 year old girl who presented with symptoms of Jervell and Lange-Nielsen syndrome (JLNS) by clinical and ECG evaluation (Patient 3, Table 3.1). A c.797T→C missense mutation in KCNQ1 (exon 6) results in the amino acid change from leucine to proline at codon 266 (p.L266P). The c.805G→A missense mutation in KCNQ1 (exon 6) results in an amino acid change from glycine to serine at codon 269 (p.G269S). These findings are consistent with a diagnosis of
JLNS, the homozygous, recessive form of LQTS. Both the p.L266P (Splawski et al. 2000) and the p.G269S mutations have been previously reported in the literature in association with LQTS (Ackerman et al. 1999; Rozich et al. 2003; Choi et al. 2004). Both mutations occur in the 5th transmembrane–spanning segment of the K+ channel. This segment flanks the pore region of the ion channel. Given their close proximity to the pore region of the channel, both mutations are expected to alter ion channel function (Ackerman et al. 1999).

An intronic variant, IVS9-3C→A in SCN5A (exon 10) was also identified in this patient.

3.3.3.4 Patient 4: p.Y315C KCNQ1 mutation

A c.944A→G missense mutation in KCNQ1 (exon 7) was detected in a patient who presented with symptoms of LQTS by clinical and ECG evaluation after a near-drowning event (Patient 4, Table 3.1). The mutation results in an amino acid change from tyrosine to cysteine at codon 315 and has been associated previously with LQTS and has been described in the literature (Splawski et al. 1998; Chen et al. 2003). In vitro expression of the mutated KvLQT1 protein showed a severe loss of current with a dominant-negative effect on the wild type potassium ion channel (Napolitano et al. 2000). This results in prolonged ventricular repolarisation. A homozygous variant in IVS15 was also detected: KCNQ1 (exon 15) IVS15+32 G→T.

3.3.3.5 Sudden death 1: p.Q81H KCNH2 mutation

A c.243G→C missense mutation in KCNH2 (exon 2) was detected in a man who suffered a sudden cardiac death at 27 years (SD1, Table 3.1). This results in an amino acid change from glutamine to histidine at codon 81 (p.Q81H). The p.Q81H mutation has been associated previously with a sudden cardiac death in a 21 month old girl by the CIDG (Gladding et al 2010), but it has not been described elsewhere in the literature. It was not found in 100 control individuals (200 alleles) and clustal alignments reveal that the
glutamine at codon 81 is conserved in mammals. The mutation is located in the N-terminal of the KCNH2 protein. Given the conservation of the glutamine residue in mammals and the absence of the substitution in 100 control individuals, it is quite possible that the p.Q81H mutation affects ion channel function, however, it has yet to be characterised using established biophysical techniques.

In addition to the detected alteration (described above), the following polymorphisms were identified. They are unlikely to have any pathogenic consequence: 

- **KCNQ1** (exon13) c.1638G→A, p.S546S; (exon13) IVS13+36G→A; **KCNH2** (exon 8) c.1953C→T, p.Y652Y; **SCN5A** (exon 10) IVS9-3C→A.

### 3.3.3.6 Patient 5: p.W568C KCNH2 mutation

The c.1704G→C (W568C) KCNH2 mutation was detected in a 56 year old woman (Patient 5, Table 3.1). The predicted protein effect is a change from tryptophan to cysteine at position 568 (p.W568C). The mutation has been previously reported in the literature in association with LQTS (Lupoglazoff et al. 2001).

### 3.3.3.7 Patient 6: p.G572S KCNH2 mutation

A c.1713G→A missense mutation in KCNH2 (exon 7) was identified in a 74 year old woman with a prolonged QT interval and clinical LQTS (Patient 6, Table 3.1). This mutation results in an amino acid change from glycine to serine at codon 572 (p.G572S) and has been reported in the literature (Tester et al. 2005). Other substitutions at the same locus, p.G572C and in particular p.G572R have been associated with a high rate of sudden death. p.G572S was not found in a control population study of 48 individuals (96 alleles). It occurs in the S5 domain/pore region of the KCNH2 protein.
3.3.3.8 Sudden death 2: p.S621R KCNH2 mutations

A c.1861A→C missense mutation in KCNH2 (exon 7) was detected in a DNA sample extracted from a Guthrie card from a 28 year old woman who died suddenly after being startled by an alarm clock (SD2, Table 3.1). The nucleotide substitution changes the amino acid from serine to arginine at codon 621 (p.S621R). The p.S621R mutation has been associated previously with LQTS and has been described in the literature (Napolitano et al. 2005). This mutation is located in the pore region of the KCNH2 gene encoded protein and most likely affects ion transfer across the potassium channel, and prolongs ventricular depolarisation (Napolitano et al. 2005). An ECG ordered some months earlier by the family doctor was reported in the notes as normal, but in retrospect showed overt QT prolongation. ECGs performed on the father and sister showed normal QTc of 417 and 360 ms respectively, and they are genotype negative. The daughter (age 3) of the proband had a QTc of 500 ms and tested positive for the mutation. She is being treated with a beta-blocker. Several other more distant relatives have since also been identified as affected gene carriers through cascade screening at LabPlus.

3.3.3.9 Patient 7: p.F627fs89x mutation

An 11bp duplication mutation in KCNH2 (exon 7) was detected (c.dup1871-1881; p.F627fs/89) in a female patient who presented with symptoms of LQTS by clinical and ECG evaluation (Patient 7, Table 3.1). This results in a frameshift from the phenylalanine at codon 627 followed by 89 altered amino acids before the appearance of a stop codon resulting in premature termination of the protein. The mutation p.F627fs/89 is a novel mutation and is not described in the literature (Figure 3.1). The size and position of the duplication was determined by cloning and sequencing of 10 colonies to confirm the sequence of each allele. It was not found in a control population study of 94 individuals (188 alleles). It occurs in the pore region of the KCNH2 protein and would lead to the loss
of all 3’ protein function which very likely results in drastically altered channel function. Parents of this patient were available for cascade testing and both tested negative. If correct paternity is assumed, this would be a *de novo* mutation.

In addition to the 11bp duplication, the following polymorphisms were identified in this patient: *KCNQ1* (exon 2) IVS2+9 G→T; *KCNH2* (exon 1) IVS1+42 G→C, (exon 7) c.1692A→G (homozygous), p.L564L and *SCN5A* (exon 24) IVS24+116 G→A.

### 3.3.3.10 Patient 8: p.A242D mutation

A c.725G→A missense mutation in *SCN5A* (exon 7) was identified in a patient who presented with symptoms of progressive cardiac conduction disorder (PCCD) by clinical and ECG evaluation (Patient 8, Table 3.1). This results in an amino acid change from alanine to aspartic acid at codon 242 (p.A242D). The *SCN5A* p.A242D is a novel alteration and is not described in the literature (Figure 3.1). It occurs between the 4th and 5th segment of the first domain of the *SCN5A* encoded protein. It was not found in 88 control individuals (176 alleles) and the alanine at codon 242 is conserved in mammals. Although it is likely to be implicated in disease, the functional consequence of the p.A242D alteration needs to be established.

In addition to the detected alteration (described above), the following polymorphisms were identified in this patient: *KCNQ1* (exon16) c.1986C→T, p.Y662Y; *KCNH2* (exon 11) c.2689A→C, p.K897T; (exon 13) IVS13+22G→A; *SCN5A* (exon 24) IVS24+116G→A

### 3.3.3.11 Sudden death 3: p.F1522Y SCN5A mutation

A c.4565T→A substitution was identified in a post mortem negative sudden death infant (SD3, Table 3.1). This missense mutation results in amino acid substitution from a
phenylalanine (F) to a tyrosine (Y) (p.F1522Y). This is a novel mutation and is not described in the literature (Figure 3.1). It was not found in 92 control individuals (184 alleles). It occurs just before the first transmembrane segment of the fourth domain and putatively alters sodium channel currents.

![Sequence panel of four novel mutations identified in genes SCN5A and KCNH2](image)

**Figure 3.1.** Sequence panel of four novel mutations identified in genes SCN5A and KCNH2. The position of mutations are indicated by an arrow, and the sequence is displayed in forward direction unless marked otherwise (*).
Figure 3.2. Mutations in KCNQ1. a) Schematic diagram of KCNQ1 genomic structure and approximate location of pathogenic mutations. b) Two-dimensional schematic representation of predicted KCNQ1 polypeptide with locations of KCNQ1 mutations

Figure 3.3. Mutations in KCNH2. a) Schematic diagram of KCNH2 genomic structure and approximate location of pathogenic mutations. b) Two-dimensional schematic representation of predicted KCNH2 polypeptide, HERG, with locations of KCNH2 mutations.
Chapter 3: Mutation analysis in LQTS genes

3.4 Discussion

Over the last decade, there has been an increasing global move of genetic testing in LQTS from research laboratories into clinical practice. There is clear evidence to support the need for LQTS gene screening as a clinical test. Knowledge of genotype assists in offering appropriate therapeutic intervention to individuals at risk of life-threatening arrhythmia. For example, there is no evidence showing that subjects with LQT3 (SCN5A mutations) respond to β-blocker therapy (unlike LQT1 and LQT2), meaning ICDs are required for symptomatic individuals. Appropriate targeting of therapies according to genotype is the major factor in making LQTS genetic testing cost-effective in the clinical setting (Phillips et al. 2005). In addition, identifying the mutation in the index case enables cascade screening of family members.
Mutation analysis was carried out for five LQTS-associated genes (\textit{KCNQ1}, \textit{KCNH2}, \textit{SCN5A}, \textit{KCNE1}, and \textit{KCNE2}) in 44 cases (20 patients and 24 sudden death victims). Molecular genetic screening results carried out by the CIDG before the start of this study are reported by Chung et al. (2007). After detection of the LQTS mutations reported here and by Chung et al. (2007), genotype testing was carried out in 30 individuals.

\subsection{3.4.1 Mutation detection rate}

Thirteen mutations were identified in 44 subjects. This gives a detection rate of 27.3\% for both LQTS patients and victims of sudden unexplained death. The detection rate, however, for patients alone is 50\% (10 mutations in 20 individuals). Chung et al. (2007) reported a similar rate (52\%) in the first 84 LQTS and Brugada index patients screened in New Zealand for mutations. There were 42 LQTS mutations and three BrS mutations detected in 43 unrelated index cases. This is consistent with the findings of Tester et al. (2005), but is slightly lower than previously published PCR-based studies (Splawski et al. 2000; Napolitano et al. 2005). The detection rate is likely to depend on the screening criteria and on the degree of pretest clinical suspicion (Tester et al. 2005).

Substantial allelic heterogeneity seems to be the common feature of global LQTS data sets (Liu et al. 2002; Tester et al. 2005; Napolitano et al. 2005; Splawski et al. 2000 and Jongbloed et al. 2002) with the exception of Finland, where four \textit{KCNQ1} and \textit{KCNH2} founding mutations account for 73\% LQTS cases. (Fodstad et al. 2004). This allelic heterogeneity makes LQTS gene mutation screening an expensive and challenging process. Hierarchical gene screening and genotype-phenotype analysis have been considered to increase the efficiency of LQTS screening (Van Langen et al. 2003).
Hierarchical mutational analysis is based on published data of gene prevalence and sequentially targets the most likely sites of mutation. Although this approach might be more cost- and time-efficient, it is apparent from this and other studies that allelic and locus compound heterozygosity exists in LQTS cohorts (Zareba et al. 2003; Westenkow et al. 2004). Without complete coding region coverage of at least the five genes screened here (\textit{KCNQ1}, \textit{KCNH2}, \textit{SCN5A}, \textit{KCNE1} and \textit{KCNE2}), patients with two mutations might be missed. This would lead to underreporting of severely affected individuals. In addition, during family cascade testing, some individuals could be falsely reassured by the absence of a mutation within one LQTS gene, and yet remain at risk of sudden cardiac death from a mutation in another known LQTS gene.

In contrast to hierarchical screening, the genotype-phenotype approach uses targeted screening based on the relationship between the phenotype and the genotype. It is known that the majority of patients with \textit{KCNQ1} mutations have events precipitated by physical exercise, whereas \textit{KCNH2} mutation-positive patients are more likely to develop arrhythmia after emotion and \textit{SCN5A} mutation-positive patients tend to be symptomatic at rest or during sleep (Schwartz et al. 2001). Although this approach could be an attractive option for rationalisation of gene-testing, the genotype-phenotype relationship in LQTS is far from robust. It is known that clinical manifestations of LQTS are highly variable even among the carriers of the same mutation; for example patients with \textit{KCNQ1} mutations (LQT1) still may die in their sleep.

An effective mutation detection technique should be accurate, cost effective, high throughput, sensitive, provide precise information about the nature and position of the detected mutation and have a reasonable read length (Mashal and Sklar 1996). Single strand conformational polymorphism (SSCP) is one technique that has been used over the years.
for mutation detection and which has been superseded by dHPLC (Section 2.2.4). Both methods are cost effective and high throughput, but they require known homozygote and heterozygote controls for repeated variant analysis. Furthermore, these techniques do not give information regarding the location or nature of the mutation (Chan, 2005; Fan et al, 1993; Mashal & Sklar, 1996; Yu et al, 2006), and need to be confirmed by DNA sequencing. Small changes in experimental conditions can affect the sensitivity of SCCP (Fan et al. 1993) whereas dHPLC is highly sensitive at detecting variants in amenable temperature domains, but variants can be missed in regions of the amplicon that have a low or high melting domain (Yu et al. 2006). Heteroduplex analysis and denaturing gradient gel electrophoresis are two other mutation detection methods that are conceptually similar, (Mashal and Sklar 1996), but also require variant profiles to be verified by DNA sequencing.

Sanger-based DNA sequencing has a reading length less than 1000bp; greater than the other methods mentioned above; is high throughput, sensitive and provides precise information regarding the nature and position of the variation, however, cost and labour intensiveness of sequencing are the key limiting factors related to its use (Mashal and Sklar 1996; Chan et al. 2005). Direct DNA sequencing costs, however, are continuously reducing and, therefore, replacing many of the two-phased molecular screening strategies, mentioned above.

**3.4.2 Posthumous diagnosis of LQTS from Guthrie cards**

In three gene-positive families, a sudden death occurred. LQTS gene mutation analysis, together with clinical review of the family, needs to be integrated into the forensic investigation of young sudden death. Ensuring that there are pathways for this to occur is a necessary requirement in establishing a clinical service for LQTS testing. Blood was not
taken at the time of post-mortem for eight of the sudden death samples. In these cases DNA was extracted from their new-born screening Guthrie cards. Guthrie cards were retrieved up to 13 years after death. Although a sufficient quantity and quality of DNA was eventually obtained from all of the Guthrie cards, much more time and technical skill was required than was needed for routine screening of DNA samples extracted from whole blood. Standard PCR conditions needed to be amended for several amplicons. PCR cycle number was increased from 30 to 40 cycles in most cases, and several more repeat steps were required throughout all stages of the methodology.

Increasingly, population-based studies are showing that sudden natural death is far more common than previously predicted, with an incidence of more than 20 per million per year in 1- to 35-year-olds (Papadakis et al. 2005; Doolan et al. 2004; Puranik et al. 2005). Accurately assigned cause of death is necessary to obtain meaningful data to inform health service provision. The molecular autopsy can work alongside family heart screening, which itself can identify cause of death in 40%–53% of autopsy-negative sudden death cases (Tan et al. 2005; Behr et al. 2008). Obtaining adequate quality DNA from Guthrie cards is clearly feasible, and when archived, these cards could potentially act as a DNA “bank” for future testing (Tan et al. 2005). As the quality and quantity of DNA obtained from Guthrie cards is usually superior to paraffin-embedded formalin-fixed tissue blocks (Carturan et al. 2008, Doolan et al. 2008). However, due to the time consuming technical challenges described earlier, Guthrie cards should not be recommended as a form of blood storage at post mortem. It would be better to extract and store DNA (preferably from spleen or liver) early after the postmortem and to store blood or tissue at -80°C or in a medium capable of protecting cellular RNA, such as Ambion RNA later (Applied Biosystems, Victoria, Australia) (Skinner et al. 2008).
Testing residual material from stored neonatal screening cards for population genetics studies is well described, and the success of these large-scale studies, with standardised methods, suggests that testing archived cards up to 25 years old is possible (Searles et al. 2008; Wion et al. 2003). In these studies, amplicon lengths of up to 480 bp have been genotyped in samples stored for 25 years in a moderate climate (Washington State) and up to 1039 bp in tropical climates for 10 years (Chaisomchit et al. 2005). The longest amplicon in the series reported in the present study was 574 bp. Importantly, improvements in technology such as whole genome amplification may allow testing of Guthrie cards that have previously been deemed inappropriate for the isolation of DNA for subsequent testing. The present study shows that these cards provide a backup archival DNA resource for genetic interrogation in deceased individuals for disorders that have clinical implications for living family members.

3.5 Conclusions

Molecular genetic screening for LQTS identified 4 unique novel mutations and 9 different previously reported mutations causing LQTS. Combined with the results obtained in the rest of the CIDG cohort (Chung et al. 2007), 17 novel mutations in total have been identified and 24 previously reported mutations were detected. Previously reported mutations have been described in the literature with varying levels of support for mutation pathogenicity. Novel mutations identified in the CIDG cohort are currently being investigated in comprehensive collaborative studies to characterise their biophysical properties and determine their clinical significance.

At the time of generating this data, 648 independent mutations in LQTS genes had been reported (http://www.fsm.it/cardmoc). This data, with that of Chung et al. 2007, extends
the data to 672 LQTS mutations. The screening results from the work carried out here combined with that from previously screened patients in the CIDG are consistent with findings from larger cohorts, although the mutation detection rate is lower. It is recommended that any diagnostic program must use full screening in index cases of at least the three most common genes associated with LQTS; \( \textit{KCNQ1}, \textit{KCNH2} \) and \( \textit{SCN5A} \). The high proportion of novel mutations (40\%, present data combine with Chung et al. 2007) dictates a need to confirm pathogenicity for locally prevalent mutations. Careful screening selection criteria, cellular functional analysis of novel mutations, and development of locally relevant control sample cohorts all will be essential to establishing regional diagnostic services for LQTS. Genetic screening of the CIDG LQTS cohort has revealed that 48\% are mutation-negative. Attempts to identify the molecular genetic causes of LQTS-associated symptoms in the mutation-negative cohort, forms the basis for the rest of this study.
CHAPTER 4:

DETECTION OF GENE DELETIONS AND DUPLICATIONS
4 Detection of single- and multiexon gene deletions and duplications

Clinical molecular testing is getting more comprehensive, yet more complicated, with the burgeoning number of known disease-causing genes. Most clinical laboratories develop assays for mutation detection in disease-associated genes based on the reported mutation spectrum. The mutation spectrum associated with genetic disease ranges from point mutations and small deletions and insertions, which can be detected by dHPLC-type screening methods and/or direct DNA sequencing, to single- and multiexon deletions and duplications. The gene mutation spectrum and comprehensive genetic testing vary among genes; for instance, >65% of Duchenne and Becker muscular dystrophy cases are due to deletions or duplications of single or multiple exons of the dystrophin gene (Norman et al. 1990; Mendell et al. 2001; Prior and Bridgeman 2005). In contrast, >90% of cystic fibrosis cases are due to single base-pair substitutions, microinsertions, or microdeletions in the \textit{CFTR} gene (Kerem et al. 1989; Bobadilla et al. 2002); ~80% of the familial adenomatous polyposis cases are due to point mutations, whereas, only ~20% are due to \textit{APC} gene deletions or duplications (Hegde and Roa 2006); and around 75% of the Sotos syndrome cases are due to point mutations in the \textit{NDS1} gene, while 25% of cases are due to deletions and duplications (Visser and Matsumoto, 2003).

Among patients with clinically definite LQTS, approximately 25% remain genotype negative after comprehensive assessment of the three major LQTS genes (Tester et al. 2005; Splawski et al. 2000; Chung et al. 2007). Methods for mutation screening of the LQT genes involve dHPLC analyses of all intron boundaries and exons, followed by sequence analysis of all those samples/exons with abnormal dHPLC profiles. Alternatively, with the cost of sequencing dropping, the preferential direct sequencing of PCR-amplified coding regions is used. These methods do not detect large intragenic deletions or duplications.
because of the background presence of the remaining normal allele (Schouten et al. 2002). Although a number of other genetic mechanisms exist that might be responsible for the LQTS observed clinically in these patients, including mutations in other yet unidentified genes, methylation, miRNA and mitochondrial genetics, a number of cases might be attributable to large genomic rearrangements in these genes. The mutation spectrum of missense and small insertion and deletion mutations in LQTS-associated genes has been investigated by us (Chapter 3) and other groups (Splawski et al. 2000; Tester et al. 2005). However, the contribution of single or multiple exon deletions and duplications to this mutation spectrum is unclear. A tandem duplication of 3.7 kb in KCNH2 was identified in a Dutch family with LQTS (Koopmann et al. 2006). However, no large gene deletions in LQTS had been described prior to the thesis.

In LQTS, the trigger of a cardiac event, mode of presentation, disease severity (risk of sudden death), and response to therapy can vary considerably according, in part, to the genetic locus at which a mutation is present (Schwartz et al. 2001; Schwartz et al. 2006; Priori et al. 2003). LQTS mutation carriers, who lack a prolonged QT interval and, therefore, escape clinical diagnosis, have a 10% risk of a major cardiac event by age 40 years if left untreated (Priori et al. 2003; Priori et al. 2004). Therefore, reaching a molecular diagnosis in gene-negative patients is valuable in establishing preventative clinical best practice for that patient as well as allowing identification of “at-risk” family members.

The current available techniques for detecting single- and multi-exon deletions and duplications in a single gene include multiplex PCR, quantitative PCR, Southern blotting, multiplex ligation-dependent probe amplification (MLPA) (Schouten et al. 2002), detection of virtually all mutations-SSCP (DOVAM-S) (Liu et al. 1999; Buzin et al. 2000) and single condition amplification/internal primer sequencing (SCAIP) (Flannigan et al. 2003).
Clinical assays based on multiplex PCR and quantitative real-time PCR have been used traditionally to detect intragenic deletions and duplications for a small number of genes. Importantly, multiplex PCR can detect deletions but cannot detect duplications accurately or examine multiple genes simultaneously. Quantitative real-time PCR methods are more accurate than multiplex PCR, but detection of intragenic duplications is still a challenge (Heid et al. 1996; Nguyen et al. 2007). In addition, PCR efficiency and PCR product saturation may adversely impact the sensitivity and accuracy of real-time fluorescent PCR, whereas spectral overlap limits the use of real-time fluorescent PCR in multiplex assays (Nguyen et al. 2007; Abbs et al. 1992). Both multiplex PCR and quantitative real-time PCR, which use a single-probe hybridisation that usually targets a common deleted region within a specific gene, can be hindered by single nucleotide polymorphisms, and the boundaries of deletions and duplications cannot easily be determined. Southern blotting can detect relatively large deletions and duplications within a specific gene, however, Southern blotting is labour-intensive, time- and DNA-consuming, requires hazardous reagents, and is not designed for simultaneous examination of multiple genes (Charbonnier et al. 2000).

DOVAM-S is a high-throughput multiplexed scanning assay that uses single-strand conformational polymorphism to detect virtually all point mutations and small deletions and duplications (Liu et al. 1999; Buzin et al. 2000). Sequencing of all variant bands is required to distinguish polymorphisms from disease-causing mutations, which does decrease the efficiency of this assay. In contrast, SCAIP was developed as a rapid, accurate, and economical direct sequencing technique for exons and flanking intronic sequences of large, multiexon genes (Flanigan et al. 2003). Despite the improved detection of deletions and point mutations, SCAIP does not detect intragenic duplications. SCAIP can be labour-intensive, as it requires PCR amplification of all exons individually as the first step.
MLPA, a high-throughput fluorescent technique in which multiple segments can be screened simultaneously to detect intragenic deletions and duplications (Schouten et al. 2002), was used here to determine whether deletions and/or duplications of one or more exons of the main LQTS genes were present in a LQTS mutation-negative cohort. The first large multiple exon deletions in the LQTS genes \textit{KCNQ1} and \textit{KCNH2} were identified and, in addition, a second, novel large multiple exon duplication in the \textit{KCNH2} gene (Koopman et al. 2006) was detected. These findings have been published in Heart Rhythm (Eddy et al. 2008).

Recently, comparative genomic hybridisation (CGH) using oligonucleotide arrays (array CGH or aCGH) has been implemented in cytogenetic and molecular diagnostic laboratories as a robust, rapid, sensitive, and relatively inexpensive assay for detecting known and new microdeletion or microduplication syndromes (Gunn et al. 2007) and targeted gene deletions (Wong et al. 2008; Dhami et al. 2005; Staaf et al. 2008). A pilot experiment was carried out to evaluate the use of aCGH as an alternative technique to MLPA to detect copy number variation in a clinical diagnostic setting. This work was carried out under the supervision of Associate Professor Don Love at LabPLUS, Diagnostic Genetics (ADHB) and at Roche Diagnostics (Mount Wellington, Auckland) with Anthony Thrush (Systems Specialist, Roche Diagnostics, New Zealand).

As a relatively small molecular diagnostic laboratory, LabPLUS are faced with the issue of low sample throughput, which can lead to inefficiency and excessive costs when using techniques described above. Therefore, a single bespoke LabPLUS CGH array was designed to target 67 genes tested in-house, including 12 LQTS-associated genes. This approach may overcome the problems of using several expensive kits for low batch
numbers and should allow for consolidation to a single validated technique. Although this work is ongoing, the preliminary results from relatives of two of the MLPA mutation positive patients are included in this chapter.

4.1 Patients

The study included 26 index cases whose clinical family history and ECG findings supported a definitive clinical diagnosis of LQTS, and 14 family members of genotype positive patients. All index cases included in the study had a Schwartz score of 4 or greater. The Schwartz score is a commonly used criterion to clinically diagnose LQTS (Schwartz et al. 1993). The score is calculated by assigning different points to various criteria:

- **QTc (Defined as QT interval / square root of RR interval)**
  - $\geq 480$ msec - 3 points
  - 460-470 msec - 2 points
  - 450 msec and male gender - 1 point
- **Torsades de Pointes ventricular tachycardia** - 2 points
- **T wave alternans** - 1 point
- **Notched T wave in at least 3 leads** - 1 point
- **Low heart rate for age (children)** - 0.5 points
- **Syncope (one cannot receive points both for syncope and Torsades de pointes)**
  - With stress - 2 points
  - Without stress - 1 point
- **Congenital deafness** - 0.5 points
- **Family history** (the same family member cannot be counted for LQTS and sudden death)
  - Other family members with definite LQTS - 1 point
- Sudden death in immediate family (members before the age 30) - 0.5 points

With 4 or more points the probability is high for LQTS, and with 1 point or less the probability is low. Two or 3 points indicates intermediate probability.

Clinical details for the index cases are listed in Table 4.1. Subjects included for cascade screening are listed in Table 4.2. Informed consent for genetic testing was obtained in all cases, following the protocols established in our multicentre ethical approval from the Auckland regional ethics committee. Coding region and splice site mutations in the three major LQTS genes (*KCNQ1*, *KCNH2*, and *SCN5A*) previously had been excluded in the probands by dHPLC and DNA sequencing using primers flanking exonic sequences (Chapter 3, Chung et al. 2007).

### 4.2 Results

#### 4.2.1 MLPA analysis

MLPA profiles that reflected altered exon copy numbers were detected in 4 (15%) patients among the gene-negative LQTS probands. Relative peak ratios of multiplex ligation-dependant probe amplification (MLPA) products with profiles reflecting deletions or duplications are shown in Figure 4.1. A relative peak ratio of 1 implies normal copy number, 0.5 half the copy number (deletion) and 1.5 double the copy number (duplication).

An apparent deletion of exon 7 of the *KCNQ1* gene was not confirmed after sequence analysis. This apparent deletion corresponded to a previously reported missense mutation (c.944A→G, p.Y315C, NM_000218.2) (Chen et al. 2003; Napolitano et al. 2000; Splawski et al. 1998; Choi et al. 2004) in the MLPA probe recognition sequence that escaped initial detection through dHPLC/sequencing screening (Figure 4.2). The remaining two deletions appeared to encompass exon 13 of the *KCNQ1* gene and exons 5 to 14 of the *KCNH2*
gene. Exons 9 to 14 of the KCNH2 gene appeared to be duplicated in the remaining MLPA mutation-positive patient (Figure 4.3). Several unsuccessful attempts at long range PCR were made to establish the breakpoints of the deletions and the duplication. However, based on the MLPA and real-time PCR data, there is substantial evidence of large rearrangements where the nature of the breakpoints has only scientific rather than clinical value.

No large exon duplications or exon deletions in SCN5A were detected.

Figure 4.1. Relative peak ratio of multiplex ligation-dependant probe amplification (MLPA) products with profiles reflecting deletions or duplications using the SALSA MLPA kit P114. a: Female patient with apparent ex7del of KCNQ1 gene was excluded after sequence analysis showed a missense mutation (p.Y315C) in the probe recognition sequence. b: Male patient with ex13del of KCNQ1 gene. Exon 14 was shown to be included in the deletion using real-time polymerase chain reaction analysis. c: Female patient with ex5-14del of KCNH2 gene. d: Male patient with ex9-14dup of KCNH2 gene.
<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Age at Diagnosis</th>
<th>Gender</th>
<th>Ethnicity*</th>
<th>QTc intervalA</th>
<th>Torsade de pointes</th>
<th>SyncopeB</th>
<th>Identified triggers</th>
<th>OtherC</th>
<th>Total Schwartz Score</th>
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<td>NZE</td>
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<td>Pregnancy</td>
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<td>AU</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Ethnicity: NZE: New Zealand European; NZM: New Zealand Maori; OE: Other European; AU: Australian; P: Pacific

A QTc interval: 1: >430ms; 2: 410-430ms; 3: >400ms

B Syncope: 1: without stress; 2: with stress

C Other: Includes remaining Schwartz score points for other ECG features, congenital deafness, and family history

KCNH2 ex6-14del
KCNQ1 ex13-14del
KCNH2 ex9-14dup

(Schwartz et al. 1993)
Figure 4.2. Partial sequence analysis of patient with apparent ex7del in the KCNQ1 gene. The underlined sequence represents the multiplex ligation-dependant probe amplification (MLPA) probe hybridization site. Arrows show the heterozygous missense mutation (c.944A→G, p.Y315C, NM_000218.2). Left-hand panels represent sequence using the forward primer. Right-hand panels represent sequence using the reverse primer.

**KCNQ1**

```
1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16
ex13-14del
```

**KCNH2**

```
1   2   3   4   5   6   7   8   9   10  11  12  13  14  15
ex6-14del
ex9-14dup
```

- Minimum region deleted/duplicated
- Maximum region deleted/duplicated

Figure 4.3. Genomic structure of the KCNQ1 and KCNH2 genes showing the locations of the deletions and duplications detected in this study. The specific breakpoints could not be determined using long range PCR. However, the nature of the breakpoints has only scientific rather than clinical value.
Table 4.2 MLPA cascade testing of relatives of genotype positive probands

<table>
<thead>
<tr>
<th>Patient no / position on pedigree (Fig. 4.4)</th>
<th>Patient/sudden death</th>
<th>Date of birth</th>
<th>Gender</th>
<th>Mutation in relative</th>
<th>Genotype positive/negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>27 (II:2, Figure 4.4b) Patient</td>
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<td>KCNH2 ex6-14del</td>
<td>negative</td>
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</tr>
<tr>
<td>28 (III :4, Figure 4.4b) Sudden death</td>
<td>05/10/1981</td>
<td>F</td>
<td>KCNH2 ex6-14del</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>29 (IV:1, Figure 4.4b) Patient</td>
<td>22/12/1999</td>
<td>M</td>
<td>KCNH2 ex6-14del</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>30 (IV:3, Figure 4.4b) Patient</td>
<td>25/04/2007</td>
<td>F</td>
<td>KCNH2 ex6-14del</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>31* (IV:2, Figure 4.4b) Patient</td>
<td>03/01/2009</td>
<td>F</td>
<td>KCNH2 ex6-14del</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>32 (I:1, Figure 4.4c) Patient</td>
<td>11/02/1930</td>
<td>M</td>
<td>KCNH2 ex9-14dup</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>33 (II:2, Figure 4.4c) Patient</td>
<td>07/01/1959</td>
<td>M</td>
<td>KCNH2 ex9-14dup</td>
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<td></td>
</tr>
<tr>
<td>34 (II:6, Figure 4.4c) Sudden death</td>
<td>10/03/1963</td>
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<td>KCNH2 ex9-14dup</td>
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</tr>
<tr>
<td>35 (II:7, Figure 4.4c) Patient</td>
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<tr>
<td>36 (II:8, Figure 4.4c) Sudden death</td>
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<td>KCNH2 ex9-14dup</td>
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</tr>
<tr>
<td>37 (II:11, Figure 4.4c) Patient</td>
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<td>KCNH2 ex9-14dup</td>
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<tr>
<td>38 (III:6, Figure 4.4c) Patient</td>
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</tr>
<tr>
<td>39 (III:7, Figure 4.4c) Patient</td>
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<tr>
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<td>negative</td>
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</table>

* Patients used to validate the CGH array (section 4.2.5)

4.2.2 Validation of MLPA results using real-time analysis

For any diagnostic assay, like MLPA, it is desirable to perform a validation to exclude any experimental errors and to determine whether the same results are achieved with a different method with different research parameters. Real-time PCR analysis confirmed the presence of deletions/duplications in the three MLPA mutation-positive patients described (Table 4.3). In addition, this validation step allowed for some minor enhancements in establishing the deleted regions identified with MLPA. The MLPA kits lack probes for certain exons, usually because they are very close to neighbouring exons with probes. The inclusion of real-time PCR primers in KCNQ1 exon 14 revealed that the KCNQ1 deletion included exons 13 and 14. In most cases, real-time PCR primers were designed to flank the probe recognition sequences that appeared to be duplicated or deleted using MLPA analysis. The MLPA probe corresponding to KCNH2 exon 5 mapped to a downstream, noncoding
intronic sequence. Therefore, the real-time primer pair was designed to detect whether the coding sequence within exon 5 was deleted or duplicated. These results suggest that exon 5 is present in normal copy number (two) and that the deletion breakpoint must be between the distal end of the real-time PCR reverse primer and the downstream MLPA probe recognition sequence.

<table>
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<tr>
<th>MLPA mutation</th>
<th>Real-time PCR amplicons</th>
<th>Mean relative ratio</th>
<th>Range</th>
<th>Standard Deviation</th>
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<td>$KCNQ1$ ex 13</td>
<td>0.612</td>
<td>0.574 – 0.652</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>$KCNQ1$ ex14</td>
<td>0.476</td>
<td>0.441 – 0.513</td>
<td>0.036</td>
</tr>
<tr>
<td>$KCNH2$ ex6-14del</td>
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<td>0.358</td>
<td>0.309 – 0.414</td>
<td>0.052</td>
</tr>
<tr>
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<td>$KCNH2$ ex10</td>
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<td>0.396 – 0.509</td>
<td>0.056</td>
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<tr>
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<td>$KCNH2$ ex14</td>
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<td>0.393 – 0.488</td>
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<td>$KCNH2$ ex14</td>
<td>1.419</td>
<td>1.215 – 1.658</td>
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<td>1.242 – 1.789</td>
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<td>$KCNH2$ ex14</td>
<td>0.438</td>
<td>0.393 – 0.488</td>
<td>0.047</td>
</tr>
</tbody>
</table>

4.2.3 Family genotyping

The pedigrees of the families displaying the $KCNQ1$ ex13-14del, $KCNH2$ ex6-14del, and $KCNH2$ ex9-14dup are shown in Figure 4.4, panels a, b, and c, respectively. Family
members of the probands in which MLPA mutations were identified that were available for genotyping are listed in Table 4.2. The ex6-14del of KCNH2 gene was present in a sample extracted from the Guthrie card (newborn screening card) of the proband’s sister (III:4, Figure 4.4b) and in her daughter (IV:3, Figure 4.4b), but was absent in her mother (II:2, Figure 4.4b), son (IV:1, Figure 4.4b) and daughter (IV:2, Figure 4.4b), who have normal QT intervals and are asymptomatic.

The KCNH2 ex9-14dup was present in the proband’s grandfather (I:1, Figure 4.4c), mother (II:6, Figure 4.4c) who died suddenly during sleep, two uncles (II:2, Figure 4.4c) and (II:11, Figure 4.4c), who have a borderline QT interval and clearly prolonged QT interval, respectively, and an aunt (II:13, Figure 4.4b), who has a long QT interval and has suffered a resuscitated cardiac death.

4.2.4 Clinical data

The clinical information on all 26 LQTS mutation-negative patients was provided by CIDG cardiologists, with Schwartz scores (Schwartz et al 1993) determined by Dr Jon Skinner and Dr Judith McCormick (Table 4.1). The first patient (patient 1, Table 1; patient II:3, Figure 4.4a) with a deletion in KCNQ1, (KCNQ1 ex13-14del), an 11-year-old boy of Maori descent, presented after two episodes of collapse during exercise. His QTc ranges from 410 to 580 ms. Limited clinical and genetic screening was possible in the wider family. His mother (patient I:2, Figure 4.4a) had a history of seizures with exertion (QTc 460 ms); two maternal uncles also have a history of seizures. He has two brothers who are asymptomatic, but one has marked QTc prolongation on exercise testing (patient II:1, Figure 4.4a), and the other has a normal QT interval.
The female patient of European descent found to have a deletion in \textit{KCNH2} (\textit{KCNH2} ex6-14del) was clinically diagnosed with LQTS at age 22 years (patient 5, Table 4.1; patient III:2, Figure 4.4b). Her QTc interval varies from 450 to 560 ms. Before recognition of her LQTS, she had experienced several episodes of collapse and seizure from the age of 9 years. The patient's only sister (III:4, Figure 4.4b) had been misdiagnosed with epilepsy prior to her sudden unexpected death at age 36 years. Retrospective review of an ECG recorded during the sister's life revealed QTc of 510 ms. The proband's son (IV:1, Figure 4.4b) has a normal QT interval, is asymptomatic and tested negative for the \textit{KCNH2} ex6-14del. The proband has two daughters (IV:2 and IV:3, Figure 4.4b) both of whom are asymptomatic. However, the elder of the two (IV:2, Figure 4.4b) is negative for the mutation, whilst the \textit{KCNH2} ex6-14del was detected in the younger daughter (IV:3, Figure 4.4b).

The asymptomatic 12-year-old boy with a \textit{KCNH2} duplication (\textit{KCNH2} ex9-14dup; patient 19, Table 4.1; patient III:4, Figure 4.4c) initially was referred after the sudden unexpected death of his mother (age 32 years) during sleep (patient II:6, Figure 4.4c). An ECG taken during her pregnancy for a heart murmur was reported as normal at that time. However, posthumous review revealed QTc of 510 ms. A screening ECG in the proband revealed a prolonged QTc (470–550 ms), and he was started on beta-blocker therapy. His maternal family history is significant. An aunt (II:13, Figure 4.4c) had received an implantable cardioverter-defibrillator following resuscitated sudden cardiac death, and she has a long QT interval. The \textit{KCNH2} ex9-14dup was detected in subsequent cascade analysis. An uncle (II:8, Figure 4.4c) died unexpectedly at age 24 years, but the postmortem examination was negative. Another maternal uncle (patient II:9, Figure 4.4c) had a long QT interval but died of cancer. The patient's only brother is asymptomatic and has a borderline QTc interval.
Figure 4.4. Pedigrees of the families in which (a) the proband with the \textit{KCNQ1} \textit{ex}13-14\textit{del} was identified (II:3), (b) the proband with the \textit{ex}6-14\textit{del} of \textit{KCNH2} gene was identified (III:2), and (c) the proband with the \textit{ex}9-14\textit{dup} of \textit{KCNH2} gene was identified (III:4). Only those individuals marked with a plus sign (genotype positive) or a negative sign (genotype negative) were available for genotyping. LQTS = long QT syndrome; SD, PM neg = sudden death, postmortem negative.

4.2.5 CGH array results

A pilot experiment was carried out to evaluate the use of aCGH to detect copy number variation in a clinical diagnostic setting. This work was carried out under the supervision of Associate Professor Don Love at LabPLUS, Diagnostic Genetics (ADHB) and at Roche Diagnostics (Mount Wellington, Auckland) with Anthony Thrush (Systems Specialist, Roche Diagnostics, New Zealand). A Roche Nimblegen CGH Custom Targeted Array was designed to detect deletions and duplications in all exons of 67 genes tested at LabPLUS,
Diagnostic Genetics, including 12 LQTS-associated genes (Table 2.10). The design of the array is discussed in Chapter 2, Section 2.2.9).

Relatives of the probands with the $KCNH2$ ex6-14del (IV:3, Figure 4.4b) and $KCNH2$ ex9-14dup (III:7, Figure 4.4c), respectively (Table 4.2) were among the 12 patient samples used to validate the targeted gene CGH array. The remaining 10 patients carry mutations in genes unrelated to this study.

This work was carried out mainly by Anthony Thrush (Roche Diagnostics) and Elaine Doherty (Lab PLUS, Diagnostic Genetics). My contribution included the selection of LQTS-associated genes for inclusion in the array, MLPA screening of the two LQTS patients included in the array, some assistance with sample labelling and hybridisation and comparison of the results obtained for the two LQTS patients with their MLPA findings.

Normalised, mean log$_2$ ratio data over each region was obtained by Anthony Thrush (Table 4.3), where Log$_2$ above +0.2 indicates a duplication and Log$_2$ below -0.2 indicates a deletion. The results for the patients with the $KCNH2$ ex6-14del (IV:3, Figure 4.4b) and $KCNH2$ ex9-14dup (III:7, Figure 4.4c) are shown graphically in Figure 4.5. The presence of the $KCNH2$ ex6-14del is confirmed in patient IV:3, Figure 4.4b. In addition to confirmed duplication in $KCNH2$ in patient III:7 (Figure 4.4c), a small deleted region was also detected (Figure 4.5c).
Table 4.3 aCGH results. Mean Log₂ ratios for deleted and duplicated regions on the array. Log₂ above +0.2 indicates a duplication and Log₂ below -0.2 indicates a deletion. The datapoints in each region the number of probes analysed.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Mutation</th>
<th>Start¹</th>
<th>Stop¹</th>
<th>Size (bp)</th>
<th>Datapoints</th>
<th>Log₂ ratio (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 31*</td>
<td>KCNH2 ex6-14del</td>
<td>7</td>
<td>150250593</td>
<td>150283627</td>
<td>33034</td>
<td>317</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>151157894</td>
<td>151158860</td>
<td>966</td>
<td>12</td>
</tr>
<tr>
<td>Patient 39#</td>
<td>KCNH2 ex9-14dup</td>
<td>7</td>
<td>150250593</td>
<td>150275172</td>
<td>24579</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>150275345</td>
<td>150276020</td>
<td>675</td>
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<td>7</td>
<td>150276456</td>
<td>150279665</td>
<td>3209</td>
<td>118</td>
</tr>
</tbody>
</table>

* Patient no refers to Table 4.2 (IV:2, Figure 4.4b)
# Patient no refers to Table 4.2 (III:7, Figure 4.4c)
¹ start/stop co-ordinates refer to genomic location of KCNH2, accession number NC_000007.12, where KCNH2 spans 150272982-150305947.
Figure 4.5. a) A rainbow plot of Log$_2$ ratios obtained for probes of each chromosome represented on the array. b) A plot of Log$_2$ ratios obtained for exons in chromosome 7 for patient IV:3 (Figure 4.4b) with KCNH2 ex6-14del MLPA mutation. c) A plot of Log$_2$ ratios obtained for exons in chromosome 7 for patient III:7 (Figure 4.4c) with KCNH2 ex9-14dup MLPA mutation. Loss and/or gain of genomic material are depicted as a shift down and shift up of the horizontal line on the x-axis, respectively. Chromosome location numbering refers to NC_000007.12, where KCNH2 is from 150272982-150305947.
4.3 Discussion

Current methods, worldwide, for mutation screening in patients with clinical LQTS yield a diagnosis in only about 70%. These mutation detection strategies (dHPLC and/or sequence analysis of coding regions only) do not detect whole exon or multiple exon deletions or duplications. Primers are designed to amplify each exon or coding region specifically and these are analysed individually. The resulting sequence trace of each exon contains no information of exon dosage or copy number (Figure 4.6). The cardiac inherited disease group (CIDG) screening data from this study and previous screening (Chung et al 2007) yielded a detection rate of only 52%. There is, therefore, much research and clinical interest in establishing a genetic cause for LQTS in the remaining gene-negative patients. Although unidentified genes could be involved in these patients, a number of cases might be attributable to large genomic rearrangements or copy number variation in these genes. A quantitative fluorescent approach, MLPA was used to detect deletions and/or duplications of one or more exons of the genes. There are a range of disorders where such deletions and/or duplications of one or more exons of a gene account for a significant proportion of the disorder’s mutation spectrum, such as Duchenne muscular dystrophy, spinal muscular atrophy and Fanconi anaemia. As there is no plausible reason why LQTS should be an exception, MLPA and real-time PCR were used to identify two large multiple-exon deletions in the \( KCNQ1 \) (ex13-14del) and \( KCNH2 \) (ex6-14del) genes as well as an intragenic duplication in the \( KCNH2 \) gene (ex9-14dup) in 26 mutation-negative LQTS patients.

A patient with an apparent deletion of a single exon was found to carry a disease-associated missense mutation within an MLPA probe recognition sequence. This patient was among those included in mutation screening of the five LQTS genes in Chapter 3. However, due
to a limitation of dHPLC, where variants can be missed in regions of the amplicon that have a low or high melting domain (Yu et al 2006), this amplicon revealed no abnormal profile, so no sequencing was carried out for that particular exon. Taken together, the MLPA method detected mutations in 11.5% of LQTS patients who were negative for traditional missense, nonsense, or frameshift mutations involving the open reading frames of the three major LQTS susceptibility genes.

In addition to genotype and mutation location, the functional effects of these identified mutations provide additional risk information that can contribute to improved patient management. Until the biophysical function of the detected mutations is established in vitro, exactly how they will be associated with clinical severity of LQTS is unclear. However, if we were to assume that irrespective of where the actual deletion/duplication takes place, the mRNA transcript simply has the deleted exons left out or duplicated exons added in, then:
The *KCNQ1* ex13-14del would result in a frameshift and premature stop codon. Premature stop codons usually result in nonsense-mediated decay and, therefore, haploinsufficiency (Moss et al. 2007) where repolarising K⁺ current is reduced due to the inability of mutated subunits to co-assemble with normal gene products and fail to be incorporated into tetrameric channels. However, as the premature stop codon, in this case, occurs more than 35 bp from the next exon–exon boundary, it probably would not undergo nonsense-mediated decay. Therefore, it is likely to be translated and result in truncation of the last 146 amino acids and the addition of a new 14 amino acids. This region forms a subunit interaction domain for *KCNQ1* (Schwake et al. 2003). If this protein is translated, as is likely, it would lack a critical subunit interaction domain and, therefore, result in a haploinsufficiency phenotype.

The deletion breakpoints of this mutation were determined by Coleman and colleagues at Cardiff University, who, subsequent to publication of these findings, identified the same deletion in one of their patients (unpublished data). Long-range PCR was used to amplify DNA between exons 13 and 16 in patient II:3 (Figure 4.4a), in one of their patients, and in DNA from a control subject. Sequence data of the deleted PCR product revealed a breakpoint fusing intragenic sequence between exons 13 and 16 and, consequently, the deletion of exons 14 and 15. The deletion spanned 2,700bp.

The *KCNH2* ex6-14del is predicted to be in frame. The deletion would remove all of the transmembrane segments and the cyclic nucleotide binding domain, leaving the PAS domain, most of the proximal N-terminal domain, and the very distal C-terminal domain. It is conceivable that this protein could fold, and if it did, it would be unlikely to interact with wild-type subunits, given that deletion of the first 373 amino acids does not alter
subunit assembly. Therefore, this mutant most likely would result in a haploinsufficiency phenotype.

Although the KCHN2 ex9-14dup would be in frame, it would result in disruption of the cyclic nucleotide binding domain and so almost certainly could result in a misfolded protein. Whether it could co-assemble with the wild-type protein is uncertain. If it were to, it probably would do so poorly and, therefore, result in a partial dominant-negative effect rather than simple haploinsufficiency. However, the finding of an additional small deleted region within this gene on CGH array (Figure 4.5c), makes it very difficult to predict the functional effect of the mutation in these patients. Without biophysical functional studies, it is unknown whether the pathogenecity is caused by deleted or duplicated regions.

Haploinsufficiency results in no more than 50% reduction in channel function (Bianchi et al. 2000), as opposed to dominant-negative mutations, which result in dysfunctional ion channels having greater than 50% reduction in channel current (Shalaby et al. 1997). Some supportive evidence of the malignancy of these mutations comes from limited cosegregation studies, including the posthumous identification of a mutation in a victim of sudden death. The two large gene rearrangements identified in KCNH2 have been found in families with an apparent severe phenotype and in which there have been several young sudden deaths with a negative post-mortem examination.

The three large gene rearrangements of KCNH2 (present study and Koopmann et al. 2006) have been identified in families with a remarkable history of autopsy-negative sudden unexpected death in the young. It is possible that large gene rearrangements confer a more malignant phenotype, and this may have implications for post-mortem genetic testing by MLPA in cases of autopsy-negative sudden unexplained death (SUD), where traditional
LQTS- and CPVT-associated mutations have been detected in approximately one-third of SUD cases (Tester et al. 2007; Tester et al. 2004). Although these sudden deaths involved adolescents and young adults, it is conceivable that similar deletions and duplications in these cardiac channel genes could provide a mechanism of cardiac arrhythmic sudden infant death syndrome as well (Arnestad et al. 2007).

Gene dosage is a common form of pathogenic mutation in many genetic diseases. In fact, such large deletion or duplication mutations can compose more than half of all germline mutations in some disorders. The detection of deletions and duplications in LQTS is part of the emerging evidence relating to the surprising frequency of large rearrangements within, or close to, disease-associated genes. The open reading frames of the three major LQTS susceptibility genes (*KCNQ1, KCNH2*, and *SCN5A*) account for approximately 75% of the LQTS mutation detection yield. Based on our small sample size, it appears that deletions and duplications in the main LQTS genes (*KCNQ1, KCNH2*, and *SCN5A*) might confirm a clinical diagnosis in a similar number of patients (2%–3%), to those who carry missense, nonsense, or small insertion/deletion mutations in the rarer LQTS genes (*ANK2, KCNJ2, CACNA1C, CAV3, SCN4B*, and *AKAP9*).

Although MLPA has successfully identified deletions and duplications in previously “gene negative” patients, MLPA assays can be laborious, and occasionally gives false-positive and false-negative results. MLPA false-negative and false-positive findings are caused largely by the presence of single nucleotide polymorphisms (SNPs) in the targeted regions. This was observed in this study where an apparent deletion of exon 7 was, in fact, due to a missense mutation in the MLPA probe-recognition sequence (Figure 4.2a). Single-exon dosage changes (duplications in particular) detected by MLPA require validation by a
second method that has a different technical basis (such as real time PCR), which can add to both the complexity and cost of MLPA assays (Nguyen et al. 2007).

CGH arrays have been widely used to detect gross chromosomal changes, including large deletions and duplications across the human genome; however, the application of targeted gene CGH arrays for detecting intragenic deletions or duplications is still a developing field. The targeted gene CGH array has several advantages over other available molecular methods, such as quantitative PCR, Southern blotting and, indeed, MLPA. These methods can be time-consuming, labour-intensive, complex, and fail to detect all mutations accurately. Mutations detected by the currently available techniques, especially single exon deletions, require further costly confirmation by a different technique, which can affect the turnaround time for results but are essential to ensure accurate results.

Because these more common techniques depend only on one or two probes for each targeted region, false-positive results can occur. Furthermore, genomic positions of the breakpoints cannot be determined by traditional methods and would require subsequent assays to be developed such as long range PCR and sequencing. Although long range PCR can effectively establish deletion and duplication breakpoints, it can be difficult to achieve success. At least two different primer pairs were designed in this study to span each of the expected deletions and the duplication identified by MLPA. Several attempts failed to achieve results. Additional primers spaced further apart might have yielded success. However, due to cost and time restraints, and the fact that establishing the exact breakpoints would be of greater research interest than clinical significance, no further attempts were made. In contrast, the targeted gene CGH array promises to be a rapid, comprehensive, relatively inexpensive, highly sensitive, and accurate method for detecting
single- and multi-exon deletions and duplications in a large set of genes simultaneously on a common platform.

The pilot aCGH experiment confirmed the presence of a $KCNH2$ deletion. Given the density of the probes (or data points), the deleted region could be mapped with greater resolution, to occur between 150250593 and 150283627 (33034 bp) on chromosome 7 (NC_000007.12). The $KCNH2$ gene is from 150272982 – 150305947. The array data, therefore, suggests that the deleted region begins before the start of $KCNH2$. The array also revealed a smaller deletion (966 bp) outside $KCNH2$ from location 151157894 to 151158860 on chromosome 7 in this subject. As with the deletion, the duplicated region in the array from the patient with the MLPA ex9-14 duplication, had a starting point beyond the $KCNH2$ gene as well as a smaller deleted region in the middle of the duplicated region (Table 4.3 and Figure 4.5). While the array is potentially offering greater resolution and providing more information, the clinical significance of the results are difficult to interpret. It is important to note that this is a pilot experiment and has not been repeated. Before definitive conclusions can be drawn or speculations made to explain the discrepancies observed in deletion size and starting point between the array and the MLPA data, the pilot experiment should be repeated. This work is ongoing at LabPlus.

As gene CGH array technology is a recent technology, establishing its use in a molecular diagnostic setting would require additional trial assays and confirmation of deletions and duplications by a secondary method, at least at the `beginning of its implementation in diagnostics. Deletions could be easily and cost-effectively confirmed by designing primers across the breakpoints, whereas, duplications would be more difficult to confirm using an alternative technology. Methods such as Southern blot or real time PCR, could be considered.
4.4 Conclusions

The need for comprehensive mutation detection assays with a reasonable turnaround time for results is a pressing necessity. Deletions and duplications constitute a significant portion of the mutation spectrum for some genes, which now include LQTS-associated genes. Following conventional missense and small insertion and deletion mutation screening strategies, the availability of comprehensive mutation detection assays for intragenic deletions and duplications that are accurate, time- and cost-efficient will provide more comprehensive mutation spectrum information for arrhythmia-associated genes which will ultimately improve clinical diagnosis and patient management.

Despite the success of the identification of deletions/duplications, 20% to 25% of LQTS patients have unidentified genetic defects, and further research into LQTS gene-negative cohorts is required. Synonymous or “silent” (non–amino acid altering) single-nucleotide substitutions residing within the exons could conceivably disrupt splice enhancer element sequences precipitating errant RNA splicing and subsequent exon skipping or intron inclusion. Such a mechanism has already been reported for diseases like Marfan syndrome, familial adenomatous polyposis, and McArdle disease (Liu et al. 1997; Montera et al. 2001; Fernandez-Cadenas et al. 2003). Patients with disorders that present similarly to LQTS but have different genetic causes might also account for a substantial portion of LQTS gene-negative patients, for example the RYR2 gene in catecholamine polymorphic ventricular tachycardia (CPVT). The potential presence of mutations in the RYR2 gene in the LQTS gene negative cohort is investigated in the following chapter.
CHAPTER 5:

MUTATION ANALYSIS OF THE CARDIAC RYANODINE RECEPTOR GENE
5 Mutation analysis of the cardiac ryanodine receptor gene

5.1 Cardiac ryanodine receptor gene (RYR2) variants in catecholaminergic polymorphic ventricular tachycardia (CPVT) and sudden death (SD)

Up to twenty percent of post-mortem examinations of young people who were apparently healthy but have died suddenly and unexpectedly, reveal no morphologic abnormalities to explain their deaths (Anderson et al. 1994). SD is the first and only clinical manifestation in about half of such cases (Chugh et al. 2004). While cardiac K+ and Na+ ion channelopathies, such as LQTS and Brugada syndrome, underlie a substantial proportion of these cases, there is now a growing body of evidence highlighting the important role of abnormalities involving intracellular calcium (Ca^{2+}) in the pathogenesis of sudden arrhythmic death syndromes (Tester et al. 2006; Cerrone et al. 2009).

In cardiac myocytes, electrical depolarisation of the sarcolemmal membrane normally leads to an increase in Ca^{2+} and eventually causes contraction of the myofilaments. This process is called excitation-contraction coupling. Ca^{2+} plays a major role as a secondary messenger in this process that converts electrical to mechanical energy (Fabiato 1983). Ca^{2+} is released from the sarcoplasmic reticulum (SR) via a calcium release channel known as the cardiac ryanodine receptor (RYR2). Ca^{2+} released via RYR2 normally produces a change in the level of cytoplasmic calcium (Bers 2002). There are several molecules which bind and regulate the function of RYR2 and maintain normal Ca^{2+} homeostasis. These include calstabin2, calmodulin, protein kinase A (PKA), phosphatase, sorcin and calsequestrin. Figure 1.14 in Chapter 1 illustrates how some of these molecules interact. Alteration of RYR2 and associated molecules can cause functional and/or structural changes of the heart that may lead to sudden cardiac death. It is the association between mutations in genes encoding these proteins, particularly RYR2, and an inherited cardiac disorder, (CPVT), that
is improving our understanding of the crucial role of Ca\textsuperscript{2+} regulation in the development of life-threatening arrhythmias (Cerrone et al. 2009).

The disorder CPVT is characterised by adrenergically mediated ventricular arrhythmias causing syncope, cardiac arrest and SD in young individuals with structurally normal hearts and usually normal resting electrocardiogram (ECG) (Coumel et al. 1978; Leenhardt et al. 1995; Tester et al. 2004; Francis et al. 2005). Following linkage studies which localised the genetic abnormality to chromosome 1q42-1q43 in CPVT patients with autosomal dominant inheritance (Swan et al. 1999), point mutations in the cardiac ryanodine receptor gene (RYR2) co-segregating with the clinical phenotype were identified (Priori et al. 2001; Laitenin et al. 2001). RYR2 mutations account for about 50% to 65% of CPVT cases and these are annotated as CPVT (Priori et al. 2002). Mutations in the CASQ2-encoded calsequestrin, a calcium binding protein in the SR, are responsible for a rare autosomal recessive form of CPVT, annotated as CPVT2 (Lahat et al. 2001; Postma et al. 2002).

The association of arrhythmias with physical or emotional stress often leads clinicians to consider affected CPVT patients as a form of LQTS without QT interval prolongation. An important differential diagnosis, is that compared to LQTS, CPVT is more malignant, the response to medical therapy is poorer, and the incidence of SCD could reach 30% before age 40. Familial occurrence, with a mainly dominant pattern of inheritance, has also been noted in about 30% of cases, with high penetrance (Cerrone et al. 2004). Achieving the correct molecular diagnosis, therefore, has extreme value in establishing preventative clinical best-practice for patients, as knowledge of the gene involved in the condition can dictate which pharmaceutical interventions and advice on lifestyle choices are most appropriate. In addition, it would allow the identification of ‘at risk’ family members.
The close clinical resemblance between CPVT and LQTS raises the possibility that some LQTS gene-negative patients carry mutations in CPVT-associated genes. CPVT-associated RYR2 mutations have previously been discovered in 6% of genotype-negative LQTS referral cases (Tester et al. 2005). As mutations in RYR2 account for most CPVT cases, a genetic screen of the RYR2 gene in a cohort of 23 LQTS gene-negative patients and sudden death victims was undertaken to identify the spectrum of RYR2 mutations in the New Zealand population, and provide a genetic diagnosis to another percentage of gene-negative “LQTS” patients. The patients and sudden deaths included in this cohort were among the original samples that were screened for mutations in five LQTS genes and had clinical symptoms or circumstances surrounding their deaths, respectively, that were consistent with CPVT.

5.2 Results

5.2.1 Exon selection and optimisation of PCR amplification

RYR2 is one of the largest ion channel proteins known, comprising 4967 amino acids. The RYR2 gene is made up of 105 exons, and screening for mutations within this gene is, therefore, a technical and logistic challenge. Unfortunately, cost and time restrictions prevented the analyses of all 105 exons. However, since the initial discovery of a CPVT-causing RYR2 mutation (Priori et al. 2001), reports show that the mutations are clustered into discrete regions or “hot spots” (Yano et al. 2006; George et al. 2007). On the basis of a potential physiological role for these “hot spots”, these regions have been called domains I (N-terminal), II (central) and III (channel region) (Figure 5.1). Therefore, all RYR2 exons that occur within these critical domains or in which mutations have previously been identified, according to the Inherited Arrhythmia Database, Gene Connection for the Heart (www.fsm.it/cardmoc/), were targeted for screening. In total 42 RYR2 exons were
selected (exons 3, 8, 14, 15, 28, 37-51, 59, 83, 84, and 87-105). Sections 2.2.3.1 and 2.2.3.2 in Chapter 2 describe the design and optimisation of these primers, respectively.

Figure 5.1 Mutation clustering in RYR2. Mutations clustered in the RYR2 are distributed in three discrete regions or “hot spots”, called domains I (N-terminal), II (central) and III (channel region). Adapted from Yano et al. 2006 and George et al. 2007.

5.2.2 Construction of an RYR2 “pseudo-reference sequence” for automated sequence analysis using Variant Reporter® Software

As the manual analyses of the sequencing data generated for the LQTS genes, discussed in Chapter 3, proved to be an extremely time-consuming and inefficient process, the Applied Biosystems® Variant Reporter® Software was used for automated reviewing of RYR2 sequence data. The Variant Reporter® Software is designed for reference based and non-reference based analysis such as mutation detection and analysis, SNP discovery and validation and sequence confirmation. The software algorithms can call missense variants (SNPs and mutations), insertions, deletions and heterozygous insertions/deletions, compared to a reference sequence. Although, once set up, the Variant Reporter® Software is very quick, efficient and accurate, a great deal of time and effort was spent in establishing
its use in the analyses of sequence traces from the coding regions of large genes with numerous exons, such as RYR2. It is, therefore, discussed here rather than in Chapter 2, Materials and Methods.

Variant Reporter® Software is easy to use and has a simple workflow, bulleted below, which would be perfectly suitable for mutation detection in a single amplicon.

- Import and assign traces into amplicons
- Specify a reference for the project
- Analyse the project
- Review variants identified
- Export and print reports.

Variant Reporter® recognises reference sequences in GenBank (http://www.ncbi.nlm.nih.gov/) format and can, therefore, automatically assign regions of interest (ROI), usually corresponding with coding region sequence, within an analysis project. For the RYR2 project, the mRNA reference sequence (NM_001035.2) could not be used as exon flanking sequence is required for the detection of splice site variants and the genomic reference sequence (NG_008799.1) could not be used due to its impractical size (791,586bp). Therefore, a RYR2 “pseudo-reference sequence” that included sufficient exon-flanking sequence, excluded unnecessary intronic sequence, and was in GenBank format required construction.

The human genome browser at UCSC (http://genome.ucsc.edu) ‘Get Genomic Sequence Near Gene’ was used to incorporate 250bp of flanking sequence around each exon from RYR2 mRNA reference sequence (NM_001035.2). Headers were removed from the output sequence and this was saved as a .txt file. This sequence was converted into one in GenBank format using the SQUIZZ - sequence/alignment format converter program.
from the Mobyle bioinformatics framework (http://mobyle.pasteur.fr) and pasted into a previously saved .txt file of RYR2 genomic sequence (NC_000001 region: 237205702..237997288). This was opened with Gene (www.ncbi.nlm.nih.gov/gene/) from which the original sequence from the origin position “1 down” (meaning downstream from position 1) had already been deleted. This file was further modified by deleting the existing numbers in the CDS brackets that is CDS join (1..48, 389..425,…) and replacing these with a new set of values that would go on to be interpreted by Variant Reporter® Software as the ROIs within the reference sequence. These values were calculated using excel, taking into consideration the size of each exon, the 250bp of exon-flanking sequence, as well as 20bp of sequence both up- and downstream of each exon. The 20bp of exon-flanking sequence was incorporated into the calculations so that they could be recognised by Variant Reporter® as ROIs, along with the exons themselves. This is necessary for exon-specific identification of any detected splice site mutations and for the provision of a cut-off sequence trace length for quality control within the Variant Reporter® project. So, for example, for three exons of 10bp each, the new set of values, including 20bp on either side of each exon, to be pasted between the CDS brackets (join(…)), would have been 231..250,251..260,261..280,741..760,761..770,771..790,1251..1270,1271..1280,1281..1300.

Having undergone all of the above modifications, the .txt file was saved as a pseudo-reference file with a GenBank extension (.gb) and successfully imported into Variant Reporter® Software. Once opened in Variant Reporter® Software, each ROI was labelled: RYR2_exon 1_upstream, RYR2_exon 1, RYR2_exon 1_downstream, RYR2_exon 2_upstream, RYR2_exon 2, RYR2_exon 2_downstream…etc.

A second file for importing into Variant Reporter® Software containing “pseudo-primer” sequences was created using 20bp of sequence at positions -11 to -30 and +11 to +30 for
forward (primer 1) and reverse (primer 2) primers, respectively, for each exon. Reverse complement software from the Sequence Manipulation Suite (www.bioinformatics.org/SMS/index.html) was used to obtain the reverse primer sequences in the 5’ to 3’ direction. The .txt file followed the format:

RYR2EX1 Primer1 Primer2
RYR2EX2 Primer1 Primer2

This allowed sequencing trace files, which were labelled accordingly, to be imported and assigned automatically to the correct amplicon. All analyses then proceeded according to the basic workflow steps suggested by the Variant Reporter® Software.

The method described here was adapted from work undertaken by Elaine Doherty (LabPlus, Auckland City Hospital) and Dr Tamsin Eades (Applied Biosystems Ltd) in establishing Variant Reporter® Software analysis of sequence traces in the Diagnostic Genetics section of LabPLus. Given the close relationship the research laboratory has with the diagnostic laboratory at LabPlus, it was a worthwhile endeavour that ensures compatibility between laboratories.

5.2.3 Demographics of patients selected for \textit{RYR2} genetic screen

Table 5.1 summarises the demographics for the 23 unrelated patients selected for \textit{RYR2} genetic testing by group discussion among all CIDG members at weekly meetings. This cohort comprised 17 patients and six sudden death victims, all of whom previously tested negative for pathogenic variants in LQTS genes \textit{KCNQ1}, \textit{KCNH2}, \textit{SCN5A}, \textit{KCNE1} and \textit{KCNE2}. 
Table 5.1 Demographics of patients selected for RYR2 mutation screening. The targeted mutational analyses of the 42 selected RYR2 exons identified three nonsynonymous and two synonymous missense variants, typed in blue.

<table>
<thead>
<tr>
<th>DOB</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>QTc (ms)</th>
<th>Presentation / Symptoms</th>
<th>Exercise Stress Test</th>
<th>Family History of SCD</th>
<th>Most severe symptom</th>
<th>Documented arrhythmia</th>
<th>Previous history</th>
<th>Notes</th>
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<tr>
<td>1 27/03/1972</td>
<td>F</td>
<td></td>
<td></td>
<td>RSCD (VF)</td>
<td></td>
<td></td>
<td>RSCD</td>
<td>VF</td>
<td></td>
<td>TdP</td>
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<tr>
<td>2 02/06/1992</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 26/12/1960</td>
<td>F</td>
<td>Caucasian</td>
<td>0.53</td>
<td>RSCD (VF)</td>
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<td></td>
<td>RSCD</td>
<td>VF</td>
<td>4 previous events</td>
<td>ICD</td>
</tr>
<tr>
<td>4 02/11/1963</td>
<td>F</td>
<td></td>
<td></td>
<td>CPVT</td>
<td>Ectopy</td>
<td>Yes</td>
<td>RSCD (ICD)</td>
<td>PMVT (ICD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 26/07/1958</td>
<td>M</td>
<td>Maori</td>
<td>&lt;440</td>
<td>CPVT</td>
<td>Ectopy</td>
<td>Yes</td>
<td>RSCD (ICD)</td>
<td>PMVT (ICD)</td>
<td>Multiple exercise-related syncope</td>
<td></td>
</tr>
<tr>
<td>6 26/08/1994</td>
<td>F</td>
<td>Caucasian</td>
<td>&lt;440</td>
<td>Syncope</td>
<td>Ectopy</td>
<td>Yes</td>
<td>Syncope (PMVT (Reveal))</td>
<td>Multiple exercise-related syncope</td>
<td>ICD</td>
<td></td>
</tr>
<tr>
<td>7 03/10/2002</td>
<td>M</td>
<td>Caucasian</td>
<td>0.43</td>
<td>Syncope with seizure</td>
<td>PMVT</td>
<td>Yes</td>
<td>Syncope with seizure</td>
<td>PMVT</td>
<td>Multiple exercise-related syncope</td>
<td>ICD</td>
</tr>
<tr>
<td>8 11/02/1969</td>
<td>F</td>
<td></td>
<td></td>
<td>Syncope with seizure</td>
<td>SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 05/05/1981</td>
<td>M</td>
<td></td>
<td></td>
<td>Syncope with seizure</td>
<td>SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TdP – torsades de pointe; RSCD – resuscitated sudden cardiac death; VF – ventricular fibrillation; ICD – implantable cardiac defibrillator; CPVT – catecholamine polymorphic ventricular tachycardia; PMVT – polymorphic ventricular tachycardia
5.2.4 *RYR2* gene variants identified by sequencing analyses

The targeted mutational analyses of the 42 selected RYR2 exons identified three nonsynonymous and two synonymous missense variants (Table 5.2, highlighted in blue). The three nonsynonymous variants, p.R420Q, p.G1885E and p.G1886S, were present in four of the 23 samples screened (17.4%). These findings were confirmed by duplicate sequence analysis. Using *in silico* algorithms that predict the effect of amino acid substitutions on protein structure, SIFT (sorts intolerant from tolerant) (Xi et al. 2004; Ng and Henikoff 2001) and PolyPhen (polymorphism phenotyping) (Ramensky et al. 2002; Xi et al. 2004), all three of the nonsynonymous variants were predicted to be tolerated and benign, respectively (Table 5.2). Their Grantham scores (Grantham 1974), which categorise codon replacements into classes of increasing chemical dissimilarity, were designated conservative for p.R420Q and p.G1886S and moderately conservative for p.G188E according to the classification proposed by Li et al. (1984) (Table 5.2)
<table>
<thead>
<tr>
<th>Variant No</th>
<th>Exon</th>
<th>Amino Acid Change*</th>
<th>Codon Change</th>
<th>Base Position Change**</th>
<th>SNP Database Number</th>
<th>Cases Hosting Variant (n=23)</th>
<th>SIFT prediction</th>
<th>PolyPhen prediction</th>
<th>Grantham Matrix Score#</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>p.R420Q</td>
<td>CGG→CAG</td>
<td>c.1379 G→A</td>
<td>-</td>
<td>1</td>
<td>0.07 tolerated</td>
<td>0.225 benign</td>
<td>43</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>p.S453S</td>
<td>AGC→AGT</td>
<td>c.1479 C→T</td>
<td>rs3765097</td>
<td>19 (9 hom)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>p.G1885E</td>
<td>GGG→GAG</td>
<td>c.5774 G→A</td>
<td>rs41315858</td>
<td>1</td>
<td>1.00 tolerated</td>
<td>1.096 benign</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>p.G1886S</td>
<td>GGC→AGC</td>
<td>c.5776 G→A</td>
<td>rs376871</td>
<td>3</td>
<td>0.67 tolerated</td>
<td>0.371 benign</td>
<td>56</td>
</tr>
<tr>
<td>5</td>
<td>51</td>
<td>p.H2602H</td>
<td>CAC→CAT</td>
<td>c.7926 C→T</td>
<td>rs684923</td>
<td>15 (5 hom)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Amino acid position is numbered according to NP_001026.2
**Nucleotide base position is numbered according to NM_001035.2

#Grantham scores, which categorize codon replacements into classes of increasing chemical dissimilarity, were designated conservative (0-50), moderately conservative (51-100), moderately radical (101-150), or radical (≥151) according to the classification proposed by Li et al 1984
5.3 Discussion

In genes such as RYR2, novel nonsynonymous variants are being identified at a rate at which functional studies cannot keep up. It is, therefore, difficult to predict the impact these changes have on protein expression and function and, consequently, the pathogenicity of the variant. Functional studies also have their own limitations, and while they may provide a certain level of proof, failure to show an effect does not always dismiss pathogeneity. This knowledge is of great importance for the care and treatment of patients and their families. More often than not, input from a multidisciplinary team is required to determine the clinical significance of these unclassified variants. This can include assessments of the presence or absence of the variant in healthy control individuals, the segregation of the variant with clinical phenotype within a family, comparisons of allele frequencies (Shattuck-Eidens et al. 1997) and in silico algorithms like the Grantham score (Grantham 1974), SIFT (Xi et al. 2004; Ng and Henikoff 2001) and PolyPhen (Ramensky et al. 2002; Xi et al. 2004) that consider factors such as sequence conservation across species and the physiochemical nature of the amino acid substitutions.

Although in silico analyses predicted each of the three nonsynonymous variants detected here to be benign, this does not rule out their involvement in disease. Other factors that determine whether or not the variant should be considered “disease-causing”, such as previously reported findings, the proximity of the amino acid substitution to splice sites, predicted functional domains and/or structural features within the protein and the clinical presentation and family history of the patient carrying the variant are discussed below.
5.3.1 The p.R420Q variant

The p.R420Q variant has a positively charged, basic arginine (R) substituted by an uncharged polar glutamine (Q) at codon 420 (NP_001026.2). This is at the N-terminal domain of the RYR2 protein which extends into the cytosol (Figure 5.3). It is generally accepted that the induction of delayed afterdepolarisations (DADs) and triggered activity caused by uncontrolled calcium release, or leakage, is the final common effect of CPVT mutations. However, there are three predominant molecular mechanisms, influenced by mutation position, that ultimately lead to this calcium leakage (reviewed in Chapter 1, section 1.4.3). The hypothesis of defective domain-domain interactions as a molecular mechanism, first proposed by Ikemoto et al. (2002), is that a loose interaction (unzipping) between the N-terminal and central regions hyperactivates RYR2, causing diastolic calcium leakage (see Chapter 1.4.3 for more detail). Given its N-terminal position, it is plausible that the p.R420Q mutation might exert a pathogenic effect through this mechanism, that is by destabilising interdomain interactions.

The p.R420Q variant identified in this study was originally thought to be novel. However, its detection in an independent report in two cases, with a clear CPVT phenotype, and absence in 400 reference alleles, led it to be recently reported as a putative pathogenic mutation by Medeiros-Domingo et al. (2009). Their first case was an eight-year-old male with a clinical diagnosis of CPVT following episodes of exertional syncope (episodes of fainting after exertion), including a swimming-triggered event and a successful resuscitation from ventricular fibrillation. His ECG was normal and his exercise test was consistent with CPVT with PVCs in singles and in a bigeminal pattern (Medeiros-Domingo, personal communication). Other than having a clear CPVT phenotype, detailed clinical information for their second patient was unavailable.
The patient in whom the variant was detected in this study is a woman in her early sixties with several sudden deaths in her family (I:1 in the pedigree shown in figure 5.2). She suffered a cardiac arrest after the death of a son (II:3, figure 5.2) and was vague about other aspects of her medical history yet recalled a couple of syncopal episodes. Although this patient’s QTc is within normal limits, her ECG is profoundly abnormal. Her baseline ECG showed an incomplete right bundle branch block pattern, which is suggestive of Brugada syndrome, but this did not alter with Flecainide administration and, therefore, excluded a diagnosis of BS. There is some ST segment depression in the lateral V leads and the depolarisation continues long after the QRS complex with very prominent U waves. Ectopy increased with exercise testing.

The patient reports no arrhythmia-related symptoms or sudden deaths among her parents or siblings, yet has herself lost two sons and a daughter (II:2, II:3 and II:4, figure 5.2). The first son, II:2 (figure 5.2), died at age eight after playing in a park. He started having blackouts three months before his death but these were not obviously associated with exercise. His QTc at the time was 440ms. The second, II:3 (figure 5.2), had a history of seizures, with an episode of sudden collapse and convulsion after swimming at age 15. His QTc was normal (360ms). He suffered a second major blackout at age 19 in a school swimming pool and died after unsuccessful attempts at resuscitation. The patient’s daughter’s (II:4, figure 5.2) death, at age 18, is under police investigation as it occurred following an assault. Little is known of her medical history. She had one episode of syncope while at school and has apparently lost two children (III:1 and III:2, figure 5.2), although no additional information is available. The surviving son (II:1, figure 5.2) is asymptomatic, however, his ECG is abnormal with left axis deviation and a BrS-like appearance in V1. His QTc is normal (410ms).
As less than 15% of the mutations reported to date in RYR2 have been studied \textit{in vitro}, pathogenicity of newly identified variants has usually been suspected based on co-segregation with the disease and absence in control subjects. DNA samples for the deceased members of this family are unavailable at present so co-segregation of the p.R420Q variant with disease could not be determined in this family. The CIDG, however, are requesting consent for the release of deceased individuals’ Guthrie cards. If these become available at some point in the future, a molecular autopsy could be performed. So,
even if we were to speculate that the deceased family members carried the p.R420Q variant and that it contributed towards their deaths, we might have expected our proband (I:1, Figure 5.2) to have suffered more symptoms herself. It has been suggested that females show a less aggressive phenotype (Medeiros-Domingo, personal communication), possibly due to hormonal interactions. RYR2 mosaicism could also offer an explanation for her less severe phenotype. Medeiros-Domingo et al. (2009) identified a proband with a maternally inherited p.Y4149S variant. Although the proband’s mother was asymptomatic and had an unremarkable exercise ECG, germline mosaicism was suspected clinically because more than one offspring was affected (sudden death in two children) and subsequently confirmed. Although RYR2 mutations are often de novo in origin, they could also be present in a mosaic form in asymptomatic parents. This needs to be considered in genetic counseling and in selection of family members for genetic screening.

5.3.2 The p.G1885E and p.G1886S variants

The p.G1885E and p.G1886S variants are two common nonsynonymous missense polymorphisms in the RYR2 gene. Common polymorphisms in other ion channels have the potential to modify the clinical phenotype; polymorphisms in RYR2 are likely to have the same potential. The most common RYR2 polymorphism, p.Q2958R, occurs in approximately 34% Caucasians (Tiso et al. 2001). The second most common RYR2 polymorphism is p.G1886S (20% African Americans, 9% Caucasians), followed by p.G1885E (6% Caucasians) (Medeiros-Domingo et al. 2009). Interestingly, in vitro studies in heterologous systems have shown that both p.G1885E and p.G1886S polymorphisms caused a significant increase in the cellular Ca$^{2+}$ oscillation activity compared to RYR2 wild-type channels. However, when both polymorphisms were introduced in the same RYR2 subunit, the store-overload-induced calcium release activity was nearly completely
eliminated (Koop et al. 2008). Although the clinical consequence of this RYR2 loss of function in vitro phenotype is unclear, these two amino acid substitutions have been associated with arrhythmogenic right ventricular cardiomyopathy (ARVC) in a compound heterozygous fashion, where one allele carries the one variant and the other allele carries the other (Milting et al. 2006).

The location affected by the variants is part of the cardiac-specific divergent region 3 (DR3 domain), which comprises amino acid residues 1852-1890 of RYR2, and is believed to be involved in regulation of the Ca\(^{2+}\) channel (Zhang et al. 2003) (Figure 5.3). The two glycine residues affected by the SNPs lay within the FKBP12.6 binding site (residues 1636-1937) (Masumiya et al. 2003). The results of Koop et al. (2006) suggest that the residues 1885 and 1886 of RYR2 mark a sensitive spot inside the DR3 domain that is important for the control of the Ca\(^{2+}\) release properties of the channel. It is still unknown if the change in the critical spot of the DR3 domain induced by the variants affects the FKBP12.6 binding site adjacent to it and the variants exert their effect via disruption of FKBP12.6-RYR2 binding.

Section 1.4.3 outlines the role FKBP12.6 plays in RYR2 biology.

In this study the p.G1885E variant was identified in a sudden death victim, a 15 year old boy who died during a swimming race. He had a history of being “knocked unconscious” while playing games or sport. Both of his parents are asymptomatic with normal ECGs and normal exercise tests
The p.G1886S variant was detected in three cases, one of which was a sudden death. There is very little clinical and family history available for two of these cases. The third case is a female in her fifties with a clear CPVT phenotype of polymorphic ectopy and a family history of sudden death. Her identical twin sister died from SCD while playing netball at age 22. She lost another sister who died at age 26 while dancing at a wedding and her grandfather died at age 36 from what was thought at the time to have been a stroke. Given the high frequency of the p.G1886S variant in control populations (20% African Americans, 9% Caucasians), it seems unlikely that it can be responsible alone for such a malignant phenotype. The \textit{in silico} algorithms SIFT and PolyPhen predict the variant to be tolerated and benign, respectively. It is possible that this family carry another genetic mutation in one of the RYR2 exons that was not included in this screen or in another gene associated with CPVT, such as CASQ2. Common polymorphisms in other ion channels have the potential to modify the clinical phenotype or infer increased risk for disease, it
seems likely that the p.G1885E and p.G1886S polymorphisms in RYR2 have the same potential.

5.3.3 The p.S453S and p.H2602H variants

Of the five variants identified, two were synonymous SNPs, p.S453S and p.H2602H. These do not result in an amino acid substitution and are highly unlikely to be implicated in disease.

5.4 Summary

In disorders as genetically heterogenous as the inherited arrhythmia syndromes and in which reaching a molecular diagnosis is crucial for directing treatment, the drive behind just about any research endeavour becomes finding a “solution” for each gene-negative patient or family, individually. Twenty-three LQTS gene-negative patients with a clinical phenotype consistent with CPVT were screened for mutations in the RYR2 gene. Three non-synonymous missense variants, which may be contributory to disease, were detected in five cases, two of which were sudden deaths. As less than 15% of RYR2 variants have been studied in vitro, the pathogenicity of most variants detected in RYR2 is contentious. Considering several factors including co-segregation with disease, presence or absence of the variant in control subjects, position of the variant within the encoded protein and in silico algorithms, it seems likely that the p.R420Q variant can be pathogenic. The family carrying this variant may now be offered appropriate medical management. Family members had not yet consented for testing at the time of the study. If in due course they are tested, the multidisciplinary CIDG will decide on best course of medical management depending on the presence or absence of the variant in each family member as well as cosegregation of the variant with the disease in the family. Absence of mutation will not
necessarily mean absence of disease and all individuals will be counselled appropriately. Although the p.G1885E and p.G1886S variants are polymorphic, they do result in increased cellular Ca\(^{2+}\) oscillation activity and have been identified in families with severe histories of sudden death. It is likely that they are implicated in disease in these families, but to exactly what extent remains unknown. The detection of the p.G1885E and p.G1886S variants in these families helps to provide some limited understanding of the predisposition to CPVT, and we are provided with some direction for their clinical treatment and further courses of action.
CHAPTER 6:

THE ROLE OF SINGLE NUCLEOTIDE POLYMORPHISMS IN LQTS
Chapter 6: The role of single nucleotide polymorphisms (SNPs) in LQTS

6.1 TaqMan SNP genotyping assays in LQTS patients

The extreme phenotypic variation seen in patients with LQTS is poorly understood. Even among patients carrying the same pathogenic mutation, the clinical manifestation in affected individuals can range from asymptomatic throughout life to episodes of recurrent seizure and/or syncope and sudden cardiac death at a young age. SNPs in LQTS-associated genes may contribute to this heterogeneity by acting in concert as disease modifiers and be either protective or associated with an increased susceptibility to arrhythmia. To determine whether the presence or absence of any of these SNPs contribute to mutation-negative, phenotype-positive LQTS patients, TaqMan SNP genotyping assays (Applied Biosystems) were used to screen 10 SNPs in the LQTS genes, \textit{KCNQ1}, \textit{KCNH2}, \textit{SCN5A}, \textit{KCNE1} and \textit{KCNE2}, in a panel of 84 unrelated LQTS patients and 100 healthy New Zealand controls.

6.1.1 Patients

Genomic DNA samples from 84 unrelated Caucasian LQTS patients that had been referred to the CIDG for LQTS testing were used for this part of the study. Patients in whom a LQTS causative mutation had been previously identified formed the ‘mutation-positive’ group (42 individuals), whereas those in whom no causative LQTS mutations formed the mutation-negative group (42 individuals). The Department of Obstetrics and Gynaecology, University of Auckland, had previously extracted genomic DNA from whole blood, from 100 anonymous Caucasian control samples from the general New Zealand population. These samples were used for comparative purposes.
6.1.2 Results and discussion

6.1.2.1 SNP selection

Ten SNPS in LQTS genes were carefully selected for genotyping in the patient and control samples using TaqMan SNP genotyping assays (Table 6.1). Financial limitations allowed for the selection of only 10-12 SNPs. The following criteria were considered in selecting SNPS: they were previously associated with a possible functional effect in the literature and/or they were previously found in the New Zealand population and were relatively common in the Caucasian population to allow detection of the minor allele in this sample size. In addition, two promoter SNPs and one intronic SNP were selected to explore the role of variants in non-coding regions (Table 6.1).

6.1.2.2 TaqMan SNP genotyping assays

Six of the ten SNP genotyping assays were predesigned by Applied Biosystems and four assays were custom designed using File Builder software (Applied Biosystems) (Section 2.2.20.1). After setting up and optimising the assays, the laboratory work and subsequent analysis was carried out jointly with BSc Honours student, Janice Mak.

Results from the TaqMan SNP genotyping assays were displayed on allelic discrimination plots. The assays were set up so that samples carrying homozygous wildtype alleles were displayed in red, heterozygous alleles in green, and homozygous minor allele (SNP) in blue (Figure 6.1). The no template controls (NTC) represent controls reactions that did not contain DNA. Although most plots revealed distinct clusters of alleles, some allelic discrimination plots revealed more dispersed clusters that extended towards the NTCs. If this occurred, the assay was repeated to avoid ambiguous allele calls (Figure 6.1).
### Table 6.1 SNPs selected for Taqman genotyping assays

<table>
<thead>
<tr>
<th>LQTS Gene</th>
<th>SNP (dbSNP ID), Hapmap CEU allele frequencies</th>
<th>Nucleotide change</th>
<th>Exon</th>
<th>Amino acid change</th>
<th>Protein position</th>
<th>Rationale for selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNQ1</td>
<td>rs36210419 A=.985, G=.015</td>
<td>A760G</td>
<td>4</td>
<td>K218E</td>
<td>S3/S4 domain</td>
<td>Possible association with torsades de pointes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>rs7945327 A=.934, C=.066</td>
<td>A/C-1096</td>
<td>-</td>
<td>-</td>
<td>Promoter</td>
<td>Relatively common&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KCNH2</td>
<td>rs36210422 C=.989, T=.011</td>
<td>C526T</td>
<td>4</td>
<td>R176W</td>
<td>N terminal</td>
<td>Potentially pathogenic&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>rs1805123 A=.761, C=.239</td>
<td>A2691C</td>
<td>11</td>
<td>K897T</td>
<td>C terminal</td>
<td>Possible mutation modifier&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>rs7803184 G=.907, A=.039</td>
<td>A/G-484</td>
<td>-</td>
<td>-</td>
<td>Promoter</td>
<td>Relatively common&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCN5A</td>
<td>rs1805124A A=.821, G=.179</td>
<td>A1673G</td>
<td>12</td>
<td>H558R</td>
<td>DI-DII linker</td>
<td>Possible mutation modifier&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>rs11129795 G=.752, A=.248</td>
<td>A/G IVS1-3208</td>
<td>Intron-1</td>
<td>-</td>
<td>Intron-1</td>
<td>Relatively common&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KCNE1</td>
<td>rs1805127 G=.647, A=.353</td>
<td>G112A</td>
<td>3</td>
<td>S38G</td>
<td>Transmembrane domain</td>
<td>Previously identified in NZ population</td>
</tr>
<tr>
<td></td>
<td>rs1805128 G=.975, A=.025</td>
<td>G253A</td>
<td>3</td>
<td>D85N</td>
<td>Transmembrane domain</td>
<td>Possible association with QT interval&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>rs2234916 A=.957, G=.043</td>
<td>A22G</td>
<td>1</td>
<td>T8A</td>
<td>Transmembrane domain</td>
<td>Possible mutation modifier&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

References:

- a) (Mank-Seymour et al, 2006)
- b) NCBI database allele frequencies
- c) (Ackerman et al, 2003)
- d) (Bezzina et al, 2003)
- e) (Crotti et al, 2005)
- f) (Ye et al, 2003)
- g) (Friedlander et al, 2005)
- h) (Gouas et al, 2005)
- i) (Wei et al, 1999)
Figure 6.1: Example of allelic discrimination plots. Alleles are labelled on the left plot with homozygous wildtype alleles in the red cluster, heterozygous alleles in the green cluster, homozygous minor allele in the blue cluster and no template controls (NTC) shown as a black cross. The plot on the right shows an example of an assay where allele clusters extend towards the NTCs. If these extended too close for an unambiguous allele call the assay was repeated.

The analysis software SDS v2.3 (Applied Biosystems) usually distinguishes between the homozygous wildtype, heterozygous, and homozygous SNP clusters and automatically calls each sample to an allele. If the software failed to call alleles automatically, obvious clusters which were well separated from the NTCs were called manually.

Ten percent of the samples in each run, were repeated in duplicate and checked for accurate allele calling by the software. In addition, a representative sample from each cluster of the allelic discrimination plot was sequenced to confirm and validate the results. Samples that clustered with the NTCs showed very low signals, which suggest that there was possible evaporation of the sample, or the DNA was of insufficient quantity. These
samples were repeated in another assay run with known positive controls. These steps were carried out by Janice Mak.

6.1.2.3 Statistical and bioinformatic procedures

In assessment of the SNPs selected for analysis in this study, some statistical and bioinformatic steps were carried out.

1. For each SNP, the Hardy-Weinberg equation \( p^2 + 2pq + q^2 = 1 \) where \( p \) and \( q \) represent the relative frequencies of the major and minor alleles respectively, \( p^2 \) and \( q^2 \) the theoretical frequencies of the homozygous genotypes and \( 2pq \) the frequency of the heterozygous genotype) was used to calculate the expected allele frequencies. The difference between the observed and expected allele frequencies was tested using Chi-Square analysis, with no significant difference indicating that the population was in Hardy-Weinberg equilibrium. All ten selected LQTS SNPs in our study were in Hardy-Weinberg equilibrium (Table 6.1). It is essential to establish this prior to testing for significant differences in genotypic frequencies. Hardy-Weinburg equilibrium assumes that the occurrence of a genotype stays constant within a population. Factors that cause a shift in genotype frequencies from Hardy-Weinburg equilibrium include non-random mating, inbreeding, migration, natural selection and random genetic drift.

2. To compare minor allele frequencies between mutation positive probands, mutation negative probands and controls, the Fisher’s exact test was performed. The following comparisons were made:
   - Mutation positive probands were compared to controls
   - Mutation negative probands were compared to controls
   - Mutation positive probands were compared to mutation negative probands.
3. Multiple amino acid sequences were aligned using Clustal (http://www.clustal.org/) to determine whether the position of the SNP was conserved across a variety of species (Table 6.2). Five of the seven non synonymous SNPs were conserved across species, two were semi-conserved.

4. In silico algorithms that predict the effect of amino acid substitutions on protein structure, SIFT (sorts intolerant from tolerant) (Xi et al 2004, Ng and Henikoff 2001) and PolyPhen (polymorphism phenotyping) (Ramensky ey al 2002, Xi et al 2004), were used to assess the likely effect of the amino acid substitution in non synonymous SNPs (Table 6.3). For four of the SNPs both programmes predicted a benign effect, a damaging effect was predicted for one and for two of the variants the programmes offered opposing predictions (Table 6.3).

<table>
<thead>
<tr>
<th>LQTS Gene</th>
<th>SNP (dbSNP ID)</th>
<th>Amino acid change</th>
<th>SNP site conserved across species?</th>
<th>Properties of wildtype amino acid</th>
<th>Properties of SNP amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>KCNQ1</em></td>
<td>rs36210419</td>
<td>K218E</td>
<td>Y</td>
<td>Lysine - polar, basic</td>
<td>Glutamic acid - polar, acidic</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>KCNH2</em></td>
<td>rs36210422</td>
<td>R176W</td>
<td>Y</td>
<td>Arginine - polar</td>
<td>Tryptophan - non polar</td>
</tr>
<tr>
<td></td>
<td>rs1805123</td>
<td>K897T</td>
<td>Y</td>
<td>Strongly basic</td>
<td>Hydrophobic aromatic</td>
</tr>
<tr>
<td><em>SCN5A</em></td>
<td>rs1805124</td>
<td>H558R</td>
<td>Semi-conserved</td>
<td>Histidine - polar, aromatic</td>
<td>Arginine - polar,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Weakly basic</td>
<td>Strongly basic</td>
</tr>
<tr>
<td><em>KCNE1</em></td>
<td>rs1805127</td>
<td>S38G</td>
<td>Semi-conserved</td>
<td>Glycine - very small</td>
<td>Serine - polar, very small</td>
</tr>
<tr>
<td></td>
<td>rs1805128</td>
<td>D85N</td>
<td>Y</td>
<td>Aspartic acid - polar, small,</td>
<td>Asparagine - polar, small</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>acidic</td>
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</tr>
<tr>
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<td>Alanine - non polar, very</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Weakly acidic</td>
<td>small, hydrophobic</td>
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</tbody>
</table>

Table 6.2 Multiple Clustal alignments. Most of the SNPs are highly conserved with identical amino acids in all sequences in the alignment. Semi-conserved substitutions were observed where amino acid change was to another amino acid of similar properties.
Table 6.3 Predictions from SIFT and PolyPhen. SIFT predicts that R176W and T8A were not tolerated, with a score of <0.05. PolyPhen prediction uses the PSIC (Position-Specific Independent Count) score. A PSIC score of >1.5 indicates that R176W and K897T are possibly damaging SNPs.

<table>
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<tr>
<th>LQTS Gene</th>
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<th>SIFT SNP tolerated?</th>
<th>SIFT Score</th>
<th>PolyPhen Prediction</th>
<th>PolyPhen PSIC score</th>
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6.1.2.4 Taqman SNP analyses

Analysis of KCNQ1 K218E SNP

The KCNQ1 K218E SNP results in an amino acid change from lysine (K) to glutamic acid (E). The nucleotide change is: AAG → GAG. The red clusters in the allelic discrimination plots represent wildtype alleles of KCNQ1 K218E SNP (Figure 6.2). No minor allele (G) was found in either the patient or the control group. One representative sample was sequenced to confirm that the red cluster was the wildtype A/A allele (Figure 6.3a).
Figure 6.2: Allelic discrimination plots for KCNQ1 K218E SNP. Left: Mutation negative and positive probands. Right: Control samples. All samples screened from patients and controls were wildtype (red cluster).

Analysis of KCNQ1 promoter SNP

TaqMan SNP genotyping assay identified wildtype (A/A), heterozygous SNP (A/C), and homozygous SNP (C/C) alleles of the KCNQ1 promoter SNP, in the probands but no homozygous SNP in the controls (Figure 6.3). DNA sequencing confirmed all three alleles (Figure 6.19). The minor allele frequency (MAF) was highest in mutation positive probands, and lowest in mutation negative probands (Figure 6.4). Fisher’s exact test showed that there was no significant difference between these three groups (p value = 0.568).
Figure 6.3: Allelic discrimination plots for KCNQ1 promoter SNP. Mutation negative and positive probands (left) showed the presence of wildtype, heterozygous and homozygous SNP, whereas control samples (right) showed only the wildtype allele, heterozygous SNP, and no homozygous SNP.

Figure 6.4: Minor allele frequencies of KCNQ1 promoter SNP in proband patients and controls. Mutation positive probands had the highest minor allele frequency at 7.1%, compared to controls at 5.5% and mutation negative probands at 4.8%. Fisher’s exact test showed no significant difference between these three groups.
Analysis of KCNH2 R176W SNP

The KCNH2 R176W SNP results in an amino acid change from arginine (R) to tryptophan (W). The nucleotide change is: CCG → TGG. The minor allele (T) was not detected in patients or controls. The red cluster in the allelic discrimination plots represents the wildtype allele of the KCNH2 R176W SNP (Figure 6.5). DNA sequencing of a representative sample from the red cluster confirmed the wildtype C/C allele (Figure 6.19).

Figure 6.5: Allelic discrimination plots for KCNH2 R176W SNP. All samples screened in probands (left) and controls (right) were wildtype.

Analysis of KCNH2 K897T SNP

The KCNH2 K897T SNP results in an amino acid change from lysine (K) to threonine (T) and base pair change from A→C. Clear clusters of homozygous wild type, heterozygotes and homozygous minor alleles were detected in proband and control samples (Figure 6.6). Mutation negative probands had the highest minor allele frequency at 26.2%, controls
25.5% and mutation positive probands 23.8%. Fisher's exact test showed no significant difference between the three groups (p value = 0.782) (Figure 6.7).

![Figure 6.6: Allelic discrimination plots of KCNH2 K897T SNP](image1)

Figure 6.6: Allelic discrimination plots of KCNH2 K897T SNP. Proband samples (left) and control samples plot (right), showed clear clusters of the three alleles. SNP nucleotide change was from A → C and amino acid change from a K (lysine) to T (threonine). DNA sequencing could not be performed to validate the three clusters due to time constraints.

![Figure 6.7: Minor allele frequencies of KCNH2 K897T SNP](image2)

Figure 6.7: Minor allele frequencies of KCNH2 K897T SNP in proband patients and controls. Mutation negative probands had the highest minor allele frequency at 26.2%, controls 25.5% and mutation positive probands 23.8%. Fisher's exact test showed no significant difference between the three groups (p value = 0.782).
Analysis of KCNH2 promoter SNP

The nucleotide change for the KCNH2 promoter SNP is A→G. Clearly defined clusters were not originally obtained in the allelic discrimination plot for the proband samples (Figure 6.8). Those with low signals that clustered with the NTCs were ambiguous. These samples were repeated and were discriminated clearly (plot not shown). The KCNH2 promoter homozygous minor allele or SNP (G) was not found in the control samples. DNA sequencing confirmed the three representative samples from the allelic discrimination plot (Figure 6.19). Mutation positive probands showed the highest MAF at 13.1%, and mutation negative probands and controls showed very similar MAF (Figure 6.9). Fisher’s exact test showed no significant differences between these three groups (p value = 0.691).

Figure 6.8: Allelic discrimination plots for KCNH2 promoter SNP. Some proband samples clustered with the NTCs (left) and were repeated in another assay run which worked with clear discrimination. Homozygous SNP did not occur in control samples (right).
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Figure 6.9 Minor allele frequencies of KCNH2 promoter SNP in proband patients and controls. Mutation positive probands showed the highest frequency at 13.1% compared to mutation negative probands and controls at 10.7% and 10.5% respectively.

Analysis of SCN5A H558R SNP

The SCN5A SNP H558R has the amino acid change H (histidine) to R (arginine) and nucleotide change: CAC→CGC. Control samples had the highest MAF for the SCN5A H558R SNP (Figure 6.11). Although, some samples clustered closer to the NTCs, automatic allele calls were made successfully, and no repeats were necessary (Figure 6.10). DNA sequencing confirmed all three representative samples from the allelic discrimination plot (Figure 6.19). Fisher’s exact test showed no significant difference between these three groups (p value = 0.826).
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Figure 6.10: Allelic discrimination plots for SCN5A H558R SNP. Clusters for each allele were “stringy” in both probands (left) and controls (right), however alleles were called automatically.

Figure 6.11 Minor allele frequencies of SCN5A H558R SNP in proband patients and controls. Controls showed the highest minor allele frequency for this SNP at 25%. Frequencies for mutation positive probands were at 21.4% and mutation negative probands at 19%.
Analysis of SCN5A intron-1 SNP

The nucleotide change is an A→G. All three alleles were detected by the assay in patients and controls (Figure 6.12). The SCN5A intron SNP was slightly more prevalent in patients than in controls (Figure 6.13). MAF was highest in mutation positive probands with 21.4%, followed by mutation negative probands at 20.2%, and control samples at 17.5%. However, Fisher’s exact test showed no significant difference between these groups (p value = 0.226). DNA sequencing confirmed all three representative samples of the SCN5A intron-1 SNP (Figure 6.19).

![Allelic discrimination plots for SCN5A intron-1 SNP. All three alleles were detected by the assay in patients and controls. There are more homozygous SNP alleles in the proband patient group (left) compared to controls (right).](image-url)
Figure 6.13 Minor allele frequencies of SCN5A intron-1 SNP in proband patients and controls. Mutation positive and negative probands showed the highest minor allele frequency at 21.4% and 20.2% respectively, with controls at 17.5%.

Analysis of KCNE1 G38S SNP

The amino acid glycine (G) is substituted with serine (S) for this SNP and the nucleotide change is $\text{GGT} \rightarrow \text{A GT}$. Homozygote wild type, heterozygote and homozygous minor alleles were observed in patients and control samples. Although the allelic discrimination plot for the probands did not cluster as distinctly as for the control samples, allele calls that were made automatically were correct and no repeats were necessary (Figure 6.14). DNA sequencing confirmed the three representative samples from the three clusters on the allelic discrimination plot (Figure 6.19). The KCNE1 G38S SNP was the most prevalent out of all the SNPs screened in this study. MAF was highest in mutation positive probands with 36.9%, compared with mutation negative probands with 25% and controls with 31.5% (Figure 6.15). The Fisher’s exact test showed no significant difference between these three groups (p value = 0.392).
Figure 6.14 Allelic discrimination plots for the KCNE1 G38S SNP. A ‘stringy’ heterozygous cluster was observed in the proband samples (left) but no repeats were needed. Control samples showed clear clusters of the three alleles (right).

Figure 6.15 Minor allele frequencies of KCNE1 G38S SNP in proband patients and controls. Mutation positive probands showed the highest minor allele frequency at 36.9%, with controls at 31.5% and mutation negative probands at 25%.
**Analysis of KCNE1 D85N SNP**

![Image of Allelic discrimination plots](image)

**Figure 6.16 Allelic discrimination plots for the KCNE1 D85N SNP.** An example of manually calling the allele types where the wildtype cluster was apparent. The heterozygous SNPs were called with confidence in the presence of a heterozygous positive control. No homozygous SNP was observed in either proband samples (left) or control samples (right).

The KCNE1 SNP arises from a nucleotide change from G\(\rightarrow\)A which results in an amino acid substitution, aspartic acid (D) to asparagine (N). Manual calls were made for the allelic discrimination plots with confidence (Figure 6.16). The green cluster on the allelic discrimination plot was called heterozygous for the reason that a known positive heterozygous control was present in the assay run. No homozygous SNP was found in patients or controls. DNA sequencing confirmed the representative samples (Figure 6.19). An interesting trend of MAFs is seen, with proband patients having a higher MAF compared to controls (Figure 6.17). However, Fisher’s exact test showed no significant difference between the three groups (p value = 0.127).
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Figure 6.17 Minor allele frequencies of KCNE1 D85N SNP in proband patients and controls. The minor allele frequencies of mutation negative and positive probands were 3.6% and 2.4% respectively, compared to controls at 1.5%.

Analysis of KCNE2 T8A SNP

The nucleotide change: ACA → GCA results in amino acid substitution T (threonine) → A (alanine). The assay automatically called all samples as wildtype A/A (Figure 6.18). One sample had a high signal; however, no positive control was available therefore DNA sequencing was used to determine the allele of the sample. The unknown sample had heterozygous A/G alleles, which makes the MAF in controls 0.5%. Fisher’s exact test showed no significant difference between patients and controls (p value = 0.186).

For genetic association studies, odds ratios would give an indication of the odds of a disease or phenotype occurring in individuals with a particular gene variant (or SNP) versus the odds of a disease occurring in a group without the gene variant. As the samples sizes in this pilot study were small, odds ratios were not calculated.
Figure 6.18 Allelic discrimination plots for KCNE2 T8A SNP. All samples called were wildtype for proband patients (left) and controls (right). One sample in the controls was undetermined (circled) by the assay, and was instead determined through DNA sequencing.
Figure 6.19 Chromatogram of representative samples sequenced to confirm allele calls. a) KCNQ1 K218E, b) KCNQ1 promoter SNP, c) KCNH2 R176W, d) KCNH2 promoter SNP, e) SCN5A H558R, f) SCN5A intron 1 SNP, g) KCNE1 G38S, h) KCNE1 D85N and i) KCNE2 T8A.
6.1.3 Conclusions

The cardiac channelopathies exhibit extreme variation in phenotype expression. The mechanisms underlying such phenotypic diversity remain unknown. Through the clinical screening program, several unclassified variants or single nucleotide polymorphisms (SNPs) have been identified that have uncertain clinical significance. In addition there are very many non synonymous SNPS documented in the literature and databases that are uncharacterised. To investigate whether a selection of SNPs play any role as susceptibility loci or genetic modifiers of established mutations in LQTS, Taqman genotyping assays were used to type 10 SNPs (spread throughout LQTS genes, \textit{KCNQ1}, \textit{KCNH2}, \textit{SCN5A}, \textit{KCNE1} and \textit{KCNE2}) in 42 gene-positive and 42 gene-negative LQTS patients, respectively, and 100 healthy New Zealand control individuals. Comparable allele frequencies to those reported in the literature were found. Although no significant differences were identified in this pilot study, important trends were observed in the \textit{SCN5A}-intron-1 SNP and the \textit{KCNE1}-D85N SNP, where minor allele frequencies were notably higher in patients compared to controls.

Genome-wide association studies are a powerful and now widely-used method for finding genetic variants that increase the risk of developing particular diseases. These studies are complex and must be planned carefully in order to maximize the probability of finding novel associations. The main design choices to be made relate to sample sizes and choice of genotyping method, usually a commercially available genotyping chip, and are often constrained by cost, which can currently be as much as several million dollars.

In the design of such studies two fundamental decisions have to be made: which loci to genotype, and in how many individuals. Both decisions have practical constraints. For
example it is currently not possible to assay all known variation in the human genome at a reasonable cost and choices must be made between a set of commercially available genotyping chips. Similarly, sample sizes are often limited by the number of well characterised clinical samples. The major consideration in study design, therefore, should be the power of the study: the probability of detecting a variant assumed to be causal (Spencer et al. 2009).

Power calculations determine the actual sample size needed to find a true genotype-phenotype correlation under the study constraints. For example, in a fine-mapping study of 200 effective tests, the sample size required to detect an overall 2-fold increase in risk (assuming a co-dominant model with 1 degree of freedom) with 90% power, false positive rate of 5%, disease prevalence of 7%, disease allele frequency of 5%, and assuming a complete LD between the genotyped marker and the causative SNP ($r^2 = 1.0$) is 800 cases and 800 controls. When parameters are changed, for example, reducing the genotype relative risk from 2-fold to 1.7-fold in the same study, increases the required sample size from 800 to 1400 cases and controls. Software packages are available for such calculations such as the "Power for Genetic Association Analyses" (PGA) package (http://dceg.cancer.gov/bb/tools/pga).

While the importance of power in association studies is certainly recognised, it is acknowledged that the SNP association studies carried out here are not of statistically sufficient power to detect a true causal variant. These analyses were carried out as a pilot study, restrained by patient and control sample numbers and cost, with the hope of potentially revealing rare variants of very high effect, where power would be strengthened (Spencer et al. 2009).
These preliminary findings highlighted some SNPs that were not associated, to any great extent, with an increased or decreased risk as well as others that showed a trend towards being involved with LQTS or modifying the effects of known mutations. It is possible, however, that genuinely associated SNPs, of moderate effect, remained undetected in these analyses owing to low power. It would be worth carrying out similar studies in much larger cohorts.

6.2 LQTS SNPs in patients with post myocardial infarction (MI)

In addition to the SNP studies described in the previous section, we embarked on a collaborative study with the Christchurch Cardioendocrine Research Group, University of Otago, Christchurch, to study allele frequencies of SNPs in LQTS-related genes in a cohort of patients with post myocardial infarction. Patients with an acute myocardial infarction (AMI) are at high risk to develop ischemia-induced ventricular arrhythmias, leading to sudden cardiac death in about one third of all AMI patients. The individual susceptibility to ischemia-induced arrhythmias may be modified by polymorphisms in genes encoding ion channels. We propose that certain LQTS alleles predispose to sudden death, and that it may be possible to predict in advance of an adverse outcome, those at greatest risk of sudden death.

6.2.1 Patients

Approximately 1300 patients were recruited as part of this study, with a mean follow-up time post myocardial infarction of five years, with approximately 30 patients dying since recruitment as a result of ventricular failure. Approximately 500ng of genomic DNA was extracted from patients recruited as part of the Acute Coronary Syndromes Study (ACS), collected by the Christchurch Cardioendocrine Research Group. From July 2002, patients
admitted to either Christchurch Hospital or Auckland City Hospital were recruited into the study using the following inclusion criteria: Ischaemic discomfort plus one or more of, ECG changes (ST segment depression or elevation of at least 0.5mm, T-wave inversion of at least 3mm in at least 3 leads, or left bundle branch block), elevated levels of cardiac markers, or a history of coronary disease. The investigation and recruitment was approved by the New Zealand Multi-region Ethics Committee. Each participating patient provided written, informed consent.

The mean age at first time of admission was 67 years and 70% of participants were male. In this cohort, 74% of patients self-identified as being of European descent (2 or more European grandparents), 6% self-identified as being of Maori/Pacific Island ancestry (1 or more grandparent of Maori or Pacific Island ancestry), 3% were reported to be of Asian ancestry (1 or more Asian grandparent). Thirty-two patients subsequently died due to cardiac arrest and ventricular failure. Those along with other baseline characteristics are shown in Table 6.4.

<table>
<thead>
<tr>
<th>Table 6.4 Baseline characteristics of ACS study patients.</th>
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<tbody>
<tr>
<td><strong>Number of patients</strong></td>
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<td>ACS patients</td>
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<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
</tr>
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</tr>
<tr>
<td>Asian</td>
</tr>
<tr>
<td>Maori/Pacific Island</td>
</tr>
<tr>
<td>BMI(^1) (kg/m(^2))</td>
</tr>
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</tr>
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</tr>
<tr>
<td>(\beta)-blocker treated</td>
</tr>
<tr>
<td>Cardiac arrest or VF(^3)</td>
</tr>
</tbody>
</table>

\(^1\)BMI – body mass index, \(^2\)CHD – coronary heart disease, \(^3\)VF – ventricular failure
6.2.2 SNP genotyping

Twenty SNPs in LQTS or arrhythmia-related genes were selected for genotyping in the patients recruited as part of the Acute Coronary Syndromes Study (ACS), described above (Table 6.5). In the previous section, SNPs in LQTS genes in patients with inherited cardiac disorders, in sudden explained death victims and within a cohort of normal controls were screened using Applied Biosystems TaqMan assays. As part of this study and screening program, a number of common and rare SNPs were identified within the New Zealand population. Some of these have previously been identified by other groups, and although not classed as pathogenic mutations, have been associated with a functional change in the protein by altering the electrophysiological response. These SNPs were included for screening in this cohort as well as others from the Inherited Arrhythmia Database that are relatively common and occur in ethnic groups that might be represented in the New Zealand population. Patient DNA aliquots were sent to the Australian Genome Research Facility (AGRF) for genotyping by Sequenom MassArray on an Autoflex Spectrometer using iPLEX GOLD chemistry. Twenty SNP assays were developed and the genotyping pass rate was 94.3%. The summary results from AGRF are presented in Table 6.6. For each SNP, the difference between the observed and expected allele frequencies was tested using Chi-Square analysis, with no significant difference indicating that the population was in Hardy-Weinberg equilibrium.

6.2.3 Data analysis

The statistical analysis was carried out by statistician, Eliza Chan, in the Department of Obstetrics and Gynaecology, University of Auckland. Three questions were addressed: 1) Do any of the 20 SNPs have statistically different genotype frequencies in the group who died post MI from cardiac arrest and ventricular failure compared to the group still living post MI? 2) Do the genotype distributions differ significantly among self-proclaimed ethnic
groups (Asian, European, Maori, Middle Eastern and Pacific Island)? 3) Do any of the 20 SNPs have statistically different genotype frequencies in patients with a higher QTc interval (male QTc ≥ 460ms, female QTc ≥ 470ms) opposed to patients with a lower QTc interval (male QTc < 460ms, female QTc < 470ms)? Fisher’s exact test and Chi-Square tests were carried out. The results are summarised in Table 6.7. SNPs rs3815459 (intronic SNP in KCNH2) and rs4074536 (p.T66A in exon 1 CASQ2) are significantly different in genotype frequencies in patients who have died post MI from cardiac arrest and ventricular failure compared to those still living. However, when the Bonferroni correction is applied to adjust for multiple comparisons of 20 SNPs, by dividing the P value by the number of SNPs tested, the significance at 0.05, becomes 0.0025. This means that a SNP is said to be significant between groups only if a p-value is <0.0025. These two SNPS are, therefore, not significant after correcting for multiple comparison. CASQ2 SNPs rs10801999 and rs4074536, and SCN5A SNP rs41261344 are significantly different among ethnicities (p<0.0025). No SNP is significantly different between high and low QTc values (Table 6.7). SCN5A SNP rs7626962 was excluded from the table as all samples were homozygote for the ‘G’ allele.

In addition to the questions addressed, above, the QTc values for the group who had cardiac arrest and VF were compared to the remaining patients. The QTc distribution data between the two groups is given in Table 6.8 and illustrated in Figure 6.20. Although their differences were not statistically significant, the patients who suffered cardiac arrest and VF had QTc distributions skewed to the upper end of the range. It is possible that this observation could be due to administered drugs but this pharmaceutical information is not available.
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<td>A2691C</td>
<td>0.804</td>
<td>0.196</td>
<td>0.114</td>
<td>0.735</td>
</tr>
<tr>
<td></td>
<td>rs36210421</td>
<td>95%</td>
<td>1241</td>
<td>G3140T</td>
<td>0.965</td>
<td>0.035</td>
<td>5.138</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td>rs3815459</td>
<td>91%</td>
<td>1188</td>
<td>G/A</td>
<td>0.727</td>
<td>0.273</td>
<td>7.431</td>
<td>0.076</td>
</tr>
<tr>
<td>SCN5A</td>
<td>rs1805124</td>
<td>95%</td>
<td>1231</td>
<td>A1673G</td>
<td>0.768</td>
<td>0.232</td>
<td>1.251</td>
<td>0.263</td>
</tr>
<tr>
<td></td>
<td>rs7626962</td>
<td>97%</td>
<td>1264</td>
<td>C3305A</td>
<td>1.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.989</td>
</tr>
<tr>
<td></td>
<td>rs41261344</td>
<td>97%</td>
<td>1257</td>
<td>G3578A</td>
<td>0.993</td>
<td>0.007</td>
<td>0.065</td>
<td>0.798</td>
</tr>
<tr>
<td></td>
<td>rs11129795</td>
<td>92%</td>
<td>1198</td>
<td>A/G IVS1-3208</td>
<td>0.755</td>
<td>0.245</td>
<td>0.697</td>
<td>0.404</td>
</tr>
<tr>
<td>KCNE1</td>
<td>rs1805127</td>
<td>91%</td>
<td>1187</td>
<td>A112G</td>
<td>0.665</td>
<td>0.335</td>
<td>0.859</td>
<td>0.354</td>
</tr>
<tr>
<td></td>
<td>rs1805128</td>
<td>97%</td>
<td>1261</td>
<td>G253A</td>
<td>0.988</td>
<td>0.012</td>
<td>0.183</td>
<td>0.669</td>
</tr>
<tr>
<td>KCNE2</td>
<td>rs2234916</td>
<td>98%</td>
<td>1269</td>
<td>A/G</td>
<td>0.993</td>
<td>0.007</td>
<td>0.065</td>
<td>0.799</td>
</tr>
<tr>
<td>KCNJ11</td>
<td>rs5219</td>
<td>92%</td>
<td>1199</td>
<td>G/A</td>
<td>0.634</td>
<td>0.366</td>
<td>0.047</td>
<td>0.829</td>
</tr>
<tr>
<td></td>
<td>rs5215</td>
<td>93%</td>
<td>1212</td>
<td>A/G</td>
<td>0.632</td>
<td>0.368</td>
<td>0.004</td>
<td>0.952</td>
</tr>
<tr>
<td></td>
<td>rs1800467</td>
<td>97%</td>
<td>1263</td>
<td>G/C</td>
<td>0.958</td>
<td>0.042</td>
<td>0.025</td>
<td>0.875</td>
</tr>
<tr>
<td>CASQ2</td>
<td>rs4074536</td>
<td>92%</td>
<td>1197</td>
<td>A/G</td>
<td>0.697</td>
<td>0.303</td>
<td>0.022</td>
<td>0.882</td>
</tr>
<tr>
<td></td>
<td>rs10801999</td>
<td>97%</td>
<td>1263</td>
<td>G/A</td>
<td>0.999</td>
<td>0.001</td>
<td>0.002</td>
<td>0.966</td>
</tr>
<tr>
<td>RYR2</td>
<td>rs3766871</td>
<td>97%</td>
<td>1257</td>
<td>A/G</td>
<td>0.972</td>
<td>0.028</td>
<td>1.031</td>
<td>0.310</td>
</tr>
<tr>
<td>KCNJ2</td>
<td>rs236514</td>
<td>94%</td>
<td>1228</td>
<td>A/G</td>
<td>0.584</td>
<td>0.416</td>
<td>0.002</td>
<td>0.966</td>
</tr>
</tbody>
</table>
Table 6.7 Post MI study data analysis summary. To adjust for multiple comparisons (Bonferroni correction) of 20 SNPs, the P value needs to be divided by the number of SNPs tested (20): the significance at 0.05, becomes 0.0025. P values < 0.0025 are significant between groups.

<table>
<thead>
<tr>
<th>LQTS Gene</th>
<th>dbSNP ID</th>
<th>Cardiac arrest/non cardiac arrest genotype frequency comparison (p value)</th>
<th>Ethnicity genotype comparison (p value)</th>
<th>High/low QTc value comparison (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNQ1</td>
<td>rs1800172</td>
<td>0.8504</td>
<td>0.9989</td>
<td>0.4459</td>
</tr>
<tr>
<td></td>
<td>rs7945327</td>
<td>0.8000</td>
<td>0.9358</td>
<td>0.7826</td>
</tr>
<tr>
<td></td>
<td>rs757092</td>
<td>0.5257</td>
<td>0.3733</td>
<td>0.5239</td>
</tr>
<tr>
<td>KCNH2</td>
<td>rs1805123</td>
<td>0.8473</td>
<td>0.6967</td>
<td>0.2525</td>
</tr>
<tr>
<td></td>
<td>rs36210421</td>
<td>0.0590</td>
<td>0.9694</td>
<td>0.7165</td>
</tr>
<tr>
<td></td>
<td>rs3815459</td>
<td>0.0095</td>
<td>0.0254</td>
<td>0.4724</td>
</tr>
<tr>
<td>SCN5A</td>
<td>rs1805124</td>
<td>0.0789</td>
<td>0.4928</td>
<td>0.8508</td>
</tr>
<tr>
<td></td>
<td>rs41261344</td>
<td>0.7074</td>
<td>0.0001</td>
<td>0.5809</td>
</tr>
<tr>
<td></td>
<td>rs11129795</td>
<td>0.8816</td>
<td>0.1025</td>
<td>0.8858</td>
</tr>
<tr>
<td>KCNE1</td>
<td>rs1805127</td>
<td>0.6751</td>
<td>0.0280</td>
<td>0.3492</td>
</tr>
<tr>
<td></td>
<td>rs1805128</td>
<td>0.3737</td>
<td>0.7657</td>
<td>0.3501</td>
</tr>
<tr>
<td>KCNE2</td>
<td>rs2234916</td>
<td>0.4242</td>
<td>0.5901</td>
<td>0.2397</td>
</tr>
<tr>
<td>KCNJ1</td>
<td>rs5219</td>
<td>0.6656</td>
<td>0.7847</td>
<td>0.2046</td>
</tr>
<tr>
<td></td>
<td>rs5215</td>
<td>0.6972</td>
<td>0.8370</td>
<td>0.1087</td>
</tr>
<tr>
<td></td>
<td>rs1800467</td>
<td>0.4669</td>
<td>0.8430</td>
<td>0.5493</td>
</tr>
<tr>
<td>CASQ2</td>
<td>rs4074536</td>
<td>0.0251</td>
<td>0.0004</td>
<td>0.6695</td>
</tr>
<tr>
<td></td>
<td>rs10801999</td>
<td>0.7453</td>
<td>0.0005</td>
<td>0.9030</td>
</tr>
<tr>
<td>RYR2</td>
<td>rs3766871</td>
<td>0.2019</td>
<td>0.0151</td>
<td>0.7281</td>
</tr>
<tr>
<td>KCNJ2</td>
<td>rs236514</td>
<td>0.2631</td>
<td>0.1797</td>
<td>0.6533</td>
</tr>
</tbody>
</table>

*High QTc - male QTc > 460ms, female QTc > 470ms; low QTc - male QTc < 460ms, female QTc < 470ms
Table 6.8 QTc distribution data.

<table>
<thead>
<tr>
<th></th>
<th>Cardiac arrest patients</th>
<th>Non-cardiac arrest patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>32</td>
<td>873</td>
</tr>
<tr>
<td>Mean</td>
<td>425.63</td>
<td>426.15</td>
</tr>
<tr>
<td>Median</td>
<td>426</td>
<td>424</td>
</tr>
<tr>
<td>Std deviation</td>
<td>90.21</td>
<td>40.41</td>
</tr>
<tr>
<td>Std error mean</td>
<td>15.95</td>
<td>1.37</td>
</tr>
<tr>
<td>Range</td>
<td>380-598</td>
<td>340-561</td>
</tr>
</tbody>
</table>

Once again, consideration needs to be given to the power of these statistical analyses. Given the relatively small sample sizes of the cardiac arrest/VF deaths (n=33) and certain ethnic groups (Middle Eastern n=1, Pacific Island n=15), these analyses are underpowered. For future studies investigating the effect of arrhythmia gene-related SNPs, meta analyses from several collaborative groups should be considered to obtain levels of sufficient power.

Figure 6.20 The distribution of QTc interval values between patients who suffered cardiac arrest and ventricular failure (VF) post myocardial infarction (MI) and all post MI patients.
CHAPTER 7:

CONCLUDING REMARKS
7 Concluding remarks

There are several challenges facing the molecular diagnostics of inherited cardiac disorders, probably most important is correlating a specific genetic variant to the clinical phenotype, or in other words establishing the molecular genetic cause of observed clinical symptoms. The clinical manifestations of arrhythmia syndromes are highly variable, even among the carriers of the same mutation (Schwartz et al. 2000). However, analysis of data on genotype-screened mutation positive patients indicates that some gene-specific differences in some aspects of phenotype do exist, such as ECG pattern, the triggers for cardiac events, and age of onset. The identification and characterisation of genetic variants in arrhythmia syndromes is improving our understanding of arrhythmogenesis, and this will ultimately result in better therapy strategies and management of patients.

The work carried out in this study formed part of the research arm of the CIDG in New Zealand. The original aim of this part of the research was to complete and assess the genetic screening programme that had been established in a research setting to allow for its successful transferral to a clinical diagnostic setting at LabPlus, ADHB. The mutation screening revealed a mutation detection rate slightly lower than previous reports. This is likely to depend on the screening criteria and on the degree of pretest clinical suspicion (Tester et al. 2005).

Allelic heterogeneity makes LQTS gene mutation screening an expensive and challenging process. Hierarchical or genotype/phenotype gene screening approaches could be considered. However, these are not recommended as limitations include the possibility of
missing compound heterozygotes and screening for the incorrect gene due to lack of concordance between genotype and phenotype.

An effective mutation detection technique should be accurate, cost effective, high throughput, sensitive, provide precise information about the nature and position of the detected mutation and have a reasonable read length (Mashal and Sklar, 1996). Although dHPLC is highly sensitive at detecting variants in amenable temperature domains, variants can be missed in regions of the amplicon that have a low or high melting domain (Yu et al 2006). DNA sequencing is high throughput, sensitive and provides precise information regarding the nature and position of the variation. With continuously reducing costs, direct DNA sequencing, is replacing many of the two-phased molecular screening strategies, mentioned above. It is only a matter of time before next generation high-throughput sequencing technologies that parallelise the sequencing process, producing thousands or millions of sequences at once, will be used (Chen et al. 2010). High-throughput sequencing technologies are intended to lower the cost of DNA sequencing beyond what is possible with standard dye-terminator methods.

New born screening Guthri cards can be used as an effective back up source of DNA for this type of mutation screen. Although the methodology involved was more labour-intensive, results could be successfully obtained.

Issues remain around the problem of interpreting novel or rare missense mutations in LQTS (and in other disorders, most notably familial cancer). Approaches that have been taken have included:

1. Genetic methods
   Segregation analysis of variants through large families
Screening control populations

Assessing phenotypic and family history

2. Molecular methods

*In silico* programmes that compare the properties of amino acids

Evolutionary conservation

3. Functional analysis of biophysical properties.

Often large cohorts for population studies or specialised electrophysiology and primary cardiac cultures from animal models are unavailable in a genetic diagnostic setting. Therefore, the value of multidisciplinary interaction in interpreting the significance of these variants becomes clear.

It is important to continue investigating cases, which remain gene negative. Synonymous or “silent” (non–amino acid altering) single-nucleotide substitutions residing within the exons could conceivably disrupt splice enhancer element sequences precipitating errant RNA splicing and subsequent exon skipping or intron inclusion. Such a mechanism has already been reported for diseases like Marfan syndrome, familial adenomatous polyposis, and McArdle disease (Liu et al. 1997; Montera et al. 2001; Fernandez-Cadenas et al. 2003). Epigenetic effects on gene function that result in transcriptional silencing of the gene could be investigated. Patients with disorders that present similarly to LQTS but have different genetic causes might also account for a substantial portion of LQTS gene-negative patients.

Large cohort genome wide association studies should continue to provide information on the modifying effects of common SNPs. Despite the most common arrhythmia genes being linked to the cardiac action potential or sarcomeric function, there is also the possibility that arrhythmia in the wider population is a complex disorder where
environment interacts with a polygenic susceptibility (Arking et al. 2006; Pfeufer 2007). New candidate genes for arrhythmia will soon become apparent and are likely to involve genes that modulate ion channel function and genes that influence the quantitative multifactorial basis of the cardiac cycle intervals, the action potential kinetics, response to stimuli and myocardial dynamics.

Next-generation sequencing is currently revolutionising medical genetics through rapid and accurate assessment of a patient’s DNA sequence with minimal cost. These technologies have already led to the identification of novel mutations, however, several challenges lie ahead. The implementation of next-generation sequencing into routine molecular diagnostic practice requires in-depth understanding of the pitfalls of these technologies and a great degree of bioinformatic expertise.

There are several competing sequencing companies offering different next generation sequencing technologies focussed largely on the research market at present; Roche, Life Technologies, Illumina, Helicos and Pacific Biosciences. Most technologies established in the diagnostic market had their beginnings in research, where they were tested, improved and ultimately accepted. It is, therefore, only a matter of time before next generation sequencing is used routinely in molecular diagnostics. As it happens, one of the very few next-generation sequencing-based tests available today has been developed by GeneDx, a molecular diagnostic company based in Maryland, USA. They run a next generation sequencing panel for LQTS1-12 on Illumina’s GA platform.

In New Zealand, we should be working towards a similar molecular diagnostic approach for LQTS and other inherited cardiac arrhythmia disorders: next generation sequencing of all implicated LQTS genes and a high resolution microarray-based deletion/duplication
detection technology, followed by, our already established, multidisciplinary data interpretation team within the CIDG.

Even without next generation sequencing, advances in molecular diagnosis and mutation detection lead to an increasing number of genotype-positive patients being identified, rendering the multidisciplinary approach to data interpretation vital. LQTS is an incredible heterogeneous disorder with clinical symptoms ranging from sudden cardiac death or several cardiac episodes in childhood to asymptomatic longevity. It also has extreme genetic heterogeneity. It is, therefore, not uncommon to obtain a genotype-positive but phenotype-negative result for a patient or indeed for more than one potentially pathogenic variant to be identified in a patient. It is in these cases where extreme care needs to be taken in translating the molecular diagnostic result to practical patient care and management. Within the forum of the CIDG, these decisions are currently assessed on a case to case basis, where the likelihood of pathogenicity of the variant/s is/are assessed by several criteria. These include clinical phenotype of patient, co-segregation of the variant with disease in families, absence of the variant/s in healthy control individuals, conservation of the amino acid residue among species, in silico prediction tools, structural modelling and in vitro functional studies.

The improvements in technology in molecular diagnostics; such as the routine use of next generation sequencing, high density dosage array technology, and possibly epigenetic testing for remaining gene-negative patients; along with the round table approach to data interpretation will allow the delivery of best-possible patient care and reduce the incidence of sudden cardiac death due to inherited arrhythmia disorders.
APPENDIX
8 Appendix

8.1 Buffers and solutions

1M MgCl2

MgCl2  101.66g

dH2O  Make up to 500mL

Note: Autoclave to sterilize.

Ethylenediamine Tetra-acetic Acid (EDTA) 0.5M (pH 8.0)

Na2EDTA.2H2O  93.06g

H2O  Make up to 500mL

10M NaOH  -50mL

Note: Dissolve Na2EDTA.2H2O in water, salt of EDTA will not go into solution until the pH of the solution is adjusted to pH 8.0, by the addition of NaOH. Make up to 1 litre with H2O. Autoclave to sterilize.

1M Tris-HCl (pH 8.0)

Tris-HCl  121.0g

H2O  Make up to 1L

Note: Autoclave to sterilize.

10% SDS

SDS  10g

dH2O  100mL (autoclaved)
Saturated NaCl

NaCl * 40g
dH2O 100mL (autoclaved)

* Slowly add NaCl until absolutely saturated. Some NaCl will precipitate out. Note: Before use, agitate and let NaCl settle, use clear supernatant.

Sucrose-Triton-X-Lysing buffer

1M Tris-HCl pH8 10mL
1M MgCl2 5mL
Triton-X 100 10mL
dH2O Make up to 1L
Sucrose 5.0g (prior to use)

Note: Autoclave (if made fresh can be used without autoclaving). Keep solution chilled at 40C. Do not keep longer than 1 day.

Proteinase-K Mixture

10% SDS 400μL
0.5M EDTA pH8 16μL
dH2O 2.8mL (autoclaved)

Proteinase-K 10mg ml-1 stock 88μL (prior to use)
Note: Solution for 8 extractions.

Proteinase-K TE Mix

T20E5 52.2mL
10% SDS 4.0mL

Proteinase-K 10mg ml-1 stock 1.6mL
Note: Add 3.2ml per sample. Solution for 16 extractions.
20mM Tris 5mM EDTA (T20E5)

1M Tris-HCl pH8 20mL
0.5M EDTA pH8 10mL
Triton-X 100 10mL
dH2O Make up to 1L

Note: Autoclave to sterilize.

1X Tris EDTA buffer (TE buffer)

1M Tris-HCl pH8 10mL
0.5M EDTA pH8 2mL
dH2O Make up to 1L

Note: Autoclave to sterilize.

Agarose gel (1.5% or 2.0)%

1X TAE buffer 100mL
Agarose 1.5g or 2.0g

Note: Make up agarose gel to desired percentage. Melt in microwave until fully dissolved; allow to cool before pouring to case.

TAE Buffer 50x

Tris 242.0g
Na2EDTA 37.2g
Glacial acetic acid 57.1mL
H2O Adjust to 1L

Note 1: Glacial acetic acid at final concentration of 40mM.
Note 2: Na2EDTA final concentration of 2mM.
TAE Buffer 1x

TAE 50x 100mL
H2O 4900mL

dNTPs (dTTP, dCTP, dGTP, and dGTP) 5mM

dATP 50μL
dTTP 50μL
dCTP 50μL
dGTP 50μL
H2O 800μL

Ethidium Bromide Solution 10mg/mL

Ethidium Bromide 0.2g
H2O 20mL

Note: Dissolve ethidium bromide in water, mix well and store at 40°C in the dark or in a foil wrapped bottle.

Ethidium Bromide working stock 0.5μg/mL

Ethidium Bromide 10mg ml-1 50μL
TAE 1000mL

Loading Buffer

10% Bromophenol Blue Solution

Bromophenol Blue 100mg
H2O 1mL

Note: Weigh using a precision balance, mix well and store at 40°C. Use in preparation of stop and loading buffers. Mix directly before use.
**Loading Buffer**

**Xylene Cyanol Solution**

- Xylene cyanol 0.25% (w/v)
- Glycerol 30% (v/v)
- H2O To volume

Note: Store for use at 4°C. Used as a loading buffer in PCR reactions. Xylene cyanol runs 800bp on a 2% agarose gel.

**6X Gel Loading Buffer**

- Bromophenol Blue 0.25% (w/v)
- 10% Bromophenol Blue Solution 125μL
- Glycerol 30% (w/v) or 1.5mL
- H2O 5mL

Note: Store for use at 4°C. Used as a loading buffer in PCR reactions. Bromophenol blue runs at 100bp on a 2% agarose gel.

**1Kb plus Ladder**

- Marker DNA 50mL
- Loading Dye stock 100μL
- Glycerol 100μL
- H2O 759μL

Note: In order to quantify the 1650bp band at 25ng, 6μL of the ladder must be run.

**Ampicillin 50mg/mL**

- Ampicillin powder 1.0g
- H2O 20.0mL

Note: Dissolve ampicillin and filter sterilize through a 0.22μm filter. Store in 1.0mL aliquots at -200°C.
Ampicillin 50μg/mL

Add 1μL of 50mg/mL per mL of solution.

Glycerol stocks

Auto clave 40% glycerol in LB broth

**LB broth with cells** 0.5mL

40% glycerol in LB broth 0.5mL.

Note: Final concentration of glycerol is 20%

SOC medium

Tryptone 2.0g

Yeast extract 0.5g

NaCl 1M 1.0mL

KCl 1M 0.25mL

H2O 97.0mL

Note: Stir to dissolve and autoclave and allow to cool to room temperature

Mg2+ 2M 1.0mL

Glucose 2M 1.0mL

Note 1: Mg2+ and glucose to a final concentration of 20mM.
Note 2: Filter the complete medium through a 0.2μm filter and adjust to pH 7.0.

**1M NaOH**

NaOH 40.0g

H2O 1L

Note: Make up to 1 litre with H2O. Autoclave to sterilize.
**Ethlenediamine Tetra-acetic Acid (EDTA) 1.0M (pH 8.0)**

Na₂EDTA.2H₂O  372.2g

H₂O  700mL

10M NaOH  ~50mL

Note: Dissolve Na₂EDTA.2H₂O in water, salt of EDTA will not go into solution until the pH of the solution is adjusted to 8.0pH by the addition of NaOH. Make up to 1 litre with H₂O. Autoclave to sterilize.

**TE 1x**

Tris-Cl  10mMol/L (pH 8.0)

EDTA  100µMol/L (pH 8.0)

Note: Autoclave to sterilize.

**MgSO₄ 1M**

MgSO₄  .23g

H₂O  10.0mL

Note: Autoclave to sterilize.

**0.2mM MgSO₄**

MgSO₄ 1M  2.0µL

Absolute ETOH  7.0mL

H₂O  3.0mL

Note: Prepare solution fresh for each experiment.
8.2 Publications and presentations

8.2.1 Publications


8.2.2 Scientific meetings and conferences

NZPEG Scientific Meeting, Terrace Downs, Canterbury, New Zealand, 12-14th September 2008. (talk) C Eddy: Long QT genetic testing: What hope is there if sequencing reveals no diagnosis?


8.3 The standard 12 lead ECG

The standard 12-lead electrocardiogram is a representation of the heart’s electrical activity recorded from electrodes on the body surface.

![ECG diagram](image)

**Figure 8.1 ECG waves and intervals as well as standard time and voltage measures on the ECG paper**

8.3.1 ECG waves and intervals:

P wave: the sequential activation (depolarisation) of the right and left atria
QRS complex: right and left ventricular depolarisation (normally the ventricles are activated simultaneously)

ST-T wave: ventricular repolarisation

U wave: origin for this wave is not clear - but probably represents "afterdepolarisations" in the ventricles

PR interval: time interval from onset of atrial depolarisation (P wave) to onset of ventricular depolarisation (QRS complex)

QRS duration: duration of ventricular muscle depolarisation

QT interval: duration of ventricular depolarisation and repolarisation

RR interval: duration of ventricular cardiac cycle (an indicator of ventricular rate)

PP interval: duration of atrial cycle (an indicator of atrial rate)

8.3.2 Orientation of the 12 lead ECG

The 12-lead ECG provides spatial information about the heart's electrical activity in three approximately orthogonal directions (Figure 8.2):

Right <-> Left

Superior <-> Inferior

Anterior <-> Posterior

Each of the 12 leads represents a particular orientation in space, as indicated below (RA = right arm; LA = left arm, LF = left foot):

Bipolar limb leads (frontal plane):

Lead I: RA (-) to LA (+) (Right Left, or lateral)

Lead II: RA (-) to LF (+) (Superior Inferior)

Lead III: LA (-) to LF (+) (Superior Inferior)
Augmented unipolar limb leads (frontal plane):

Lead aVR: RA (+) to [LA & LF] (-) (Rightward)

Lead aVL: LA (+) to [RA & LF] (-) (Leftward)

Lead aVF: LF (+) to [RA & LA] (-) (Inferior)

Unipolar (+) chest leads (horizontal plane):

Leads V1, V2, V3: (Posterior Anterior)

Leads V4, V5, V6: (Right Left, or lateral)
b)

V1: right 4th intercostal space
V2: left 4th intercostal space
V3: halfway between V2 and V4
V4: left 5th intercostal space, mid-clavicular line
V5: horizontal to V4, anterior axillary line
V6: horizontal to V5, mid-axillary line

c)

Figure 8.2 a) Einthoven's Triangle. Each of the 6 frontal plane leads has a negative and positive orientation (as indicated by the '+' and '-' signs). Lead I (and to a lesser extent Leads aVR and aVL) are right <-> left in orientation. Also, Lead aVF (and to a lesser extent Leads II and III) are superior <-> inferior in orientation. b) Location of chest electrodes in the 4th and 5th intercostal spaces. c) A typical 12 lead ECG, made up of the three standard limb leads (I, II and III), the augmented limb leads (aVR, aVL and aVF) and the six precordial leads (V1, V2, V3, V4, V5 and V6).
8.4 PCR primer sequences

The PCR and real time PCR primer sequences and PCR conditions described in Chapter 2, Materials and Methods are listed in the tables below (Tables 8.1 - 8.8).
Table 8.1 Primers used for PCR of exons and flanking intron boundaries in the *KCNQ1* gene, chromosome 11, NC_000011.9 (2466221..2870340)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer sequence (5’ – 3’)</th>
<th>Reverse primer sequence (5’ – 3’)</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C) / additives</th>
<th>Location (start to finish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNQ1ex1</td>
<td>TGGCTTGCAGGCTGACGTC</td>
<td>CGCGGGTCTAGGCTACCC</td>
<td>332</td>
<td>60, DMSO</td>
<td>2466266 – 2466597</td>
</tr>
<tr>
<td>KCNQ1ex2</td>
<td>AACGAGACGAGGGTTGATGCT</td>
<td>AAGGACACTTGGCATCTGAGCG</td>
<td>244</td>
<td>60, DMSO</td>
<td>2466538 – 2466781</td>
</tr>
<tr>
<td>KCNQ1ex3</td>
<td>GTTCAGGTAGCTGGCTCTTGC</td>
<td>CGGCTTGGTGAAAGCTCTC</td>
<td>363</td>
<td>55</td>
<td>2496020 – 2499382</td>
</tr>
<tr>
<td>KCNQ1ex4</td>
<td>TGTTGCTGGGGTCTTTCCTG</td>
<td>CAGGACCCAGCTGTCGCCA</td>
<td>269</td>
<td>60, Q solution</td>
<td>2592475 – 2592743</td>
</tr>
<tr>
<td>KCNQ1ex5</td>
<td>GAGACACTGAGCCAGC</td>
<td>CGGCTTGGTGACCCACCTT</td>
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<td>55, Q solution</td>
<td>2593191 – 2593388</td>
</tr>
<tr>
<td>KCNQ1ex6</td>
<td>AAGGAGGGTCTGACGAGAG</td>
<td>GACAGGGTTGGAGAGAG</td>
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<td>2593983 – 2594363</td>
</tr>
<tr>
<td>KCNQ1ex7</td>
<td>TGGCTTGGCTGGCTTTCCTG</td>
<td>CAGGACCCAGCTGTCGCCA</td>
<td>284</td>
<td>60</td>
<td>2604545 – 2604828</td>
</tr>
<tr>
<td>KCNQ1ex8</td>
<td>CATACTGCTGGCTTCCACAA</td>
<td>AGGGCTGATGGCAACTTAACA</td>
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<td>TGAACAGGTCCTGAGAGG</td>
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<td>AGAACTGCTGGCTTGTGTC</td>
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</tr>
<tr>
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<td>ACTGATTGCTGGCTGGAAGGT</td>
<td>TTGCAAGAGGGTCCTATG</td>
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<td>AGGGAGGGTCTGACGAGAG</td>
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<td>55</td>
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<tr>
<td>KCNQ1ex13</td>
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<td>GTTCAGGGCTCCTCCTCCT</td>
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<td>KCNQ1ex14</td>
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<td>55</td>
<td>2798131 – 2798400</td>
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<tr>
<td>KCNQ1ex15</td>
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<td>GCTCCTCCTTCTGGGCTCTT</td>
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<td>Touch down PCR</td>
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Table 8.2 Primers used for PCR of exons and flanking intron boundaries in the *KCNE1* gene, chromosome 21, NC_000021.8 (35818988..35883613, complement)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer sequence (5’ – 3’)</th>
<th>Reverse primer sequence (5’ – 3’)</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C) / additives</th>
<th>Location (start to finish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNE1ex1</td>
<td>GGTAGGTTAGATCTGTCCGTTGG</td>
<td>GTTGCCCCGACTAGGCCACTT</td>
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<td>35831764 35832197</td>
</tr>
<tr>
<td>KCNE1ex2</td>
<td>GGAGGTTCCAGCTCCATTAG</td>
<td>GCAATGAGTCCCTCTGATGTC</td>
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<td>60</td>
<td>35830867 35831194</td>
</tr>
<tr>
<td>KCNE1ex3</td>
<td>ATTTGGGGTTGCATTTTTCTT</td>
<td>GGCAGGATGTGTCAGTITTTA</td>
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<td>35821508 35822008</td>
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Table 8.3 Primers used for PCR of exons and flanking intron boundaries in the *KCNH2* gene, chromosome 7, NC_000007.13 (150642049..150675014, complement)

<table>
<thead>
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<th>Primer</th>
<th>Forward primer sequence (5’→3’)</th>
<th>Reverse primer sequence (5’→3’)</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C) / additives</th>
<th>Location (start to finish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNH2ex1</td>
<td>CCACCCGAAGGCTAGTGCTG</td>
<td>GTCCCCCTCGCCAAAGCCT</td>
<td>299</td>
<td>64, DMSO</td>
<td>150674778 150675076</td>
</tr>
<tr>
<td>KCNH2ex2</td>
<td>GAGAATGTGGGGAAGGGCTG</td>
<td>CTCTTGACCCCAGCCCCCTG</td>
<td>354</td>
<td>55, Q solution</td>
<td>150671752 150672105</td>
</tr>
<tr>
<td>KCNH2ex3</td>
<td>CCACCTGCTGGTGCCCAAG</td>
<td>AATGAGACACGAACCCGCTTAGG</td>
<td>256</td>
<td>60</td>
<td>150656613 150656868</td>
</tr>
<tr>
<td>KCNH2ex4</td>
<td>CCATTTCCAGGCCTTGC</td>
<td>CAGAAGAAGCGTGAGGGCTG</td>
<td>563</td>
<td>60, DMSO</td>
<td>150655100 150655662</td>
</tr>
<tr>
<td>KCNH2ex5</td>
<td>CTGATCTATGTTGAGCTCGT</td>
<td>CCCCTCCAAGCCTCCGCA</td>
<td>337</td>
<td>55</td>
<td>150653426 150654662</td>
</tr>
<tr>
<td>KCNH2ex6</td>
<td>GTCACCATGGCCTGCTCA</td>
<td>CTGGCCCTCTCTCCTCCTCCTCAC</td>
<td>521</td>
<td>55, Q solution</td>
<td>150649467 150649987</td>
</tr>
<tr>
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<td>CTTGCCCATCAACGGGAAT</td>
<td>TCCTCAACTTTTGCTTCCTCC</td>
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<td>55, Q solution</td>
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<tr>
<td>KCNH2ex8</td>
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<td>ACTGTAGCCGCTCGAGACTT</td>
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<td>60, Q solution</td>
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<tr>
<td>KCNH2ex9</td>
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<td>AGCCCGATGACTGCAATATT</td>
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<td>58, Q solution</td>
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<tr>
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<td>CAGCTGAGACAGAGGAGGATG</td>
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<td>60, Q solution</td>
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<tr>
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<td>TGCTCCCCAAGCAGCTGAA</td>
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<td>KCNH2ex13</td>
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<td>58, Q solution</td>
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<tr>
<td>KCNH2ex14</td>
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<td>58, Q solution</td>
<td>150643922 150644200</td>
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<td>KCNH2ex15</td>
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Table 8.4 Primers used for PCR of exons and flanking intron boundaries in the **KCNE2** gene, chromosome 21, NC_000021.8 (35736323..35743440)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer sequence (5’ – 3’)</th>
<th>Reverse primer sequence (5’ – 3’)</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C) / additives</th>
<th>Location (start to finish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNE2ex1</td>
<td>TGTGGGTTCAAAAGCATAGG</td>
<td>TCATTTCCTGAAAAGGCACA</td>
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<td>60</td>
<td>35736267 35736527</td>
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<tr>
<td>KCNE2ex2</td>
<td>TCCCTCCCACCTTTACATAG</td>
<td>CTTGCATCTTTACATGTCTGG</td>
<td>521</td>
<td>60</td>
<td>35742684 35743204</td>
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<td>KCNE2 3’UTR</td>
<td>TGTAGAGGACTGGCAGGAAPA</td>
<td>CTTTCTATCCCACAGCCACT</td>
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Table 8.5 Primers used for PCR of exons and flanking intron boundaries in the **SCN5A** gene, chromosome 3, NC_00003.11 (38589553..38691163, complement)

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<th>Reverse primer sequence (5’ – 3’)</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C) / additives</th>
<th>Location (start to finish)</th>
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<td>CTCCTTCGCCCTGCTCATT</td>
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<td>38674451 38674913</td>
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<tr>
<td>SCN5Aex3</td>
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<td>60</td>
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<tr>
<td>SCN5Aex4</td>
<td>GGTAGCAGCTGCTGCTGAGTGT</td>
<td>CCTGACACACAAGCCCCCTTC</td>
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<tr>
<td>SCN5Aex5</td>
<td>TGACACCTGTAAGGACACATGG</td>
<td>ATGTGGACTGCGAAGGGAGAAGC</td>
<td>324</td>
<td>55</td>
<td>38662216 38662539</td>
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<tr>
<td>SCN5Aex6</td>
<td>CTTTCTCCCCCTCTGACTGTGTT</td>
<td>GGTATTCTGGTGACACAGCCATT</td>
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<td>SCN5Aex7</td>
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<td>Exon</td>
<td>DNA Sequence</td>
<td>Protein Sequence</td>
<td>Start</td>
<td>End</td>
<td>Length</td>
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<tr>
<td>3</td>
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<td>GGAAGCGCAGAGAGCATGAGACACC</td>
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<td>5</td>
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<td>60</td>
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<td>AGCCATTCACAACATATACAGTCTTGG</td>
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<td>565</td>
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</table>
Table 8.6 Primers used for PCR of exons and flanking intron boundaries in the RYR2 gene, chromosome 1, NC_000001.10 (237205702..237997288)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer sequence (5’ – 3’)</th>
<th>Reverse primer sequence (5’ – 3’)</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C) / additives</th>
<th>Location (start to finish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RYR2ex3</td>
<td>GCTGGGTCTGGATGCTTGA</td>
<td>GTTCCTATAGTGACTGAGC</td>
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<td>58, Q solution</td>
<td>237494118 – 237494341</td>
</tr>
<tr>
<td>RYR2ex8</td>
<td>TTGTGTGTGGGAAATCATT</td>
<td>GTCAATTAGCATATCAGCA</td>
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<td>58</td>
<td>237540539 – 237540782</td>
</tr>
<tr>
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<td>AGTACACATCTCCCTAACT</td>
<td>TAACGTTTCTGCTGACTTA</td>
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<td>58</td>
<td>237608628 – 237608957</td>
</tr>
<tr>
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</tr>
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<td>TGTTCATCAGGGAAACAGGG</td>
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<td>60, Q solution</td>
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<tr>
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<td>263</td>
<td>58, Q solution</td>
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<td>TTCCATTGAAAGTGCTTGAG</td>
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<td>RYR2ex46</td>
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<td>GATCAGCTGAGGCTTGAG</td>
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RYR2ex48 GAGATTCGAAATTCGAGATTTCC TAGGAGATGGGAGATGGTTGG 309 60 237806850 237806850
RYR2ex49 ACAGCCAATTGACACAAAAAT ACCATGGCTTACCTGAAAA 349 60 237811707 237812055
RYR2ex50 CATGTTAACCTCCCTTAAG AGGCCACATTGAAATATTCTG 354 58 237813107 237813460
RYR2ex51 AGAAACATTTCCTTGGCATTATGA AAAGTCAGAGAAGCGAGTAATCAA 186 60 237814667 237814852
RYR2ex59 CTATGCTGTGTTCTTGTCTGTA TCATTGAATTCTATTCTAGCTTT 251 58 237837363 237837613
RYR2ex83 AATGGAAAGCCTGGTTTTGGT AACCCTGAATTGAGAACCDDC 205 58 237922989 237923193
RYR2ex84 TGCTCTTCTGAGCAAAAGAAA GAAGGGACTGAGTAATCCTGCTG 182 60, Q solution 237924188 237924369
RYR2ex87 CTTCCTCAGGATAAAGTCCAAG TGCTACAGATAGCAGCCATGTT 250 62, Q solution 237936732 237936981
RYR2ex88 GAAGGAATAATCGCTTTTG AATGAGGAATGCGCAATTGGTAC 300 60 237941930 237942299
RYR2ex89 TCCTGCTGATAGTATTTCCTGC TCCATAAGCCCATGCTGATC 203 89 237944812 237945014
RYR2ex90a GGGACATATCCTTGATTCAGATG TTTCATTCTCATCCGTCTCC 375 60 237946867 237947241
RYR2ex90b GAGCCAATAAGCAGATACGC ATAGACCCCTCCTGAATGCGTT 289 60 237947171 237947459
RYR2ex90c ATTAGCAGAGAGCCTGCTGTA TATGCTGCACTTGCTGCTGTT 293 58 237947372 237947664
RYR2ex90d GGACACCATCTTGTGAAATGC CATGTCCTCGAGCTGATCT 256 60 237947594 237947849
RYR2ex90e GGGACAGACTCTTCTGATTCGAA GGGCAATCGACTCTTAACCTC 255 60, Q solution 237947798 237948052
RYR2ex90f AACATGCGGACTGCTACAT CAGAGGATAATGCTGCTGAC 300 60, Q solution 237948009 237948308
RYR2ex91 AATTCATTCAAAGGTGATGG GATGACCAAGAAGAGATG 193 58 237949211 237949403
RYR2ex92 CCACAACCCCGTTTAGTTC AGGGAGAGTGCTGCAACAT 299 60 237951224 237951522
RYR2ex93 AGGTTTCAAGCTCTGTGATTTC GCCTAGGCACCAGTATTCCA 273 60 237954676 237954948
RYR2ex94 TGCTTTTTGCTTGGCAATGTT CAGTACCTCCTGGCTCTACT 313 60 237955339 237955651
RYR2ex95 TGACACAAAGATATGCGACTAA ACAATCCTGCAGAATCTCCA 274 60 237957106 237957379
RYR2ex96 TCAATGTAAGTTTACCTGGCAG AATGGAGACATTAATAGCCAG 294 60 237958460 237958753
RYR2ex97 AATGGTTGAAGCGCAAACAA GGAAGGAAATCAATGCGAC 265 60 237961296 237961560
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<th>Exon</th>
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<th>Genomic Coordinates</th>
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<td>CTGTACATTGTCATTTGAGG</td>
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<td>TCAGCTCTAGTGGAGGAGAT</td>
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Table 8.7 Optimal partial denaturing temperatures for dHPLC analysis of LQTS genes KCNQ1, KCNH2, SCN5A, KCNE1 and KCNE2, determined using interpretation of the DNA melting properties by the Navigator version 1.5.1 software.

<table>
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<th>Gene (exon)</th>
<th>dHPLC temperatures (°C)</th>
<th>Gene (exon)</th>
<th>dHPLC temperatures (°C)</th>
<th>Gene (exon)</th>
<th>dHPLC temperatures (°C)</th>
<th>Gene (exon)</th>
<th>dHPLC temperatures (°C)</th>
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<td>KCNQ1ex1</td>
<td>64</td>
<td>KCNH2ex1</td>
<td>66.3, 68.9</td>
<td>SCN5Aex2</td>
<td>62, 65.5</td>
<td>SCN5Aex17</td>
<td>61.9, 64.5</td>
</tr>
<tr>
<td>KCNQ1ex2</td>
<td>62, 65</td>
<td>KCNH2ex2</td>
<td>65.1, 67.3</td>
<td>SCN5Aex3</td>
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<td>SCN5Aex18</td>
<td>63.4, 65.6</td>
</tr>
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<td>KCNQ1ex3</td>
<td>65.2, 67.2</td>
<td>KCNH2ex3</td>
<td>62.2, 65.2</td>
<td>SCN5Aex4</td>
<td>62.5, 64.2</td>
<td>SCN5Aex19</td>
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</tr>
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<td>SCN5Aex5</td>
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<td>SCN5Aex20</td>
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<td>KCNH2ex5</td>
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<td>SCN5Aex6</td>
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<td>61.5, 62.6</td>
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<td>KCNH2ex6</td>
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<td>SCN5Aex7</td>
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<td>KCNH2ex11</td>
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<td>62.4, 65.6</td>
<td>SCN5Aex28a</td>
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<td>KCNH2ex13</td>
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<td>SCN5Aex14</td>
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<td>KCNH2ex14</td>
<td>64.9, 66.1</td>
<td>SCN5Aex15</td>
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<td>SCN5Aex28c</td>
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<td>58.2, 66.2</td>
<td>KCNH2ex15</td>
<td>65.8, 66.6</td>
<td>SCN5Aex16</td>
<td>61.4, 62</td>
<td>SCN5Aex28d</td>
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KCNE1ex1    | 64                       | KCNE2ex1    | 68.2, 68.8               |
KCNE1ex2    | 64.2                     | KCNE2ex2    | 63.1, 64.2               |
KCNE1ex3    | 62.5, 64                 | KCNE2 3'UTR | 64                       |
Table 8.8 Summary of primer pairs used for quantitative real time -PCR analyses.

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<th>Primer name (gene/exon)</th>
<th>Forward primer sequence (5’ – 3’)</th>
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<th>Fragment length (bp)</th>
<th>Chr</th>
<th>Start</th>
<th>Finish</th>
<th>Amplification efficiency</th>
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