Development of a cardiac channel molecular autopsy in a South African cohort of sudden unexplained deaths in the young

by

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Declaration

I, the undersigned, hereby declare that the thesis submitted to the University of Pretoria for the degree PhD (Medical Criminalistics), and the work contained therein, is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree. Where previously published work was used, acknowledgement to the author(s) is provided in the reference list at the back of each section. Procedures were carried out in accordance with the ethical rules prescribed by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria.

Signature: 

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List of abbreviations

A

AB	Allele balance
ACE	Angiotensin-converting enzyme
ACM	Arrhythmogenic cardiomyopathy
ACMG	American College of Medical Genetics
AF	Allele frequency
AHA	American Heart Association
ALTE	Acute life threating event
AMP	Association for Molecular Pathology
APHRS / HRS	Asia Pacific Heart Rhythm Society / Heart Rhythm Society
Arg (R)	Arginine
ARVC	Arrhythmogenic right ventricular cardiomyopathy
Asn (N)	Asparagine
Asp (D)	Aspartate

B

BAM	Binary alignment / map
bp	Base pairs
BLOSUM	Blocks Substitution Matrix
BrS	Brugada syndrome
BWA - MEM	Burrows - Wheeler Alignment - Maximal Exact Match

C

Ca^{2+}	Calcium
CAD	Coronary artery disease
CDC	Center for Disease Control and Prevention
CNS	Central nervous system
CL	Cytoplasmic loop
CPVT	Catecholaminergic polymorphic ventricular tachycardia
CSol	Central solenoid
C-terminus	Carboxyl-terminus
Cx40	Connexon 40
Cys	Cysteine
CVD	Cardiovascular disease

D

DCM	Dilated cardiomyopathy
DNA	Deoxyribonucleic acid
DNF	Death notification form
DOD	Delayed onset depolarisation
DoH	Department of Health
DP	Read depth
DGC	Dystrophin-associated glycoprotein complex

E

ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EMS	Emergency medical services
EPV	Estimated predictive value
ESC	European Society of Cardiology

F

FFPE	Formalin fixed paraffin embedded
FKBP12.6	FK506 binding protein

G

GERP	Genomic evolutionary rate profiling
GOF	Gain of function
GP	General practitioner
GQ	Genotype quality

H

HCM	Hypertrophic cardiomyopathy
HGVS	Human Genome Variant Society
HDCM	Hypokinetic non-dilated cardiomyopathy

I

ICD	Implantable cardiac defibrillator
IKr	Rapid delayed rectifying potassium current
IKs	Slow delayed rectifying potassium current
INDEL	Insertion / deletion

K

K^+	Potassium

L

LB	Likely benign
LP	Likely pathogenic
LQT1	Long QT type 1
LQT2	Long QT type 2
LQT3	Long qt type 3
LQTS	Long qt syndrome
LV	Left ventricular
LVNC	Left ventricular non-compaction
Lys	Lysine

M

MAPQ	Mapping quality
MQ	Mapping quality

N

Na+	Sodium
NCD	Non-communicable disease
NGS	Next generation sequencing
N - terminus	Amino - terminus

P

Pretoria MLL	Pretoria Medico - Legal Laboratory

Q

QD	Quality by depth

R

RCM	Restrictive cardiomyopathy
RV	Right ventricular

S

SA	South Africa
SAM	Sequence alignment map
SAM / BAM	Sequence alignment map / binary alignment map
SCA	Sudden cardiac arrest
SCD	Sudden cardiac death
SD	Sudden death
SIDS	Sudden infant death syndrome

SUD	Sudden unexpected death
SUDI	Sudden unexpected infant death
SUID	Sudden unexplained infant death
SR	Sarcoplasmic reticulum
SSA	Sub - Saharan Africa
T	
TMD	Transmembrane domain
TMEM43	Transmembrane protein 43
TOD	Time of death
Trp	Tryptophan
U	
UCSC	University of California Santa Cruz
UK	United Kingdom
USA	United Sates of America
V	
VCF	Variant call format
W	
WES	Whole exome sequencing

List of publications

van Deventer BS, du Toit-Prinsloo L, van Niekerk C. Cardiovascular deaths: What do the genes say? *S Afr Med J* 2022;112(12):1. doi.org/10.7196/SAMJ.2022.v112i12.16801

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B Stroh van Deventer,1 BSc, MSc; L du Toit-Prinsloo,2 MBChB, MMed (Path) (Forens); C van Niekerk,3 BSc, PhD. Postmortem genetic testing in young individuals: What clinical medical practitioners need to know. *S Afr Med J* 2022;112(12):1-4. doi.org/10.7196/SAMJ.2022.v112i12.16800

List of international conference contributions

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Thesis outline

Chapter One serves as an introduction to this thesis, providing a summary on the topic of cardiovascular diseases in the young, with an emphasis on the underlying genetics thereof. This chapter follows the format of an article which was published in the South African Medical Journal. It highlights research findings of inherited cardiac arrhythmogenic disorders as a major cause of sudden unexpected deaths in the young, and emphasizes the importance of the early clinical diagnosis, treatment and ultimate prevention thereof. The chapter concludes with the relevance, aim and objectives of this study.

Chapter Two reviews the published literature on sudden unexpected deaths in the young, focussing on the cardiac causes which comprises the majority of them. Not all SCDs in the young have an obvious cause of death that can be determined at autopsy, and only through postmortem genetic testing has it been shown that inherited cardiac arrhythmogenic disorders are the cause of a large number of these unexplained cases. The international implementation of a targeted molecular screening in all suspected sudden cardiac deaths in the young, and the clinical benefits thereof, are discussed. Finally, the lack of similar research into the genetic causes of sudden unexpected deaths in the young South African population, is addressed.

Chapter Three provides a more focused literature review on LQTS, its association with sudden unexplained infant deaths, and the postmortem genetic analysis thereof. This chapter followed the format of an article that was published in the International Journal of Clinical Pathology. Generally, sudden unexplained infant deaths (SUID) are discussed as a separate entity from those occurring in the young (between the ages of one and 45 years old), with research showing a different genetic profile between these two categories. This chapter specifically discusses the most prevalent cardiac arrhythmogenic disorder linked to SUID, with an overview of the underlying genetics thereof. The results of various genetic studies conducted on SUID cases, including that of our pilot study, are compared, and applicable recommendations are provided.

Chapter Four discusses the proof-of-concept post mortem genetic study that was conducted on a two-month-old SUDI cases, which were admitted to the Pretoria Medico-Legal Laboratory for a medico-legal autopsy. This chapter follows the format of an article which was published in the Cardiovascular Journal of Africa. The circumstances of death, relevant medical history and autopsy results are described in detail, followed by the experimental procedure that was

followed for post mortem genetic testing of the *SCN5A* gene. The chapter ends with a full discussion on the genetic results, the implications thereof and further recommendations for a broader study to be conducted.

Chapter Five discusses the importance of molecular diagnostics in the forensic medical practice, particularly in the medico-legal investigation of sudden unexplained deaths in the young. A brief overview on the current international guidelines and implementation thereof, is discussed, with the lack of similar studies and / or implementation in SA providing the rationale behind our study. Next generation sequencing of 49 arrhythmogenic-related genes was performed in a South African cohort of unexplained sudden deaths in the young, and the methods, results, and interpretation thereof, discussed in this chapter.

Chapter Six provides final concluding remarks on the lack of molecular diagnostics in the investigation of South African unexplained sudden deaths and highlights the implications of the results obtained from this study. The increase in South African cardiovascular deaths is emphasised, and the critical need for genetic research is highlighted. The potential public health benefits of genetic testing in these deaths are discussed, and practical recommendations on the implementation thereof, are provided.

Chapter 1
Introduction

This chapter was published as an editorial article, entitled "Cardiovascular deaths: What do the genes say?"in the South African Medical Journal (S. Afr. Med. J.) December 2022;112(12):x.https://doi.org/10.7196/SAMJ.2022.v112i12.16801. A pdf copy of this publication is available as Appendix L.

This chapter followed the editorial style of the S. Afr. Med. J.

1.1 Introduction

Cardiovascular diseases (CVDs) are ever-increasing, and as such are considered to be one of the most concerning public health burdens worldwide. They remain the leading cause of death across the world (~17.7 million deaths were reported in 2015), accounting for 31% of all global deaths.[1]

More than 75% of these cardiovascular deaths occur in low- and middle-income countries, and although CVD is an acknowledged health concern in Africa, this priority area should receive much more attention than it currently does.[2]

Up to 50% of all cardiovascular deaths are a result of a sudden cardiac death (SCD), defined as 'a natural death due to cardiac causes, heralded by abrupt loss of consciousness within 1 hour after the onset of symptoms. The consequences of these deaths, particularly in the young, have a greater impact and health burden in terms of life years lost than all individual cancers and other leading causes of death. The fact that nearly 90% of SCDs are caused by an inherited disorder justifies the international focus on, and prioritisation of, the underlying genetic causes of these cardiac disorders.[3]

Disorders linked to SCDs vary greatly between different age groups, with ischaemic heart disease being the most common cause of death in the older population. In comparison, the majority of SCDs in the younger population (≤45 years) are due to inherited cardiomyopathies and arrhythmogenic disorders. Unfortunately, there is a lack of clinical symptoms or warning signs, with research showing that in 75% of SCD cases, death is the first 'symptom'.[4]

Inherited cardiomyopathy- and arrhythmogenic-related SCDs result from lethal arrhythmias. These are caused by alterations (genetic variations) in genes that all play a role in cardiomyocyte excitability and contractility. Cardiomyopathy-related genetic variations affect the structure and function of the heart muscle, whereas cardiac arrhythmogenic genetic disorders are generally associated with isolated electrical dysfunction. The four most common inherited arrhythmogenic disorders include long QT syndrome, Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia and short QT syndrome. Although each of these has a characteristic electrocardiogram (ECG) profile when experiencing an arrhythmic episode, their spontaneous and sporadic nature results in a difficult clinical diagnosis.[5,6]

The same gene can be altered in different ways (different genetic variations), which can lead to vastly different clinical manifestations of a disorder. These differences are important when considering available and effective treatment for patients, as each treatment is designed to target a certain defect and/or function. Fortunately, there are various types of treatment available, which can range from antiarrhythmic medications to implantable cardioverter defibrillators and pacemakers.[1,7]

Therefore, even for general practitioners (GPs), there is clinical importance in determining the underlying genetics of an inherited cardiac disorder, to allow for effective and individualised treatment of a patient and/or affected family members. Since diagnosis can be challenging, be it due to an absence of ECG abnormalities, overlap of clinical phenotypes or lack of symptoms, genetic testing in all individuals at risk for an inherited cardiomyopathy or arrhythmogenic disorder is crucial. This is reiterated by the marked reduction in mortality associated with the administration of proper treatment.[7]

The general medical practitioner, in particular, is in a central position in SCD prevention, and plays an essential role in the multidisciplinary team tending to affected family members. GPs have a greater personal connection to the community, and often care for different generations of the same family, which allows for earlier recognition of subtle warning signs suggestive of an inherited cardiomyopathy or arrhythmogenic disorder. Clinical practitioners should especially be cognisant of any family history of syncope, epilepsy, sudden death, deafness, heart failure or pacemaker implantation at a young age (<50 years). Primarily, the GP will be the first to recognise a possible inherited cardiac disease in an individual or family,

and through appropriate genetic testing may provide the only opportunity for an early diagnosis and proper clinical management.

1.2 Relevance of this study

One of the major current international focus areas involves the use of molecular diagnostics in the field of forensic medicine. In accordance with the prescribed minimum guidelines, the application of post mortem genetic testing as routine investigation in all unexplained sudden death (SD) cases has been internationally adopted in the practice of forensic pathology. Due to rapid developments in technology and its decrease in cost, many international forensic pathology centres now use a multigenic cardiac next generation sequence (NGS) panel in all SCD cases suspected to be of a genetic nature, to evaluate numerous genes associated with cardiomyopathies and channelopathies as a possible cause of death. Despite all international studies done in this field, there is a lack of similar studies conducted on the South African population. To our knowledge, no post mortem genetic study has ever been conducted on South African SCD cases in the young (between the ages of zero and 40 years old).

According to the South African Department of Health (DoH), South Africa (SA) has a health burden attributed to different aspects, one of which is evidence of a rapidly increasing prevalence of non-communicable diseases. One of the greatest challenges currently experienced in the South African health system is not accredited to communicable diseases, but rather to the increased impact of chronic diseases, which include cardiovascular diseases. There is no evidence pointing to a decline in the number of SCD's in the young, thus raising great concern. Currently, a top priority in the field of SCD investigation is the development and enhancement of any possible risk stratification techniques, which will lead to more accurate diagnosis and treatment for affected patients in the general South African population.

Establishing the correlation between post mortem genetic results and an accurate understanding of the pathogenic basis associated with SCD's, enables the possibility of developing more meaningful and relevant risk stratification techniques. This study played a vital role in determining the associated gene and its clinical significance associated with inherited cardiac arrhythmogenic disorders underlying SCD's in a young South African population. Hopefully, the results of this study will contribute to a more specific screening and subsequent effective treatment for all individuals at high risk.

This study seeked to provide a realistic and applicable interpretation of genetic variants linked to SCD cases in the young, specific to the South African population. The goal / outcome of this study was to aid in the prevention of future such deaths.

1.3 Aim

The aim of this study was to determine the prevalence of identified variants in 49 major genes linked to inherited cardiac arrhythmogenic disorders in sudden unexpected death cases in the young (which for the purpose of this study was considered all cases below the age of 45), that were admitted and subjected to a medico-legal autopsy at the Pretoria Medico-Legal Laboratory.

1.4 Objectives

The objectives of this study were:

- To analyse and compare the 49 multigene cardiac panel next generation sequence results of all sudden unexpected death (SUD) / sudden unexpected death in infant (SUDI) cases to those of controls and database reference sequences, to identify variants linked to inherited cardiac arrhythmogenic disorders.

- To determine the specific targeted regions in each of the 49 genes associated with SUD / SUDI cases unique to the South African population.

- To retrospectively compare the targeted regions associated with these 49 genes in the South African SUD / SUDI population (obtained from the results of this study), to that of international populations.

- To evaluate the feasibility of a routine post mortem genetic test for all SUD / SUDI cases in SA;

- To suggest a multidisciplinary approach to be followed in all SUD / SUDI cases, in order to successfully serve the South African public at risk and to suggest effective testing that will in the long run lead to prevention of such deaths.

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Chapter 2

Introduction

A commonly shared goal between countries worldwide, is to attain the highest standard of well-being and to increase the population's life expectancy, for which the monitoring of health trends and the identification of priority areas are vital.[1] Statistics on mortality is one of the most effective sources of information on a population's health status. The importance of determining the cause of death, is best described by Ye *et al*.[2] who stated that one of the best ways to help the living, is by counting the dead, establishing the characteristics of those who died, and the underlying causes based on data from civil registration systems.

2.1 Death in South Africa

South Africa (SA), which is still considered a low-and middle-income country, currently faces a global health burden due to its high rate of premature mortality. The worldwide shift from communicable to non-communicable diseases, which is also evident in SA, now causes a double burden of infectious and non-infectious diseases.[3] Furthermore, SA has one of the highest crime rates in the world, leading to a high burden of non-natural deaths as well. In order to relieve such an increased health burden, the importance and critical need for a well-established death investigation system, the thorough reporting thereof, and the effective implementation of policy making in SA, is imperative.[1, 4]

In SA, the Department of Home Affairs (DHA) is the custodian of the civil registration system responsible for information on the cause of death, such as diseases, injuries, or complications, provided on a death notification form (DNF). According to the latest release (May 2020) on mortality statistics by Statistics South Africa (Stats SA), a key stakeholder who collects their information from the DHA, the estimated population of SA in 2017 was 56,52 million. The total number of deaths recorded by Stats SA for the same year was 446 554. Of these deaths, 41.1% occurred in individuals below the age of 50 years old.[1]

A total number of 395 380 deaths (88.5%) were recorded as natural deaths in 2017, whereas 52 164 (11.5%) of the deaths were recorded to be from unnatural causes. A total of 3 986 DNF's had no cause of death indicated on them. Of these 3 986 forms, 2 079 (52.2%) had a doctor indicating a natural death without a specific cause given, with the remaining 1 907 (47.8%) cases indicating that the death was still under investigation or that the doctor was not in a position to specify a cause of death. Research has shown that a reasonable number of these

cases, where no cause of death could be indicated on the DNF, is a result of a sudden unexpected death (SUD), which forms the focus of this research study.[1]

2.2 Sudden unexpected death

The terms "sudden death" and "sudden unexpected death" are often used interchangeably in articles reporting on the incidence of these types of deaths. Both terms will be used throughout this study. Byard [5] proposed that the term "unexpected" should rather be avoided and the focus should be on all deaths which occurred suddenly. He divides cases of sudden death into three categories:

- Apparently well individuals who died suddenly or were found dead in the bed [which can include congenital cardiac abnormalities and sudden infant death syndrome (SIDS)];
- Mildly unwell individuals who present similarly than the abovementioned. These can include viral infections and in certain SIDS cases could be an infant with the history of a low grade fever;
- People with a known serious condition, but who were stable on treatment and then suddenly died (such as sudden deaths in epilepsy).

The definition of a sudden death varies between available published literature, with different authors setting different time intervals of zero, one, six and 24 hours between the onset of symptoms and the time of death.[5, 6] For the purpose of this study, the sudden unexpected death of a person is defined as "a natural, unexpected fatal event occurring within one hour of the beginning of the symptoms, in an apparently healthy subject, or one whose disease was not severe enough as to predict such an abrupt outcome". Sudden death is a leading cause of mortality in the young and considered a public health problem worldwide.[7-9] It is considered one of the most significant challenges faced by healthcare professionals, due to its prevalence and significant impact on society.[10, 11]

The true global incidence of SUD's remains vague due to a lack of standardized published literature on this matter.[12] Many sudden deaths are not witnessed which contribute to the underestimation on the true incidence.[13, 14] Most recent reports indicate the global incidence of SD in the young to range between 1.3 and 8.5 per 100 000 person years.[9] Italy, (Australia and New-Zealand) and (England and Wales) reported similar SD incidences of 1, 1.3 and 1.8 per 100 000 person years, respectively.[15, 16] The Portuguese population recorded a SD incidence of

2.4 per 100 000, whereas Denmark recorded an incidence of 2.8 per 100 000 person years.[17] Statistics regarding the African incidence of SD remain scarce. Senegal reported an incidence of 4.1 per 100 000, whereas a few other studies only reported on selective study cohorts inclusive of SD cases.[18] A study conducted in Nigeria (2012), over a seven-year period, reported that 30.3% of their total deaths comprised of SD's.[19] Two South African studies, one conducted in Pretoria, and one conducted in Cape Town (Stellenbosch), recorded that a respective 14% and 20.4% of SUD's contributed to their total case load.[20, 21]

2.2.1 Sudden unexpected death in infants

The incidence of sudden unexpected death in infants [SUDI (between zero and one year old)] is not included in the statistics provided in section 1.2 above, as these deaths are classified as a separate entity. Sudden unexpected death in children is defined as "the death of a child which was not anticipated as a significant possibility, 24 hours before the death, or where there was a similarly unexpected collapse leading to, or preceding the events which led to the death".[22] This includes sudden unexpected deaths in infancy and unexpected death in older children. Sudden deaths in infants are still considered one of the leading causes of infant mortality worldwide and have also been reported to be an extraordinarily high burden in sub-Saharan Africa (SSA).[23, 24] According to Duncan *et al*.[25] for most countries, the rate of sudden unexplained infant deaths (SUIDs) [or the previously termed sudden infant death syndrome (SIDS) cases] is reported at approximately 0.2–0.5 per 1 000 live births. The most recent published incidence rate for South African SUID cases was 1.06 per 1 000 live births for the white population and 3.41 for infants from the mixed-ancestry population group, respectively.[26] The investigation into SUDIs and child mortality remains a high-priority research area in SA.

2.3 South African legislation pertaining to sudden expected deaths

According to South African legislation, a sudden unexpected death of a person is classified as an unnatural death. Unnatural deaths are defined in the Regulations Regarding the Rendering of Forensic Pathology Service R636, drafted in terms of Chapter 11 of the National Health Act 61 of 2003 [27], as:

- "Any death due to physical or chemical influence, direct or indirect, or related complications.

- Any death, including those deaths which would normally be considered to be a death due to natural causes, which in the opinion of a medical practitioner, has been the result of an act of commission or omission which may be criminal in nature; or
- Where the death is sudden and unexpected, or unexplained or where the cause of death is not apparent".

In addition, the so-called procedure related deaths, which are defined by the Health Professions Act 56 of 1974 (section 56 as amended by the Health Professions Amendment Act 29 of 2007) as "the death of a person undergoing, or as a result of, a procedure of a therapeutic, diagnostic or palliative nature, or of which any aspect of such a procedure has been a contributory cause, shall not be deemed to be a death from natural causes as contemplated in the Inquests Act, 1959 (Act No. 58 of 1959), or the Births and Deaths Registration Act, 1992 (Act No. 51 of 1992).[28-30]

These unnatural deaths must be investigated in the medico-legal environment, according to statutory provisions. It is the duty and responsibility of the forensic pathologist to conduct a thorough autopsy of these cases in order to determine a cause and mechanism of death. The Inquests Act 58 of 1959 "provides for the holding of inquests in cases of deaths or alleged deaths apparently occurring from other than natural causes and for matters incidental thereto." According to Section 3, subsection (ss.) of the Inquests Act "if the body of a person who has allegedly died from other than natural causes is available, it shall be examined by the district surgeon or any other medical practitioner, who may, if he deems it necessary for the purpose of ascertaining with greater certainty the cause of death, make or cause to be made an examination of any internal organ or any part or any of the contents of the body, or of any other substance or thing". Additional legislative documents are also available which further aid the Inquests Act in providing the importance and legal requirements to be met, for the conduction of a medico-legal autopsy. This includes the National Code of Guidelines for Forensic Pathology Practice in SA as well as the National Health Act 61 of 2003 and the Regulations Regarding the Rendering of Forensic Pathology Service R636.[27]

2.4 Causes of sudden unexpected death

Underlying causes of SUD can be classified according to the anatomical system involved, and generally divided into cardiovascular causes and non-cardiovascular causes. From a clinical point of view, this subdivision is a result of the fact that a significant proportion of

cardiovascular causes is of a hereditary nature, whereas non-cardiovascular causes tend to be less hereditary.[16, 31] Non-cardiovascular abnormalities mainly include disease processes of the central nervous system (CNS), respiratory system, gastrointestinal system as well as the genito-urinary system.[16]

The majority (up to 90%) of SD's are cardiac-related [termed sudden cardiac death (SCD)], which can be further divided into cases where structural abnormalities are identifiable at autopsy, and cases, especially in the young, where the heart is grossly (no anatomical/structural abnormalities noted at autopsy) and histologically normal.[9, 32] Coronary artery disease (CAD), cardiomyopathies and myocarditis are recorded as the cause of death in the majority of the cases in which identifiable changes are predominant.[33] In a significant number of cases where no structural changes are found, a channelopathy, described as an inherited disease of the cardiac ion channels, is suspected to be the cause of death.[16, 34] Additionally, some cases without any evidence of structural cardiac abnormalities might carry cardiomyopathy-related variants, which are then called 'concealed cardiomyopathy'. A schematic presentation on a brief summary of the underlying classification of causes of sudden unexpected death is shown below in Figure 2.1.

Cardiovascular disease is a leading cause of death and currently deemed a major public health concern, worldwide. Globally, approximately 17 million cardiovascular deaths are recorded each year, and given the fact that up to 50% of these are due to SCD's, the importance and relevance of this topic cannot be overstated.[35, 36] The consequences of SCD are now considered to have a greater impact and health burden in terms of life years lost, than all individual cancers and other leading causes of death combined.[37]

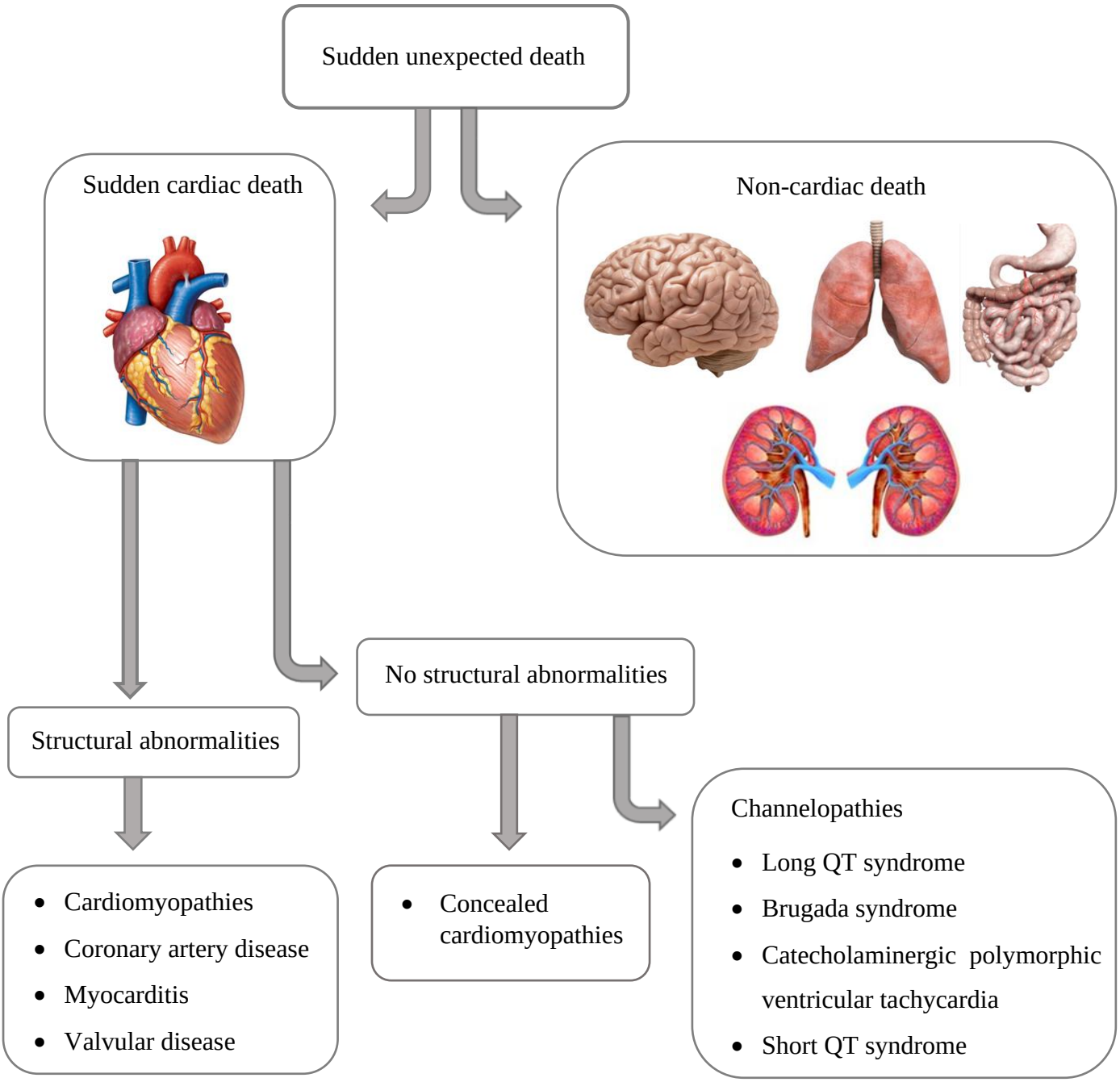

Figure 2.1 Summarised schematic representation on the classification of causes of sudden unexpected death

2.5 Sudden cardiac death

Sudden cardiac death is defined by the American Heart Association (AHA) as "the sudden abrupt loss of heart function in a person who may or may not have diagnosed heart disease whereby the time and mode of death are unexpected and the death occurs either instantly or after symptoms appear".[38]

Globally, an approximated 5 million SCD's are recorded each year, with an estimated 450 000 in the United States of America (USA) alone.[39] Europe records around 300 000 SCD's per annum.[12] Various studies report varying incidences of SCD, whether it be attributed to different age ranges, sizes of study cohorts or different study designs being implemented. Internationally, the incidence of SCD varies between 15 and 59 per 100 000 persons, which constitutes up to 20% of all deaths.[10] It is important to note that these SCD incidences, as well as the associated causes thereof, also vary between different age groups. It is generally known that with an increase in age, the incidence of SCD also increases. Sessa *et al*. reported a SCD incidence rate of 1 per 1 000 subjects in the 35 to 40 year-age group, and an increased incidence rate of 2 per 1 000 by the age of 60 years old. For the elderly age group, the reported incidence rose to 200 per 1000 subjects per year.[6] Although the vast majority of these deaths include the elderly, 20% of all deaths in the young (generally between zero and 40 years of age) are still attributed to SCD.[40] The incidence of SCD among children, adolescents and young adults occur rather infrequently, with a global estimated rate between 1 and 8.5 per 100 000 person years.[7] Italy reported an annual incidence rate of 1 per 100 000, Australia a rate of 1.3 per 100 000, England a rate of 1.8 per 100 00 and Denmark reported a rate of 2.8 per 100 000 person years.[7, 15, 41] Japan recorded an estimated 1 500 SCD's for each year.[42] Unfortunately, there are not enough published reliable data pertaining to SCD in SA. A study conducted by Roman *et al*.[20] 2021, reported on a total of 707 SUD cases, admitted over a one-year period to the Western Cape Forensic Pathology Service (FPS) Tygerberg, of which the majority were cardiac-related. Approximately 2,000 young and healthy South Africans die each year as a result of SCD.[43]

2.5.1 Causes of sudden cardiac death

The causes of SCD are greatly dependent on the age of the deceased. Although the incidence of cardiac-related death increases with age, the proportion of SD's due to an inherited disease is much higher in the young population.[6] In individuals over the age of 40 years, ischaemic heart disease is the most common cause of SCD, whereas in individuals younger than 40, cardiomyopathies and primary arrhythmogenic disorders (channelopathies) are ranked above

ischaemic heart and valvular disease, with 75% as a result of the former.[39, 41] Thiene[16] reported that in 93% of their cardiac-related cases, the mechanism of SCD was arrhythmic, with the majority ascribed to cardiomyopathies and channelopathies. Furthermore, research have shown that up to 90% of SCD's are potentially caused by inherited cardiac diseases, of which cardiomyopathies and channelopathies are the most prevalent.[16, 36]

Although a large proportion of SCD's are found to have a clearly stated pathological cause of death, it is important to note that not all SCD in children, adolescents and young adults have an obvious cause of death that can be determined at autopsy.[44] Research has indicated that an estimated 3% to as high as 53% of these SCD cases have no identifiable abnormal morphological findings at autopsy and remain unexplained.[34, 44] Post mortem molecular screening has diagnosed the above-mentioned channelopathies as a cause of approximately 40% -50% of unexplained SD's, due to its characteristic absence of structural abnormalities to the heart.[45] Although cardiomyopathies are generally categorised by a structurally abnormal heart, more and more evidence to the contrary has been reported. Numerous cases of unexplained SD's, especially those in infants, which presented with structurally normal hearts at autopsy, have been shown to carry likely pathogenic variants in cardiomyopathy-related genes.[46-48] Although different cardiomyopathies and channelopathies may all result in the same type of SCD, it is important to remember that each of them represents distinct clinical features, genetic aetiologies and management strategies.[41]

2.6 Cardiomyopathies

Cardiomyopathies are generally described as disorders which affect the myocardium, thereby leading to both functional and structural abnormalities of the heart.[49] Depending on the type of cardiomyopathy, the myocardium may become hypertrophied, dilated, or more rigid / stiff, which ultimately leads to heart failure and even SCD. Cardiomyopathy is defined by the American Heart Association as "a heterogeneous group of diseases of the myocardium, usually with inappropriate ventricular hypertrophy or dilatation".[49]

Cardiomyopathies can be classified as either primary (genetic, mixed, or acquired causes), or secondary, where an infiltrative, toxic or inflammatory cause is implicated. They are divided into five main structural subtypes; hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), arrhythmogenic cardiomyopathy (ACM), and the less common restrictive cardiomyopathy (RCM) and left ventricular non-compaction cardiomyopathy (LVNC).[50, 51] The majority of non-ischaemic cardiomyopathies have a genetic aetiology with

a dominant inheritance, which currently involves more than 100 genes. These genes however are characteristic of heterogenicity, incomplete penetrance as well as variable expressivity, and commonly overlap between the different types of cardiomyopathies (Figure 2.2).[52]

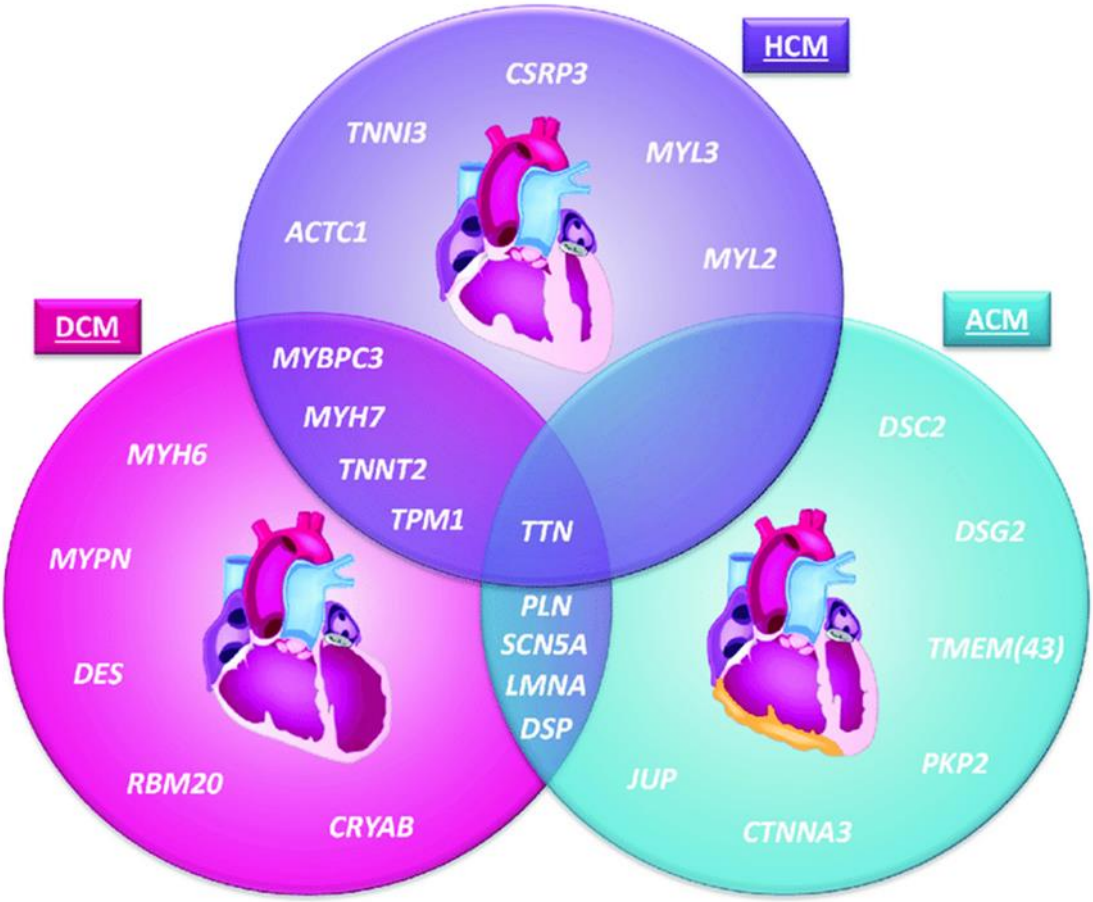

Figure 2.2. Overlap of genes associated with cardiomyopathies. Each of the three circles demonstrate the most prevalent genes associated with its respective cardiomyopathies. The blue circle illustrates the 10 most prevalent genes associated with hypertrophic cardiomyopathy, overlapping with 14 genes mostly representing dilated cardiomyopathy (shown in pink). The green circle shows 11 prevalent genes associated with arrhythmogenic cardiomyopathy, overlapping with genes in the blue and pink circle.[53]

2.6.1 Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy is considered the most prevalent inherited cardiac disease, commonly associated with hypertrophy of the ventricular walls and / or septum of the heart.[31] An estimated prevalence of 1 in every 500 individuals has been reported.[31] Although HCM-diagnosed patients generally receive a good prognosis, a significant number of cases however experience life-threatening complications, such as SCD and end-stage heart failure.[54] Although the exact mechanism of HCM-related SCD's remain poorly understood, abnormalities in the myocardial structural design are said to play a role in both left ventricular (LV) modelling and arrhythmogenesis. Morphological expression of the disease does vary, with some affected individuals experiencing symptoms from an early age, whereas others may live asymptomatically for decades.[55]

Hypertrophic cardiomyopathy can be defined as "a genetic disorder of cardiac myocytes that is characterized by cardiac hypertrophy, unexplained by the loading conditions; a nondilated left ventricle; and a normal or increased ejection fraction".[56] At a cellular level, three different groups of distinct features can be observed, namely the disarray of cardiomyocytes and myofibrils, intramural microvasculature abnormalities, and interstitial fibrosis.[31] The disarray of cardiomyocytes, which is patchy and not localised to a specific area, present with hypertrophied myocytes of unusual shapes, with abnormal connections and alignment. The disarray of myocytes has particularly been observed in patients who died at a relatively young age.[54] Where abnormalities of the microvasculature are concerned, a reduction in arteriolar density and small vessel dysplasia will be evident, with a reduced luminal cross-section area.[54] Myocardial fibrosis may vary in its degree of scarring and interstitial fibrosis and differs from the fibrosis observed in DCM.[54] These abnormalities can result in diastolic dysfunction, impaired coronary reserve, atrial fibrillation, ventricular arrythmia, heart failure and SCD. Symptoms of the disease may include syncope, exercise intolerance, chest pain, shortness of breath and dizziness.[49] Figure 2.3 and 2.4 shows images of hypertrophic cardiomyopathy detected at autopsy and the histological examination thereof.

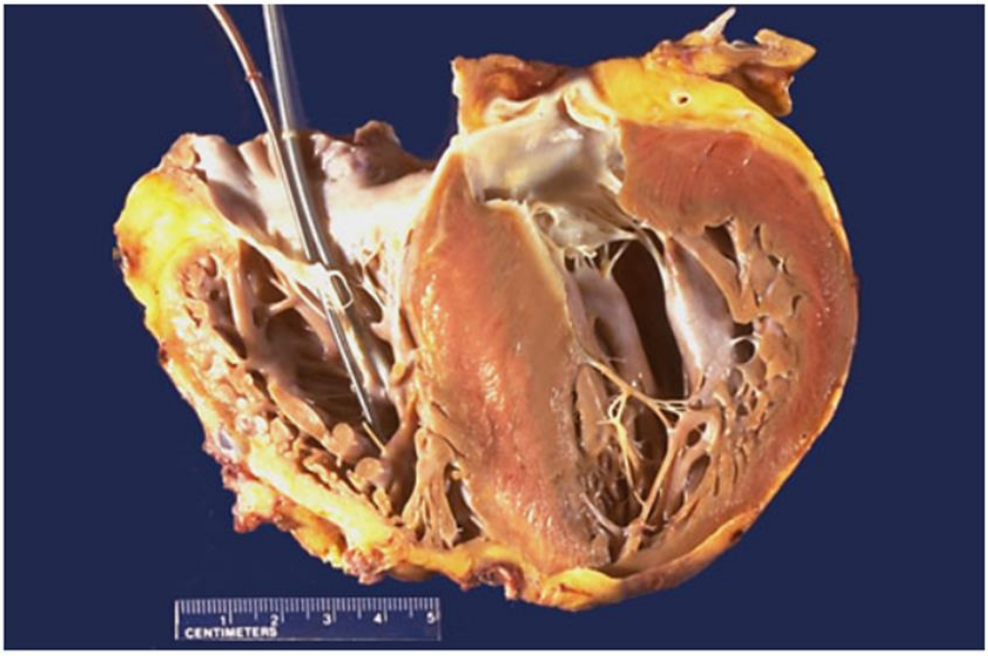

Figure 2.3. Hypertrophic cardiomyopathy of the heart at autopsy. At autopsy, the examination of an HCM-diagnosed deceased individual's heart reveals a left ventricle with marked hypertrophy. Asymmetric bulging of a large interventricular septum into the left ventricle chamber can also be observed.[57]

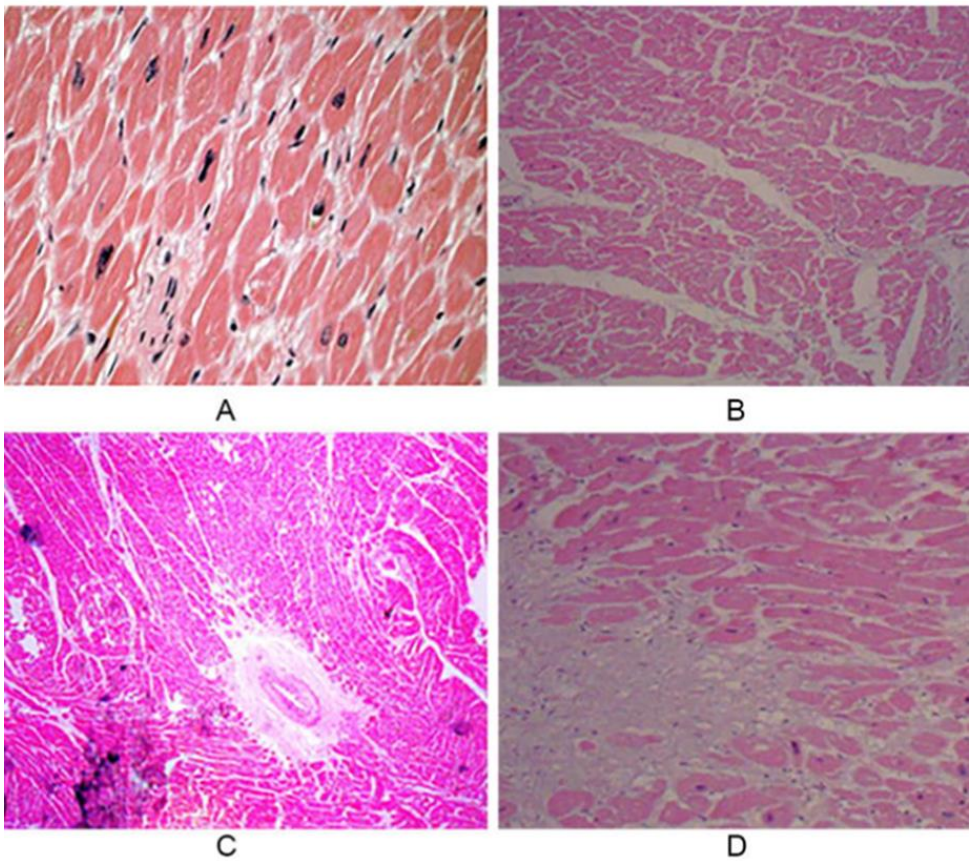

Figure 2.4. Histopathology of an HCM-diagnosed heart. Slide A shows hyperchromatic nuclei of cardiomyocytes. B shows interstitial diffuse fibrosis where bundles of collagen surround the cardiomyocyte individually. Slide C reveals perivascular fibrosis spreading radially around capillaries and small arteries. D shows replacement fibrosis characterised by areas of fibrosis not considered to be an infarct.[58]

2.6.1.1 Genetic profile of hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy is an autosomal dominant inherited cardiac disease, characterised by genetic heterogeneity.[59] Studies have shown a link between HCM and genetic variants in genes encoding for sarcomere and sarcomere-associated proteins.[59, 60] Up to 60% of HCM-patients demonstrate a clear recognisable familial disease.[59. 60] Numerous genes have been associated with HCM, however only nine of them are consistently implicated in research studies.[52, 56] Approximately 50% of HCM-linked variants are identified in the β-myosin heavy chain (*MYH7* gene) and myosin-binding protein C (*MYBPC3* gene).[56, 61] Approximately 10% of HCM-variants can be found in *TNNT2*, *TNNI3* and *TPM1*, whereas *ACTC1*, *MYL2*, *MYL3* and *CSRP3* occasionally reveal variants.[56, 61] Table 2.1 shows a list of the nine most common genes identified with HCM-related variants. The majority of pathogenic HCM-variants are missense, followed by insertions and deletions (INDELS).[52. 56. 61] The effects of such variants may include direct effects on protein structure, impairment of signalling pathways as well as inactivation or activation of signalling pathways.[62]

Table 2.1. List of the nine most prevalent genes identified with variants associated linked to hypertrophic cardiomyopathy. [56]

Gene	Protein name
MYH7	B-myosin heavy chain
MYBPC3	Myosin-binding protein C
TNNT2	Troponin T type 2
TNNI3	Troponin I type 3
TPM1	Tropomyosin α-1 chain
ACTC1	Actin α cardiac muscle 1
MYL2	Myosin light chain 2
MYL3	Myosin light chain 3
CSRP3	Muscle LIM protein

2.6.1.2 Clinical diagnosis and treatment of hypertrophic cardiomyopathy

The diagnosis of HCM mainly involves minimally invasive techniques. Two-dimensional doppler echocardiography and magnetic resonance imaging (MRI) are useful imaging techniques to measure the left ventricular (LV) wall thickness, where a measurement of ≥ 15 mm are indicative of HCM.[55] Assessments on ventricular dimensions, systolic and diastolic function and mitral valve function also prove to be valuable.[55] Genetic screening may diagnose at-risk family members unaware of having the disease. The main goal of treatment is to relieve symptoms and prevent SCD.[63] Medication may include β-blockers, calcium channel blockers, heart rhythm drugs (Amiodarone) and blood thinners such as Warfarin.[63] An implantable cardiac defibrillator (ICD) may be recommended in patients with severe HCM.[55, 63]

2.6.2 Dilated cardiomyopathy

Dilated cardiomyopathy is a disorder of the heart muscle, whereby the ventricles of the heart become thin and stretched, leading to enlarged and weakened ventricles and thus a reduced ability to pump blood.[52] It is the leading cause of both heart failure and need for transplantations, with an estimated prevalence of 1 in every 250 individuals.[52. 64] Approximately 10 000 deaths, as a result of DCM, and 46 000 hospitalisations are recorded annually in the USA.[64] Although DCM can present with a clinical syndrome of systolic heart failure, other manifestations have increasingly been reported, including cardiac arrhythmias and SCD. The absence of any clinical symptoms is also not uncommon in DCM-patients.[65]

Dilated cardiomyopathy can be defined by "the presence of LV or biventricular dilatation and systolic dysfunction in the absence of abnormal loading conditions (hypertension, valve disease) or coronary artery disease sufficient to cause global systolic impairment".[65] Concerns regarding this definition have been raised, due to the increase in evidence of patients having long preclinical phases, with no clinical symptoms and only minor cardiac alterations. A new definition, by updating the diagnostic criteria for family members of HCM-patients, has been proposed by the European Society of Cardiology (ESC) working group on myocardial and pericardial diseases. This new definition creates a category for hypokinetic non-dilated cardiomyopathy (HNDC), which reads as follows: "Left ventricular or biventricular global systolic dysfunction without dilatation (defined as LVEF < 45%), not explained by abnormal loading conditions or coronary artery disease".[65] Although it almost always present late in its clinical course, DCM results in a reduced systolic function due to myocardial remodelling, which furthermore results in an increase in end-systolic and end-diastolic volumes. In case of

progressive dilation, insufficiency of both the tricuspid and mitral valve can occur, which will increase the stress on the ventricular walls, thereby lowering the ejection fraction even more.[66]

Gross pathology of the heart of a DCM-diagnosed patient may appear globular-shaped with ventricular or four-chamber dilation. Generally, the heart weight will be increased, with normal or reduced LV wall-thickness.[58] Histological features of the myocardium may reveal hypertrophy of the myocytes with enlarged and irregular hyperchromatic nuclei.[58] Mild interstitial fibrosis and perivascular fibrosis may also be observed. The presence of focal interstitial lymphocytes and macrophages, without myocyte damage, has also been observed.[58] Figure 2.5 illustrates the gross pathology, at autopsy, of the heart of a SCD victim who's cause of death was DCM. Figure 2.6 shows three common histopathological features observed in the myocardium of a DCM-patient.

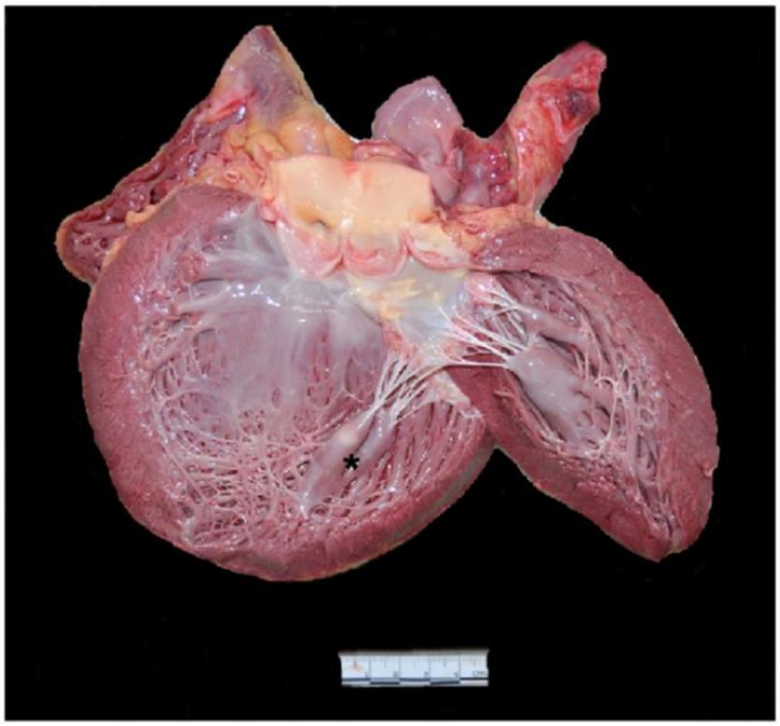

Figure 2.5. Gross pathology of the heart observed at autopsy of a sudden cardiac death due to dilated cardiomyopathy. Cardiomegaly and dilation of the heart chambers were noted, with fibrosis noted in the papillary muscle.[67]

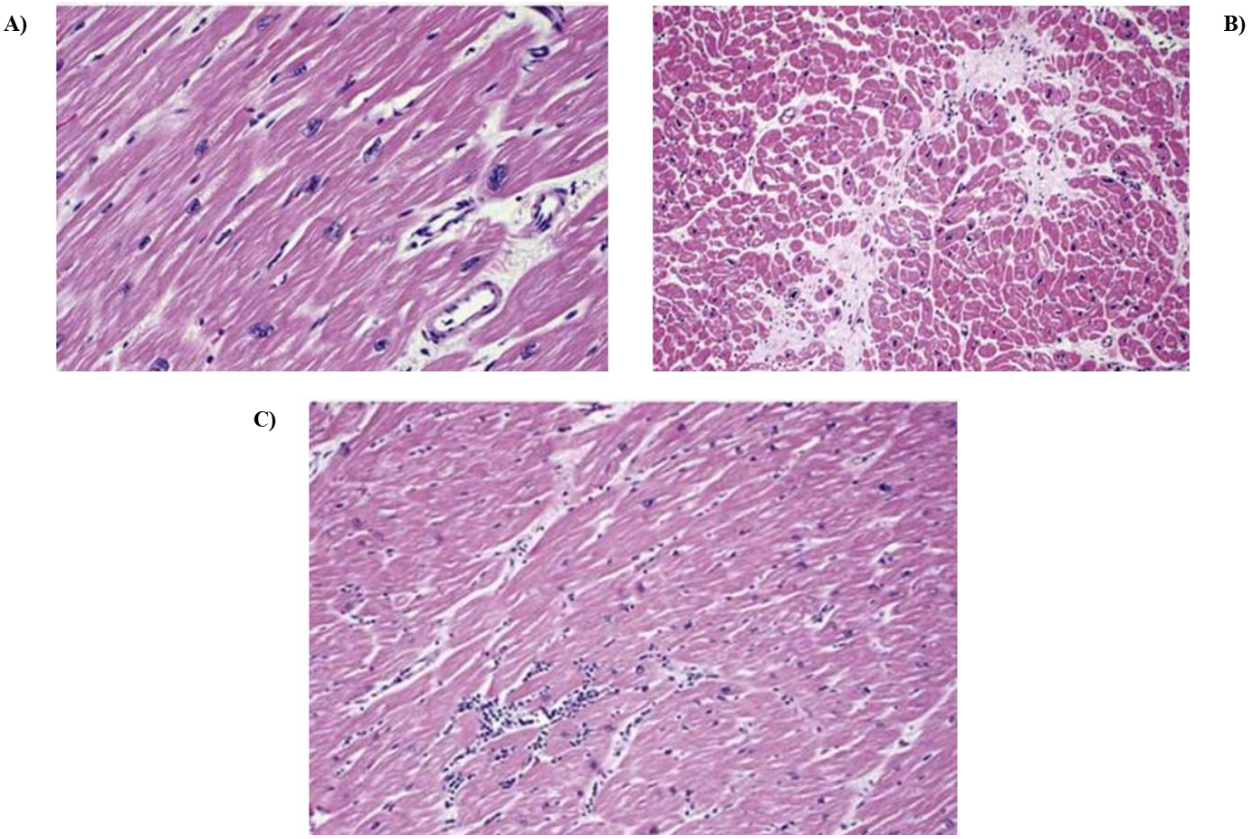

Figure 2.6. Histological examination of the myocardium of a DCM-diagnosed heart. A) Hypertrophic myocytes with enlarged irregular, hyperchromatic nuclei can be observed. B) Focal interstitial fibroses. C) Lymphocyte infiltration.[58]

Causes of DCM can be classified as either primary or secondary, of which all affect the ventricular function by some degree. Numerous causes of secondary DCM have been identified, including ischaemic disease, infectious myocarditis, hypertension, medication-induced and alcohol-induced.[52, 65] Peripartum DCM is also considered to be secondary to complications experienced during the last months of pregnancy.[66] Contrary to secondary DCM, primary DCM is generally associated with an unknown cause, which is then termed idiopathic DCM. The majority of DCM cases are deemed idiopathic, accounting for at least 50% in the adult population, with an even higher incidence observed in children.[52] However, research has shown that most idiopathic DCM cases are in fact caused by genetic variants in genes linked to DCM.[68] The genetic aetiology behind DCM is commonly underestimated, although familial studies have proven that up to 50% of idiopathic cases are inherited.[52, 68]

2.6.2.1 Genetic profile of dilated cardiomyopathy

Dilated cardiomyopathy demonstrates an autosomal dominant inheritance pattern, with genetic overlap between other cardiomyopathies, especially that of HCM.[68] Variants linked to DCM have been identified in more than 80 genes, encoding a wide range of proteins involving the sarcomere, cytoskeleton, nuclear lamina, desmosomal complexes, ion channels as well as transcription factors.[51, 69] Variants in these genes result in numerous negative effects, including on the mechanism of muscle contraction, the functioning and sensitivity of ion channels to cellular homeostasis and the generation transmission of the mechanistic force in the myocardium.[70] Although such a large range of genes are implicated in familial DCM, studies have shown an accumulation of LP variants, linked to DCM, in 12 different genes.[52, 68] Table 2.2 provides a list of these 12 genes. The *TTN* gene, encoding a sarcomeric protein, is the most prevalent gene associated with DCM, with approximately 25% of patients identified with such a variant.[52] Both *LMNA* and *SCN5A*-variants are linked to an increase in life-threatening ventricular arrhythmias and SD.[64, 68]

Table 2.2. List of the 12 most prevalent genes implicated in dilated cardiomyopathy.[68]

Gene	Protein name
TTN	Titin
BAG3	BCL-associated athanogene 3
LMNA	Lamin A/C
TNNT2	Troponin T2
PLN	Phospholamban
SCN5A	Sodium voltage-gated channel α-subunit 5
RBM20	RNA-binding motif protein 20
MYH7	B-myosin heavy chain
FLNC	Filamin C
TNNC1	Troponin C1
DES	Desmin
DSP	Desmoplakin

2.6.2.2 Clinical diagnosis and treatment of dilated cardiomyopathy

Individuals suffering from DCM may experience symptoms such as paroxysmal nocturnal dyspnoea, orthopnoea, chest pain, leg swelling, shortness of breath and palpitations.[66] The diagnosis of DCM is based on both a physical examination and the obtainment of a personal and family medical history. Physical tests can include echocardiography, blood tests, chest x-ray, electrocardiogram and an MRI. Genetic testing may also be requested.[65, 68] Treatment is available, which highly depends on the cause of DCM. The goal of treatment is to relieve symptoms and prevent further heart damage. Medications such as β-blockers, diuretics, Digoxin, and anticoagulants can be described.[71] Some cases may necessitate ICD or pacemaker implantation, and in the event of severe heart failure, a cardiac transplant may be required.[71, 72]

2.6.3 Arrhythmogenic cardiomyopathy

Arrhythmogenic cardiomyopathy (ACM), also known as arrhythmogenic right ventricular cardiomyopathy (ARVC), is an inherited cardiac disease categorized by progressive fibrofatty replacement of the myocardium in either the right, left, or both ventricles.[73, 74] With a prevalence ranging between 1 in 2 500 to 1 in 5 000 in the general population, it has been shown to affect more men than women, especially young competitive athletes.[75] Although the disease was first characterised by structural changes of the RV, with or without LV involvement, left dominant arrhythmogenic cardiomyopathy has increasingly been identified, which led to the preferred use of the term 'ACM'.[51, 76] Fibrofatty replacement in the myocardium results in the impaired transmission of electrical signals through the heart, ultimately causing heart rhythm disorders in ACM patients.[77] A decrease in heart function may also occur, which can lead to an increased risk of heart failure. Disease expression is variable, with a lack of symptoms and an increased prevalence of SCD, often documented.[70, 73] During early stages of the disease, structural changes to the myocardium may be absent, or subtle, which will be more confined to a localised area of the RV.[73] Called the "triangle of dysplasia", this localised area generally involves the inflow and outflow tract, as well as the apex of the heart.[77] Histological examination of the triangle of dysplasia typically shows fatty infiltration, fibrosis and myocyte loss (Figure 2.7).

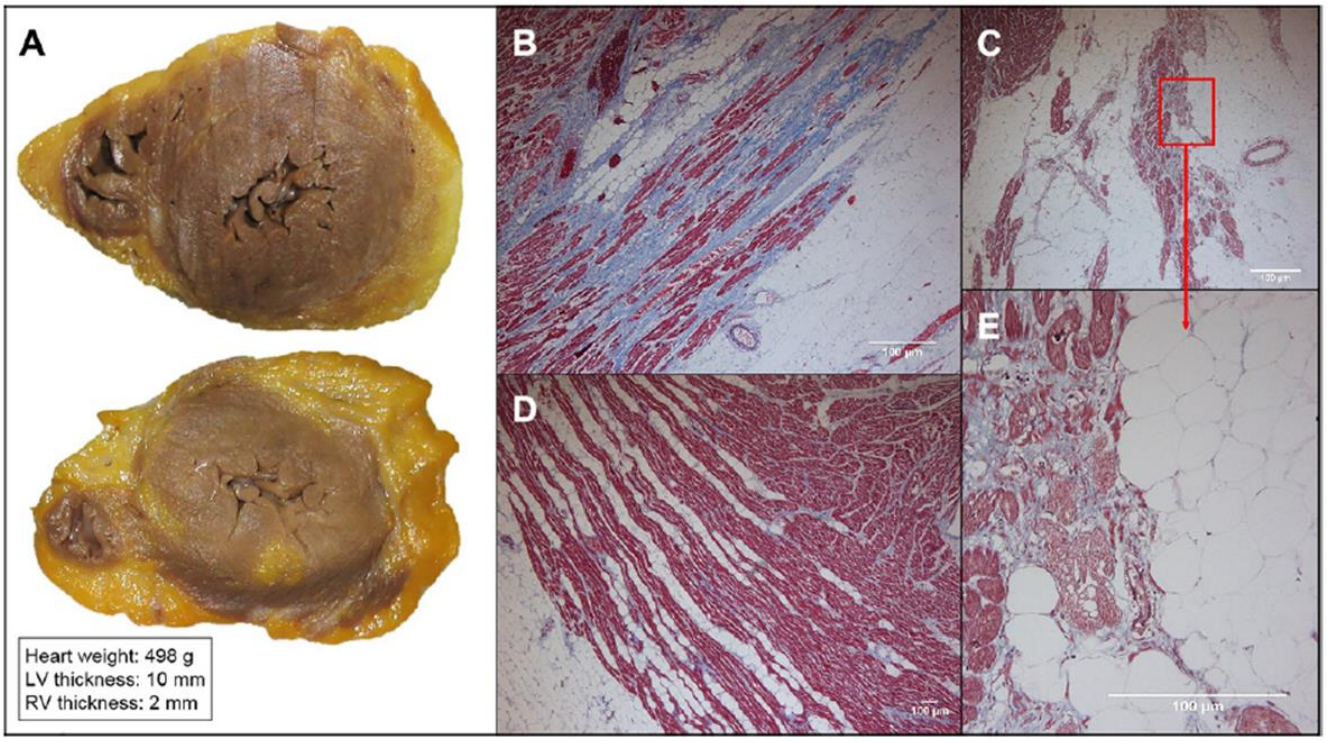

Figure 2.7. Macroscopic and microscopic examination of an ACM-diagnosed heart. A) Macroscopic examination at autopsy shows a cross section of the heart near the apex and the immediate above segment. Marked epicardial adipose infiltration with a thick epicardial band at the left ventricle is observed. B and C shows microscopic images of the posterior and lateral wall of the left ventricle, respectively, illustrating subepicardial fibrofatty infiltration with different amounts of fibrosis. D illustrates the right ventricle with mild lipomatosis in the external third myocardium without degeneration or replacement fibrosis. E shows myocyte degeneration.[76]

2.6.3.1 Genetic profile of arrhythmogenic cardiomyopathy

Inherited arrhythmogenic cardiomyopathy is caused by variants in genes encoding structural components of cardiac desmosomes and adherens junctions, which provide both functional and structural integrity of cardiomyocytes.[78] Although desmosomes play an intergral role in cell to cell binding, evidence have shown its role in regulating electrical channels and cell signalling as well.[79] Desmosomes, together with adherens junctions and gap junctions, form important intercalated discs (Figure 2.8) which connect adjacent cardiomyocytes, and is critical in maintaining mechanical and electrical connections for proper cardiac contraction.[80, 81] Variants in genes encoding these proteins have all been linked to cardiac arrhythmias, syncope and SCD.[78]

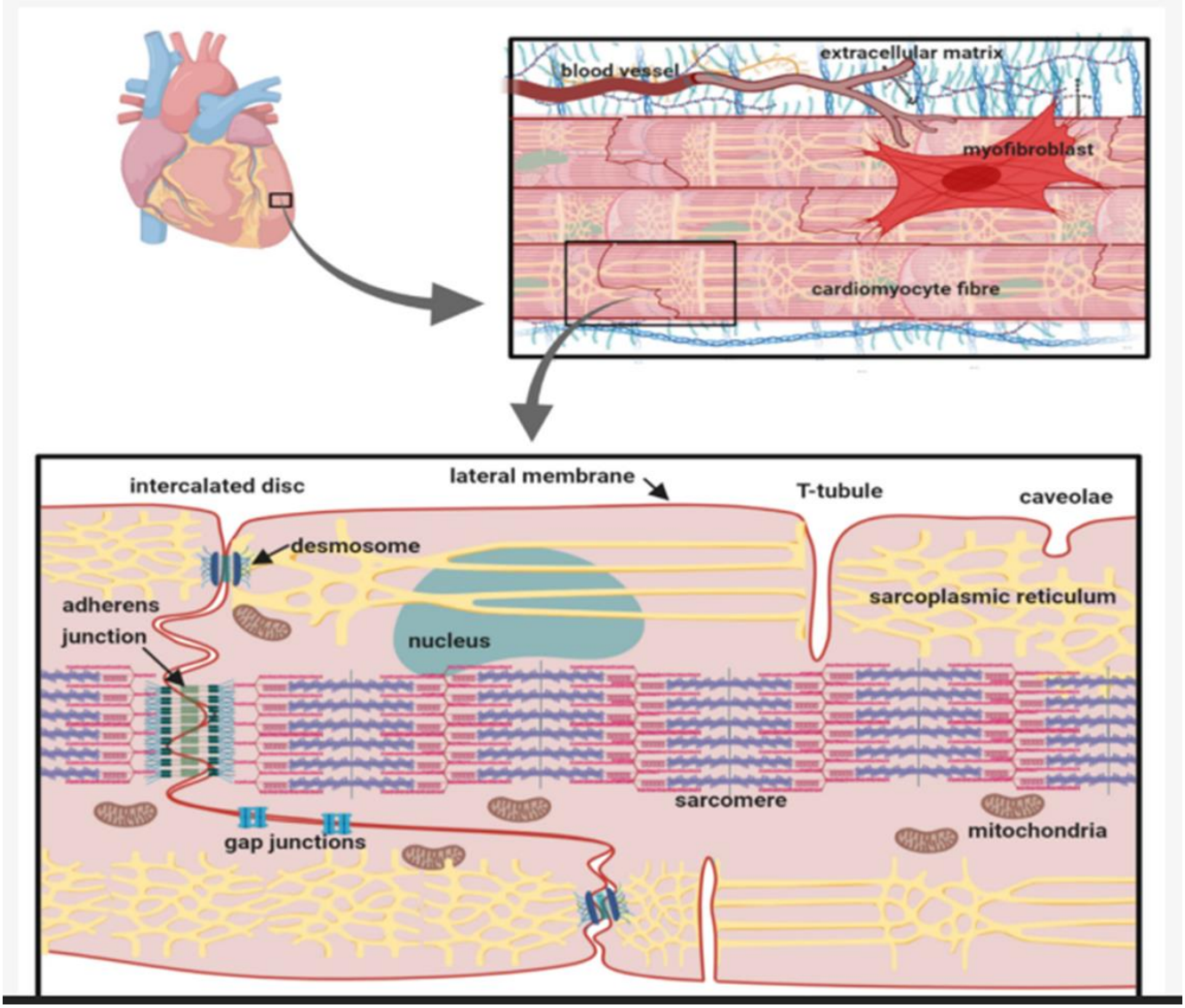

Figure 2.8. Graphical illustration of an intercalated disc between adjacent cardiomyocytes. The three protein components (desmosome, adherens junction and gap junction) form an intercalated disc which is responsible for the connection between two adjacent cardiomyocytes.[80]

Research studies have associated ACM with variants most commonly identified in 13 different genes (Table 2.3), of which the majority (up to 80%) occur in desmosomal genes.[82, 83] The desmosome comprises of five major desmosomal proteins, including plakophilin-2, desmoplakin, desmocolin-2, desmoglein-2 and plakoglobin, encoded by *PKP2*, *DSP*, *DSC2*, *DSG2* and *JUP*, respectively.[79, 81] Both desmocolin-2 (*DSC2*) and desmoglein-2 (DSG2) are transmembrane molecules and members of the cadherin family, responsible for cellular adhesion at desmosomal junctions.[78] Armadillo proteins, encoded by *JUP* and *PKP2*, are linkers in the desmosome-intermediate filament complex, which are also involved in signalling

pathways and modulation of cell behaviour.[78] Finally, desmoplakin (*DSP*; a member of the plakophilin-linker family), serve as a linker between plakoglobin and the intermediate filament complex.[78] Non-desmosomal genes include *DES*, *TMEM43*, *CTNNA3*, *LMNA*, *TTN*, *PLN*, *TGFB3* and *SCN5A*.[78] The *DES* gene codes for the main intermediate filament (desmin) in cardiomyocytes. Transforming growth factor β-3, a cytokine encoded by *TGFB3*, plays an important role in both cell adhesion and expression of desmosomal genes.[78] Transmembrane protein 43 (TMEM43) may play a role in the adipogenic pathway and be responsible for myocardial fibrofatty replacement. The *CTNNA3* gene codes for a molecule critical in maintaining tissue dynamics.[78]

Table 2.3. List of the 13 most prevalent genes implicated in arrhythmogenic cardiomyopathy.[82]

Gene	Protein name
PKP2	Plakophilin-2
DSP	Desmoplakin
DSC2	Desmocolin-2
DSG2	Desmoglein-2
JUP	Plakoglobin
TMEM43	Transmembrane protein 43
TGFB3	Transforming growth factor β-3
CTNNA3	Catenin α-3
DES	Desmin
LMNA	Lamin A/C
TTN	Titin
PLN	Phospholamban
SCN5A	Voltage-gated type 5 α-subunit

2.6.3.2 Clinical diagnosis and treatment of arrhythmogenic cardiomyopathy

Symptoms of ACM may include occasional or rapid palpitations, dizziness, arrhythmia, shortness of breath, chest pain and syncope.[73, 74] The diagnosis of ACM uses a set of major and minor criteria, based on structural, histological, ECG, arrhythmia, and familial/genetic features of the disease, which were proposed and updated in 2010 by an International Task Force.[77] Physical examination may include an ECG, chest X-ray and two-dimensional echocardiography. A full family medical history, complimented with genetic testing of at-risk family members, is also recommended.[74, 77] The main goal of treatment is to alleviate symptoms and prevent further progression of the disease. Treatment can include the use of various medications, including angiotensin-converting enzyme (ACE) inhibitors, antiarrhythmic medication, β-blockers, anticoagulants, and blood thinners. Some cases may require catheter ablation or implantation of an ICD.[74, 82]

2.7 Primary arrhythmogenic disorders (channelopathies)

Channelopathies, characterised by a lack of structural changes to the heart, are generally described as inherited arrhythmogenic disorders, associated with isolated electric dysfunction.[41, 84] This dysfunction is caused by variants in genes encoding cardiac ion channels and regulatory protein receptors, which are involved in the ionic control of the cardiac action potential.[41, 85] The cardiac action potential, which is generated by the activation and deactivation of ion channels, is responsible for the coordination between heart expansion and contraction and is therefore essential for proper heart function.[86] Ion channels are multimeric transmembrane proteins, consisting of pore-forming α-units and regulatory β-subunits, present in every cell type.[87] The continuous flux of ions across the plasma membrane, and the membranes of intracellular organelles, are carefully regulated through the ion channels.[87, 88] All the basic cellular and tissue processes like transepithelial transport, hormone secretion as well as muscle cell contraction depend on ion channel function, which in turn is largely affected by membrane potential and trans-axonal ionic concentration.[89] Ionic fluxes result in electrical currents across the membranes and determine the transmembrane electric potential differences, which is responsible for the initiation of cardiac action potentials.[86, 89]

Although ion channels are expressed in all tissue and organ cells throughout the body, the most recognized channelopathies are found to exist in neuro-muscular and cardiovascular disorders. This can be explained by the primordial roles the excitable cells, containing the voltage-gated ion channels, play in these two systems.[86] In the heart, the voltage-gated ion channels,

conducting Na^+, K^+ and Ca^{2+}- ions, play a major role in generating the action potential which coordinates the contraction of the upper and lower chambers of the heart, therefore maintaining a normal heart rhythm.[86] Disturbances in this regulated electrical-contraction pattern lead to unregulated electrical impulse propagation, with subsequent uncoordinated muscle contraction, ultimately resulting in a loss of pumping action.[86] A link between many human diseases and the dysfunction of ion channels (channelopathies) has been established, either as a result of genetic variants or acquired malfunctions of ion channels.[90] Figure 2.9 illustrates the role of cardiac ion channels in generating an action potential, as well as the negative effect a channelopathy has on the coordination of an action potential, and subsequently - the rhythm of a heartbeat.

Genetic variants linked to channelopathies are commonly identified in genes which code for the pore forming alpha subunit and regulatory proteins, such as enzymes or beta subunits, which in turn regulate the alpha subunit.[84, 91] Variants in hundreds of genes have been linked to channelopathies, and proved to predispose a person to the development of life-threatening arrhythmias and sudden death.[87] In fact, studies have shown that genetic alterations in channelopathy-related variants may account for up to 50% of unexplained SUD, especially in the young.[92, 93] The three most prevalent and epidemiologically relevant cardiac channelopathies, of which the majority of these variants are linked to, include long QT syndrome (LQTS), catecholaminergic polymorphic ventricular tachycardia (CPVT) and Brugada syndrome (BrS).[87, 88, 94]

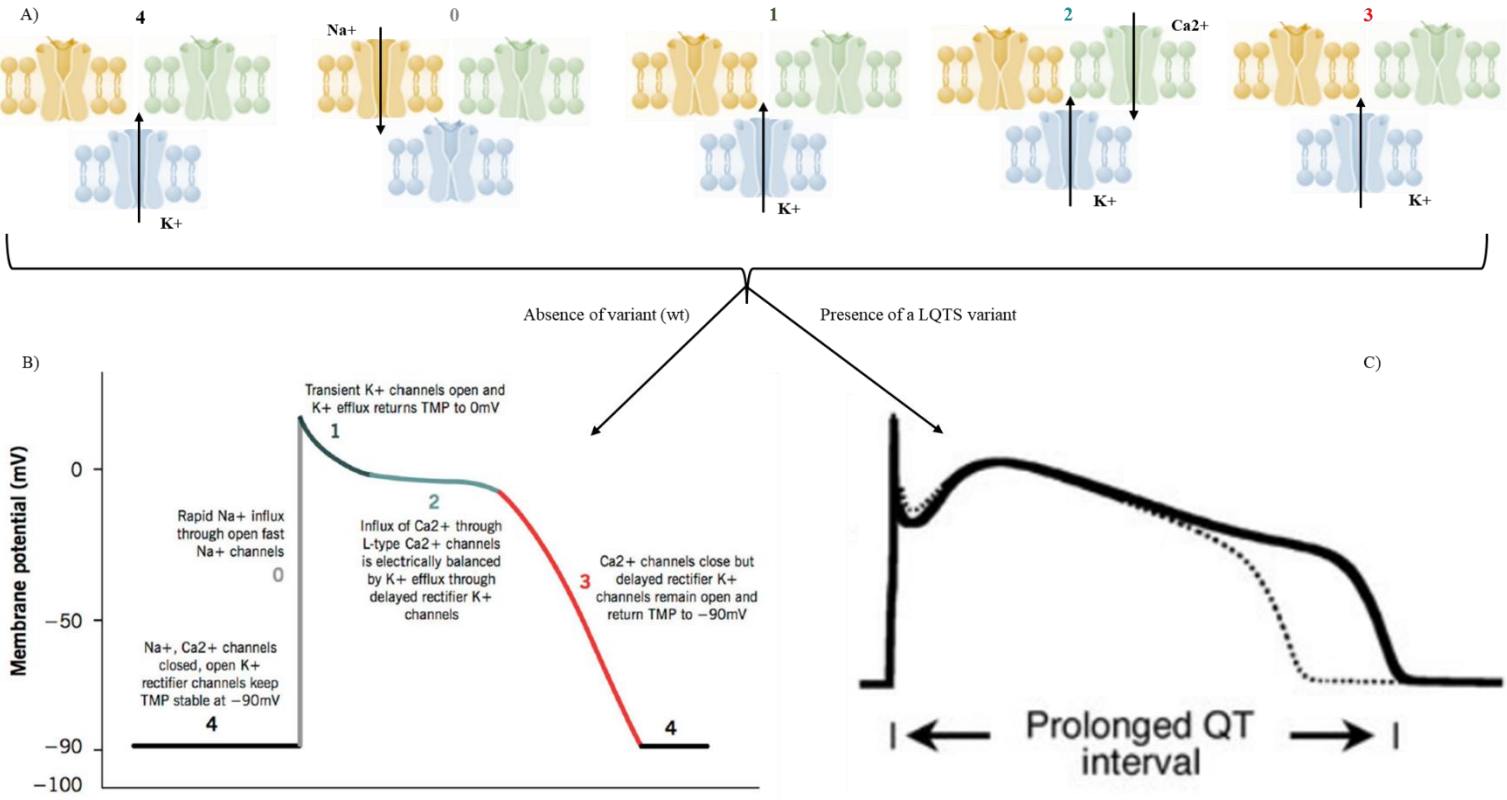

Figure 2.9. Summarised output of different types of ion channels leading to a cardiac action potential

A) Illustrates the different states of the Na^+, K^+ and Ca^{2+}-channels during each of the four phases of a cardiac action potential. B) Normal cardiac action potential. Phase 4 shows all gated Na^+ and Ca^{2+} channels are closed, while the K^+ channel remains open, creating a resting state with a membrane potential of -90 mV; phase 0 illustrates the depolarizing phase as a result of Na^+-influx due to the opening of these channels, causing the membrane potential to become rapidly positive at 40 mV; phase 1 shows the inactivation of the Na^+-channels and the opening of the K^+-channels during the repolarizing phase, resulting in the membrane potential to return negative due to the efflux of K^+-ions; phase 3 illustrates the hyperpolarizing phase created by the influx of Ca^{2+} whilst the K^+ channels remain open and the Na^+-channels remain inactivated, resulting in the membrane potential to become slightly more negative before returning to the resting membrane potential. C) Illustrates an abnormal (prolonged) cardiac action potential as a result of variants in a gene encoding a LQTS-relates channel, ultimately affecting the normal heart rhythm (in this case LQTS).[95]

2.7.1 Long QT syndrome

Long QT syndrome is an inherited primary arrhythmogenic disorder, characterised by prolonged cardiac repolarisation, which causes syncope, seizures and sudden death.[96] It mostly occurs in young and apparently healthy individuals, and is considered the most prevalent channelopathy and leading cause of sudden death in the young.[97, 98] The most common characteristic of LQTS is represented by a delayed repolarization of the ventricular cells.[98] This is attributed to either a result of the reduction in repolarizing (outward) currents, or an increase in depolarizing (inward) currents, and is associated with ECG manifestations of prolonged QT intervals, and T wave abnormalities.[98]

There are three main gene-specific triggers of LQTS manifestations, which include emotional stress, physical stress and rest/sleep without arousal.[91, 96] It is a genetically heterogenous condition, with the majority of LQTS cases inherited in an autosomal dominant manner.[97, 99] The less common recessive forms of LQTS are associated with severe cardiac phenotypes and congenital deafness.[97] The prevalence of inherited LQTS is estimated to be 1 per every 2,000 individuals.[88] However, reports have indicated that this might be an underestimation, given the heterogeneity of the disease leading to a miss-diagnosis in approximately two thirds of LQTS patients.[88, 98] In addition, an estimated 10-35% of patients present with a normal QT interval when measured on a resting 12-lead ECG.[96] This further contributes to the underestimated prevalence of inherited LQTS in the general population.[96, 100] The onset of symptoms usually occurs at a mean age of 12 years. Earlier onset of symptoms is typically associated with more severe outcomes.[98, 101]

2.7.1.1 Genetic profile of long QT syndrome

To date, a significant number of genetic variations, which varies between published literature, have been associated with LQTS.[97] Approximately 60-75% of clinically diagnosed patients test positive for variants in either one or more of the 17 different genes associated with LQTS.[100, 102] A list of these 17 genes is provided in Table 2.4 below.

Table 2.4. List of the 17 different genes associated with long QT syndrome.[102]

Gene	Protein name
AKAP9	A-kinase anchoring protein 9
ANK2	Ankyrin 2
CACNA1C	Calcium voltage-gated channel, subunit α1C
CALM1	Calmodulin 1
CALM2	Calmodulin 2
CALM3	Calmodulin 3
CAV3	Caveolin 3
KCNE1	Potassium voltage-gated channel, subfamily E, regulatory subunit 1
KCNE2	Potassium voltage-gated channel, subfamily E, regulatory subunit 2
KCNH2	Potassium voltage-gated channel, subfamily H, member 2

Gene	Protein name
KCNJ2	Potassium voltage-gated channel, subfamily J, member 2
KCNJ5	Potassium voltage-gated channel, subfamily J, member 2
KCNQ1	Potassium voltage-gated channel, subfamily Q, member 1
SCN4B	Sodium voltage-gated channel β-subunit type 4
SCN5A	Sodium voltage-gated channel α-subunit type 5
SNTA1	Syntrophin α-1
TRDN	Triadin

Studies have shown that three major genes are responsible for 75-90% of these variants: the potassium voltage-gated channel subfamily Q member 1 (*KCNQ1*), the potassium voltage-gated channel subfamily H member 2 (*KCNH2*) and the sodium voltage-gated channel α-subunit type 5 (*SCN5A)* gene.[96, 97] Loss-of-function variants in *KCNQ1*, encoding for the ion channel which mediates the I_{Ks} potassium current, cause long QT type 1 (LQT1) syndrome.[98, 102] The majority of arrhythmias experienced in LQT1 patients are triggered by exercise related stress.[70, 98, 102] Loss-of-function variants in *KCNH2*, encoding for the ion channel generating the I_{Kr} potassium current during repolarization, cause long QT type 2 (LQT2) syndrome.[102] In LQT2 patients the majority of events are triggered by emotional stress.[96, 102] Gain-of-function variants in *SCN5A*, encoding the sodium channel which generates the depolarizing I_{Na} sodium current, cause long QT type 3 (LQT3) syndrome.[97, 102] The cardiac events in LQT3 patients, which is considered the most lethal among LQTS, occur during a period of sleep/rest and are also associated with cases of SIDS.[89, 102] The higher lethality rate can be best shown by the 20% increased risk of sudden death presenting as the first clinical manifestation in LQT3 patients, vs. the 4% risk among LQT1 and LQT2 patients.[89, 102]

All three genes code for ion channel subunits which are involved in the generation of the cardiac action potential of the ventricular myocytes.[70, 89] Gene-suggestive ECG patterns for the three major types of LQTS have been described, although it does not serve as a pre-genetic test prediction.[89] Figure 2.10 illustrates the association between broad-based T waves and LQT1, notched or biphasic T waves and LQT2 and a long isoelectric segment followed by a narrow-based T wave associated with LQT3, recorded on an ECG.[103]

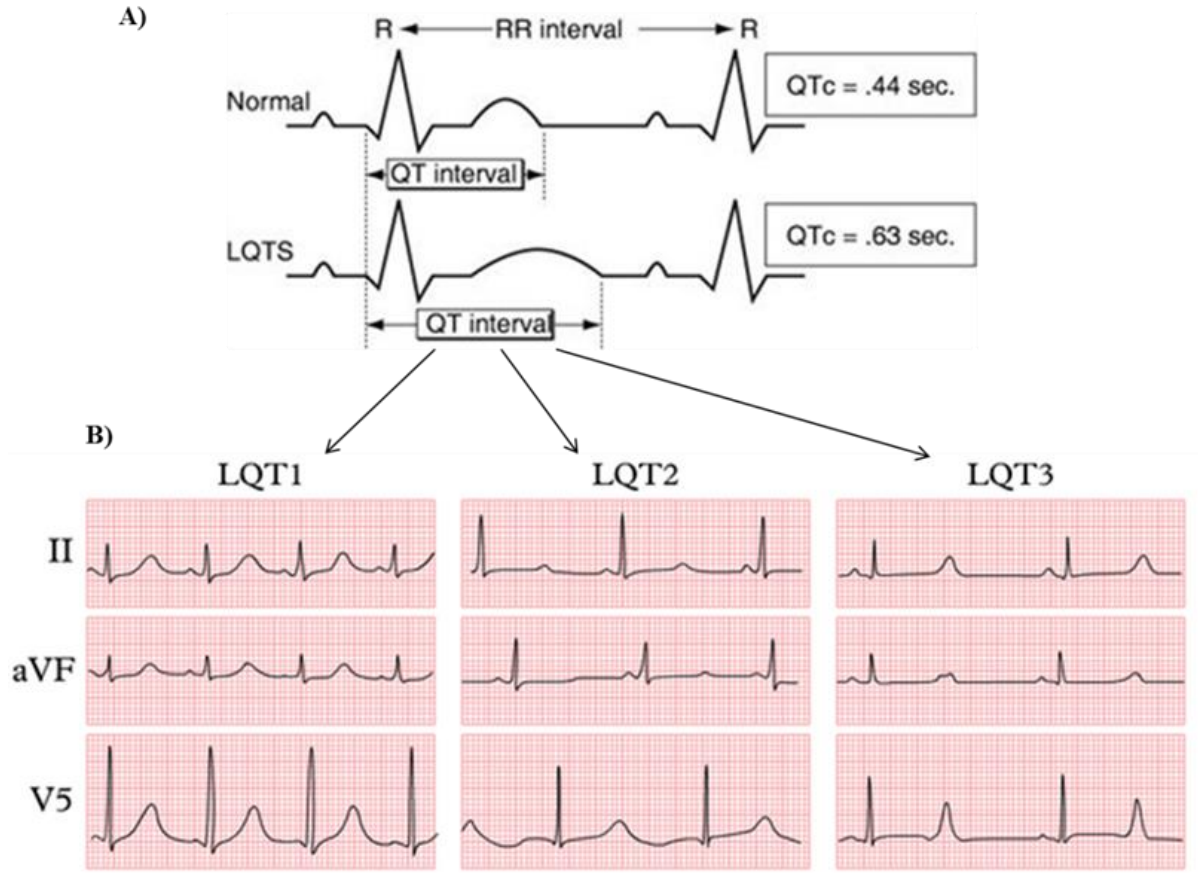

Figure 2.10. Comparison of ECG patterns between a normal QT interval and the three most common types of long QT syndromes. A) Shows an ECG pattern of a LQT1 patient, characterised by prolonged QT intervals associated with a broad-based T-wave. b) Shows an ECG pattern of a LQT2 patient, characterised by low amplitude and notched T-waves. C) Shows an ECG pattern of a LQT3 patient, characterised by long ST-segments with a late-appearing T-wave.[103]

2.7.1.2 Diagnosis and treatment of long QT syndrome

Long QT syndrome patients may experience symptoms such as palpitations, syncope, seizures and SCD.[96] A clinical diagnosis of LQTS is based on a personal and family medical history as well as 12-lead ECG of the patient. The duration of the heart-rate corrected QT (QTc) interval in unaffected individuals measures between 350 ms and 440 ms.[96, 97] Patients diagnosed with LQTS generally present with a prolonged QT interval above 470 ms, in combination with the above-mentioned symptoms, and the absence of other QT prolongation causes.[97, 99] Numerous LQTS-patients have a shorter QT interval, which reiterates the importance of a family medical history.[97] Patients diagnosed with LQTS should avoid QT-prolonging medications.[96] Treatment is available to alleviate symptoms and to prevent SCD, and may include β-blockers, which

have been shown to efficiently suppress cardiac events. Lifestyle changes, such as avoidance of high-intensity exercises and extremely loud noises, are recommended. The implantation of ICD's is also not uncommon in patients diagnosed with LQTS.[96, 98]

2.7.2 Brugada syndrome

Brugada syndrome (BrS) is a widely recognised inherited arrhythmogenic disorder, associated with a high risk of life-threatening cardiac arrhythmias and SCD.[104, 105] This syndrome is associated with one of three specific abnormal ECG patterns (see Figure 2.11), characterized by ST-segment elevations recorded in the anterior precordial leads.[105, 106] A global estimated prevalence of three to five per 1 000 people has been reported, with a higher incidence in South Asian populations.[106, 107] Brugada syndrome mostly affects young males, with the risk of lethal arrythmias highest in the third and fourth decades of life. Approximately 20% of SCD's, in which no structural cardiac abnormalities will be noted, can be attributed to BrS.[107-109]

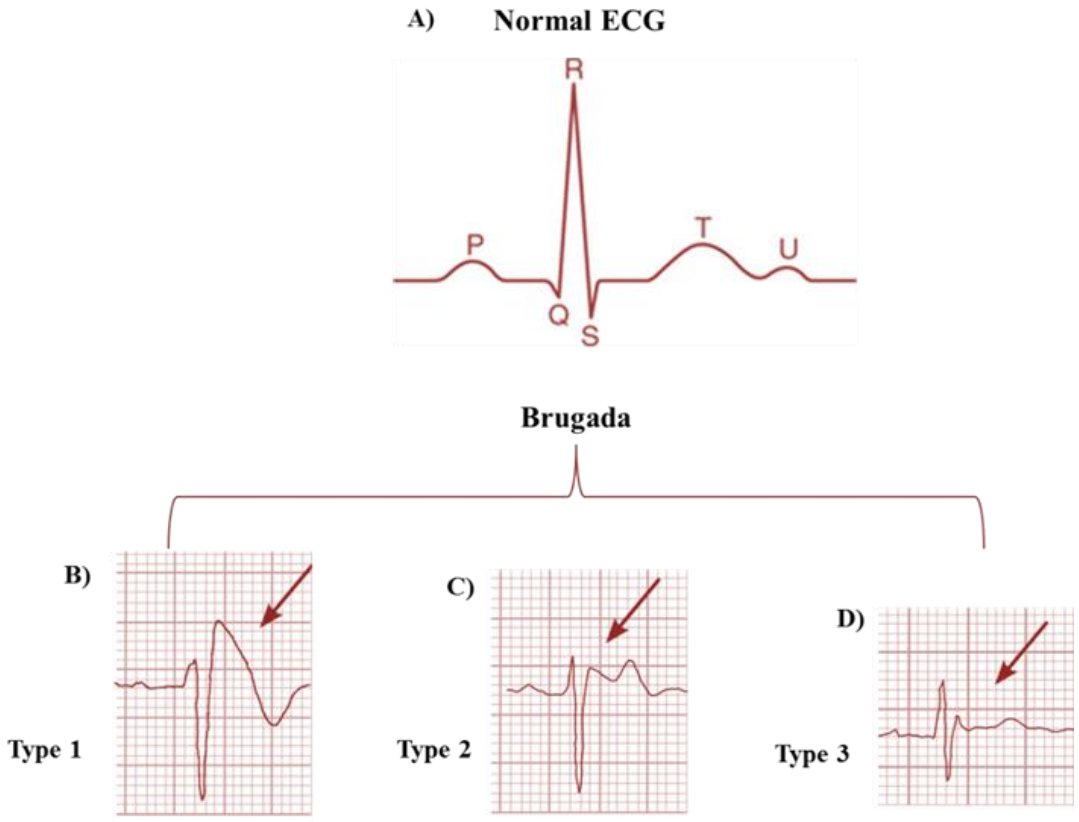

Figure 2.11. The three types of Brugada ECG patterns compared to a normal ECG pattern. A) Normal ECG pattern. B) Type 1 ECG pattern with pronounced elevation of the J point (indicated by arrow), a coved-type ST segment with an inverted T wave. C) Type 2 ECG pattern indicating a saddle-back ST segment elevated by >1mm. D) Type 3 ECG pattern in which the terminal ST segment is elevated <1mm.[110]

Lethal arrhythmias associated with BrS have been shown to originate in the right ventricular outflow tract (RVOT), starting with a short coupled extra systole, followed by a rapid polymorphic ventricular tachycardia.[105, 106, 110] As shown in Figure 2.11 above, type 1 ECG, which is the only pattern considered to be of diagnostic value, presents with a non-ischaemic coved-type ST segment elevation (≥2 mm) as a result of delayed sodium exit during repolarization. This ST-segment elevation is also followed by a recorded negative T-wave in the anterior precordial leads V_1-V_3.[105, 110] A type 2 ECG pattern, characterised by a saddle-back ST-segment elevation (≥2 mm) followed by a positive T-wave, as well as a type 3 ECG pattern with either a coved or saddle-back type ST-segment elevation (≤1 mm), are not considered to be of diagnostic value for BrS.[104, 106] The most common clinical manifestations associated with BrS are syncope, seizures and sudden deaths as a result of ventricular arrhythmias, usually occurring during a period of rest/sleep.[108, 110]

2.7.2.1 Genetic profile of Brugada syndrome

Brugada syndrome is inherited in an autosomal dominant pattern, with incomplete penetrance and variable expression, currently identified in only 30% to 35% of BrS-diagnosed patients.[111, 112] Due to its genetic heterogeneity, up to 24 genes encoding calcium, sodium and potassium channels, have been linked to BrS. Table 2.5 provides a list of these 24 genes.[108, 111] Although more than 450 different BrS-variations have been identified up to date, the majority (75%) is found in the *SCN5A* gene and are associated with a loss of function of the sodium channel.[104, 107] Furthermore, DCM-patients carrying *SCN5A* variants, in comparison to other genetic variants, have been linked with a higher susceptibility to cardiac arrhythmias and an earlier, more severe onset of symptoms.[108, 112] Currently, due to the lack of genotype-phenotype associations with BrS, the inclusion of genes other than *SCN5A* in the diagnostic panel is not recommended.[108, 112]

Table 2.5. List of 24 genes associated with Brugada syndrome.[108]

Gene	Protein name
ABCC9	ATP Binding Cassette Subfamily C Member 9
CACNA1C	Calcium Voltage-Gated Channel Subunit Alpha1 C
CACNA2D1	Calcium Voltage-Gated Channel Auxiliary Subunit Alpha2 Delta 1
CACNB2	Calcium Voltage-Gated Channel Auxiliary Subunit Beta 2
FGF12	Fibroblast growth factor 12
GPD1L	Glycerol-3-Phosphate Dehydrogenase 1 Like
HCN2	Hyperpolarization Activated Cyclic Nucleotide Gated
HCN4	Hyperpolarization Activated Cyclic Nucleotide Gated
KCND3	Potassium Voltage-Gated Channel Subfamily D Member 3
KCNE3	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 3
KCNE5	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 5
KCNH2	Potassium Voltage-Gated Channel Subfamily H Member 2
KCNJ8	Potassium Inwardly Rectifying Channel Subfamily J Member 8
PKP2	Plakophilin 2
RANGRF	RAN Guanine Nucleotide Release Factor
SCN5A	Sodium Voltage-Gated Channel Alpha Subunit 5
SCN10A	Sodium Voltage-Gated Channel Alpha Subunit 10
SCN1B	Sodium Voltage-Gated Channel Beta Subunit 1
SCN2B	Sodium Voltage-Gated Channel Beta Subunit 2
SCN3B	Sodium Voltage-Gated Channel Beta Subunit 3
SCN4B	Sodium Voltage-Gated Channel Beta Subunit 4
SEMA3A	Semaphorin 3A
SLMAP	Sarcolemma Associated Protein
TRPM4	Transient Receptor Potential Cation Channel Subfamily M Member 4

2.7.2.2 Diagnosis and treatment of Brugada syndrome

Although many patients diagnosed with BrS may remain asymptomatic, typical symptoms include palpitations, syncope, seizures or SCD, usually occurring under vagal triggers such as a period of rest and / or sleep.[105, 106] The diagnosis is based on a physical examination along with a thorough medical history, including that of family members.[110] Diagnostic tests typically include an ECG (sometimes accompanied with the administration of flecainide), echocardiography and / or electrophysiological testing and mapping.[110, 111] The implantation of an ICD is most effective in alleviating symptoms and preventing SD.[111]

2.7.3 Catecholaminergic polymorphic ventricular tachycardia

Characterised by adrenergic-induced bidirectional and polymorphic ventricular tachycardia, in the absence of any structural heart disease, catecholaminergic polymorphic ventricular tachycardia (CPVT) is considered one of the most lethal primary arrhythmogenic disorders, mostly affecting the young.[113, 114] Its prevalence has been estimated at one in every 10 000 individuals, with the first symptoms usually presenting between the ages of four and 12 years old.[115, 116] However, the onset and presentation of symptoms have been observed in older patients (40 years of age), as well.[115] Given its rarity in comparison to LQTS, the fact that the proportion of SCD's due to CPVT nearly equal those caused by LQTS, the severity and lethality of this disease cannot be overstated.[117] Research has shown that, without proper treatment, the mortality rate can rise up to 50% by the age of 35 years.[115, 116] It typically causes exertional syncope, seizures and sudden death.[116] It is heritable in an autosomal dominant manner similar to LQTS, but different in the fact that CPVT presents with a normal ECG at rest.[115, 116] Ventricular arrhythmias only arise when the heart frequency exceeds 120 to 130 beats per minute, caused by exercise or catecholamine stress.[115, 117] Catecholaminergic polymorphic ventricular tachycardia-related arrhythmias typically results from delayed after-repolarisations due to excessive calcium leak from the sarcoplasmic reticulum (SR).[117, 118] Figure 2.12 below illustrates the typical bidirectional ventricular tachycardia of an ECG recording for a CPVT patient during an exercise stress test.

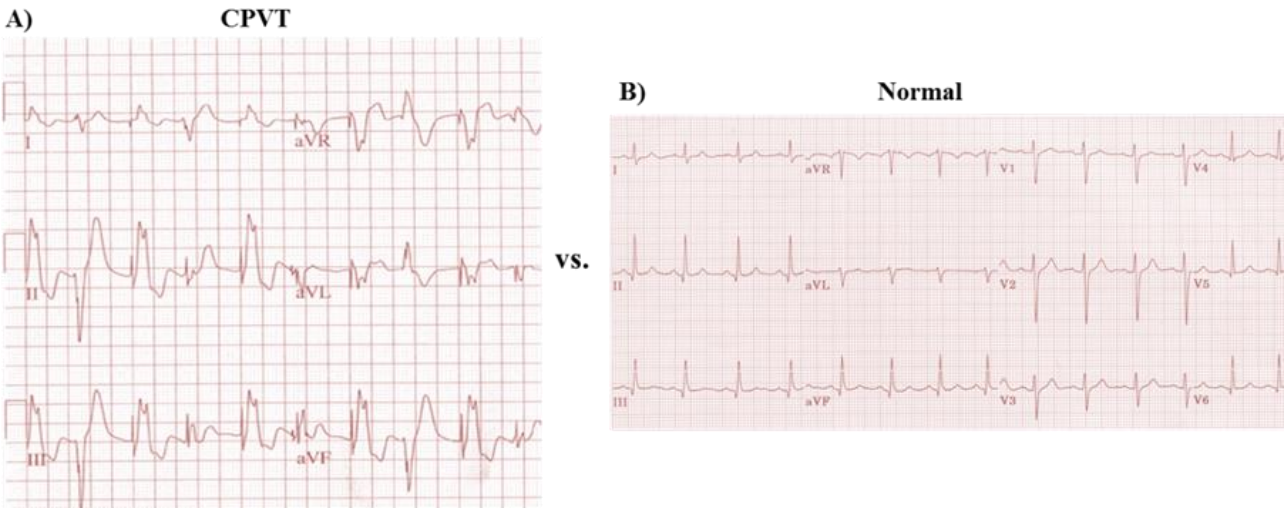

Figure 2.12. Comparison of electrocardiogram recordings between a normal individual and a patient diagnosed with CPVT. A) Shows an ECG recording illustrating typical bidirectional ventricular tachycardias in a CPVT patient during an exercise stress test. B shows a normal ECG recording in a patient without any heart disease.[116]

2.7.3.1 Genetic profile of catecholaminergic polymorphic ventricular tachycardia

Molecular studies revealed that the most common variants linked to CPVT are found in the *ryanodine receptor 2* (*RyR2*)-encoded cardiac ryanodine receptor/calcium release channel.[115] Inherited in an autosomal dominant pattern, this is annotated as type 1 CPVT (CPVT1).[115, 117] The ryanodine receptor is a component of calcium channels found in the membrane of the smooth sarcoplasmic reticulum, which is in charge of calcium release for electromechanical coupling.[114, 115] The majority of *RyR2* variants result in an increase in RyR2 channel function, causing a greater release of Ca^{2+} ions into the cytoplasm, and an ultimate delay in after-depolarisation during the action potential.[115, 118] Type 2 CPVT (CPVT2) is considered a rare form that arises in an autosomal recessive manner, with genetic variants in calsequestrin, encoded by the calsequestrin2 (*CASQ2*) gene.[115] Calsequestrin is a calcium storage protein responsible for the regulation of storage and release of Ca^{2+} ions into the SR. Variations in the *CASQ2* gene negatively affects the storage of Ca^{2+}, ultimately affecting its release into the cytoplasm.[115, 118] Approximately 65% of CPVT-diagnosed patients carry one or more variants in these two genes.[116] Some studies, although less than 1%, have recorded CPVT-associated variants in the *CALM1* and *TRDN* genes (Table 2.6).[116, 117]

Table 2.6. List of the four genes associated with inherited catecholaminergic polymorphic ventricular tachycardia.[116]

Gene	Protein name
CALM1	Calmodulin 1
CASQ2	Calsequestrin
RyR2	Ryanodine receptor 2
TRDN	Triadin

2.7.3.2 Diagnosis and treatment of catecholaminergic polymorphic ventricular tachycardia

Although CPVT patients usually remain asymptomatic, with the first onset of symptom being a SCD, symptoms such as dizziness, palpitations and syncope may occur.[117] Following the exclusion of a structural heart disease, along with a normal resting 12-lead ECG, an exercise stress test may reveal bidirectional or polymorphic ventricular tachycardias on an ECG recording.[115, 116] Genetic testing may also be recommended.[115] In order to prevent SD, treatment is critical for all diagnosed patients. Therapeutic medications include β-adrenergic receptor inhibitors and antiarrhythmics, such as flecainide.[115] In patients with a history of syncope or cardiac arrest following therapeutic treatment, the implantation of an ICD is recommended.[115, 117]

2.8 Importance of molecular screening in sudden unexpected deaths

Forensic molecular biology, also known as forensic molecular pathology, uses a molecular approach in not only studying, but also diagnosing, the underlying genetic basis of human disease and death processes.[119-121] International reports show that no other scientific discipline has embraced the application of molecular biology techniques for diagnostic purposes more than the field of forensic science and pathology, particularly in the medicolegal investigation of SD's in the young.[119, 122] Research has established that the majority (85%) of SD in the young are due to SCD, of which most (75%) are completely asymptomatic prior to death.[7, 10] Furthermore, approximately 90% of these SCD's can be attributed to inherited cardiac arrhythmogenic disorders, such as the previously discussed

cardiomyopathies and channelopathies.[7, 10, 123] Unfortunately, up to 53% of aforementioned cardiac disorders cannot be identified at autopsy, and post mortem molecular screening has proven to be invaluable in determining the cause of death.[92, 124]

The importance and benefit of such testing has far reaching consequences, beyond establishing the cause of death.[23, 125] Over 95% of inherited cardiac arrhythmogenic disorders are inherited in an autosomal dominant manner, implicating a 50% chance for first-degree relatives to inherit the same disease-causing variant.[125] Literature shows an approximate 40% to 50% of family members of SCD cases test positive for an inherited cardiac disorder.[126, 127] Considering the fact that these family members are often unaware of carrying disease-causing variants, along with the absence of any symptoms prior to a sudden death, the importance of post mortem genetic testing in SCD's cannot be overemphasised.[92, 125] The confirmed marked reduction in mortality associated with the administration of proper treatment, leaves no ethically arguable justification for allowing potential patients/family members at risk to remain undiagnosed and untreated.[125, 128]

International guidelines have been established with the aim to prevent criticism of case analysis in the medico-legal setting and protect surviving family members with possible genetic disorders.[10, 123] According to the 2020 published Asia Pacific Heart Rhythm Society / Heart Rhythm Society (APHR/AHR) expert consensus statement[10], genetic testing should be implemented in all unexplained SUD's and all SCD's suggestive of an inherited cardiomyopathy. The minimum requirements involve only targeted genetic testing of the major genes. However, the use of commercial panels consisting of a combination of up to 100 cardiomyopathy and channelopathy genes is becoming more common.[10, 123, 129] The improvement in technology and decrease in cost allows for the use of next generation sequencing (NGS), a high throughput sequencing technology capable of a more rapid and broader analyses of hundreds of genes.[130, 131] As a result, the yield of causative variants in SCD has increased, however the amount of generated data, and the expertise needed for the analysis thereof, has become more complex.[130]

2.9 Post mortem molecular screening in South Africa

Cardiovascular disease is one of the leading causes of death worldwide, with a reported ~85% occurring in low- and middle-income countries.[3, 7, 37] Cardiovascular disease is not only the second biggest killer in Africa, but also the mean age of these deaths has been recorded to be the youngest in the world.[37, 132] Considering the heritable nature of these deaths, one of

the greater health priorities in Africa should be a focus on preventing and treating cardiac diseases. There are key gaps in the knowledge base, especially on research priorities that focus on genetic causes of cardiac diseases specific to the African population.[37, 132, 133]

Approximately 2 000 young South Africans succumb to a SCD each year, with the majority of these deaths remaining unexplained after a full medico-legal autopsy had been conducted.[132] No forensic mortuary in SA has implemented post mortem genetic testing yet, with no data available pertaining to the prevalence and genetic aetiology of SCD's in the young. As discussed in this chapter, this has potentially dangerous implications, not only towards family members at risk, but also forensic pathologists who are mandated to establish the cause and mechanism of these SD's.

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Chapter 3

Long QT syndrome and sudden unexpected infant death

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Abstract

Long QT syndrome (LQTS) is an inheritable primary electric disease of the heart characterised by abnormally long QT intervals and a propensity to develop atrial and ventricular tachyarrhythmias. It is caused by an inherited channelopathy responsible for sudden cardiac death in individuals with structurally normal hearts. Long QT syndrome can present early in life, and some studies suggest that it may be associated with up to 20% of sudden unexplained infant death (SUID), particularly when associated with external stressors such as asphyxia, which is commonly seen in many infant death scenes. With an understanding of the genetic defects, it has now been possible to retrospectively analyse samples from infants who have presented to forensic pathology services with a history of unexplained sudden death, which may, in turn, enable the implementation of preventative treatment for siblings previously not known to have pathogenic genetic variations. In this viewpoint article, we will discuss SUID, LQTS and postmortem genetic analysis.

3.1 Sudden unexpected infant death

In most countries, sudden and unexpected death cases will be referred for routine medicolegal autopsy. Unfortunately, 70% to 80% of sudden unexpected deaths in infants (SUDIs) will remain unexplained, even after thorough investigation, which include a detailed postmortem examination including macroscopic examination with evisceration of all organs and all ancillary investigations such as histology, microbiology, virology and toxicology.[1-3] The Centre for Disease Control and Prevention (CDC) estimated in 2016 that 3500 infants die suddenly and unexpectedly each year in the USA.[4] A review study conducted in Wales reported the approximate prevalence of SUDI was 14% of all infant deaths recorded over a 2-year period (2010– 2012).[5] These unexplained deaths were previously defined as sudden infant death syndrome (SIDS).[6-9]

In 2013, Byard indicated a possible diagnostic shift in SIDS cases. During the 1990s, the continued monitoring of diagnostic practices and trends in infant deaths revealed the extent to which pathologists contributed to this diagnostic shift.[10,11] An increased awareness of the infant's position in relation with many of these sudden deaths enabled the pathologists to identify more cases of accidental asphyxia in relation to unsafe sleeping environments. Furthermore, Byard also documented an opposing component of the diagnostic shift, which involved the subjective reassignment of causes of death.[12] A specific trend was detected where many pathologists refrained from attributing the cause of death to SIDS and rather used terms such as undetermined cause of death or asphyxia-related death.[12] Reasons for this shift include the absence of pathognomonic diagnostic features for SIDS and the insufficient findings that may be present in cases of accidental or intentional smothering.[12-15] Pathologists have rather taken to determining these deaths as sudden unexplained infant deaths (SUIDs), which are defined as 'the death of an infant less than one year of age in which investigation, autopsy, medical history review and appropriate laboratory testing fail to identify a specific cause of death. SUID includes cases that meet the definition of sudden infant death syndrome.[16]

3.1.1 Aetiology of SUID

Studies show SUID occurred more frequently in infants between the age of 2 and 4 months and rarely after the age of 8 months.[1,3,17-20] Death apparently occurs during periods of sleep, suddenly and without warning.[1,17] A uniformly accepted triple-risk model was first introduced in 1994 by Filliano and Kinney, and highlighted the interaction of multiple risk factors that increase the probability of SUID.[21] These risk factors are divided into three groups: a

vulnerable infant, a critical developmental stage and exogenous stressors.[21] Current theories still suggest that SUID is a complex event and infants may die when risk factors in each of these groups occur at the same time: a vulnerable infant (which can include an underlying genetic mutation/predisposition) in a critical developmental stage (peaks at 3 months) with an exogenous stressor such as asphyxia challenges from unsafe sleeping practices, soft bedding, the exposure to second-hand smoke as well as bacterial and viral infections.[3,17-22]

In the 1990s, there was a decrease in the number of SUID cases, which could probably be attributed to the introduction of the 'back-to-sleep' campaign. However, since then, the SUID rate has remained stable and is the number one cause of death in postneonatal infants in most developed countries.[3,18,19] The large number of published studies strongly suggests that SUID may be multifactorial and may include metabolic and genetic disorders, as well as deficits in serotonin receptors in the brainstem,[23,24] which motivates for the continuous research into possibly preventable causes.[1,3,17-19] Fortunately, with the rapid development in technology and continued studies on genetic risk factors, postmortem molecular analysis proved to be an invaluable tool in determining a possible cause of death in many SUID cases.[25,26]

A postmortem genetic study conducted by Wang *et al.* showed that in their cohort of infants, African-Americans had the highest risk of dying suddenly, followed by Hispanics and Caucasians, with the Asian population at smallest risk.[25] Arnestad *et al* suggested an intriguing hypothesis with regard to possible modulating factors involving specific genetic variants and the associated ethnicity of the individual.[18] Comparing the ethnic/racial differences as described above with the occurrence of SUIDs indicates that the rate of SUIDs among lower income/socioeconomic deprived racial and ethnic groups showed an increase compared with groups within a higher income bracket.[11] American Indians, African-Americans, Maoris from New Zealand as well as Aboriginals in Australia all have a higher incidence of SUID.[1,17] No definitive explanation for this increased occurrence could be found; however, a complex interaction between genetic and environmental risk factors may be the underlying basis—in keeping with the triple-risk model.

3.2 SUIDs and channelopathies

Numerous studies have been done on the association of serotonin receptor deficits in SUIDs.[2,3,8,27] In addition to serotonin receptor deficits, other studies, which have also received increased attention over the past few years, have shown that one of the possible preventable causes of SUIDs is that of inherited, life-threatening cardiac arrhythmic disorders, commonly

referred to as cardiac channelopathies.[26,28-30] These channelopathies, which include long QT syndrome (LQTS), Brugada syndrome (BrS) and catecholaminergic polymorphic ventricular tachycardia (CPVT), are a result of pathogenic variants in genes that code for cardiac ion channels.[25,26,28,30] These genes play a role in the cardiac electrical conduction physiology, thus affecting the normal heart rhythm.[8,31-33]

The first evidence pertaining to cardiac conduction disorder in SUIDs is that of Keeton *et al*.[34] who in 1977 reported on the diagnosis of severe conduction disorders in six cases of acute life-threatening events (ALTE) in infants. These infants received proper treatment before any fatalities occurred.[34] Data obtained from six separate studies indicate that the overall prevalence of pathogenic variants in cardiac ion-channel-related genes in SUID victims may be 20%. These variants seem to have a fatal outcome when coinciding with certain stressors/triggers such as fever and asphyxia,[18,35-39] which is especially relevant when considering that asphyxia is commonly encountered in SUID especially in a so-called unsafe sleeping environment. The American National Society of Genetic Counselors,[40] Ackerman,[35] Michaud *et al.*, [33] Arnestad *et al.*[18] and Davis *et al.*[29] all reported that an average of 15% of SUID cases occurred due to inherited cardiac arrhythmic disorders. It was suggested that the putative cause of death in one of every five SUIDs may be the result of pathogenic variants in a cardiac ion-channel-related gene.[13,36]

The 'peak' age of SUIDs is commonly accepted as 3 months.[1,3,17-20] However, in infants identified with a channelopathy, the age range at time of death varies greatly between each study cohort, with no peak age of death noted among all the studies. Some recorded a range between 4 days and 12 months while others recorded median ages at death varying from 2 months up to 6 months.[18,20,25] The exact mechanism to which this relatively broad span of age range can be attributed to is still unknown. It should be kept in mind that the broader definition of SUID includes all infants up to the age of 1 year.

Some variants in genes linked to the different channelopathies seem to be more prevalent in certain population groups while rare in others.[18,25] A number of studies indicate a higher prevalence of certain genetic variants among the Maori population,[1,17,20] whereas other specific variants, especially the SCN5A H558R amino acid replacement, are associated with a higher prevalence in the Caucasian population group.[41] In contrast, certain common variants found in the Hispanic and Asian populations are identified as disease-causing variants in the Caucasian

population.[18] The SCN5A-A572D variant, which has previously been described as disease-causing, is a common variant found in the Norwegian population.[18]

3.2.1 Long QT syndrome

The channelopathy that has the strongest link to SUIDs is LQTS.[2,26] LQTS is an inherited arrhythmogenic disorder associated with the ionic control of the cardiac action potential. Clinical outcomes include syncope, seizures and sudden death, especially in young and apparently healthy individuals.[2, 26] Of note, all LQTS features, including a postmortem examination that remains unexplained, are similar to SUID.[2,26]

LQTS is a genetically heterogeneous condition, with the majority of cases inherited in an autosomal dominant manner.[26, 42] The less common recessive forms of LQTS are associated with severe cardiac phenotypes and congenital deafness.[31,42,43] The characteristics of LQTS are represented by a delayed repolarisation of the ventricular cells. This is attributed to the reduction in repolarising (outward) currents, or an increase in depolarising (inward) currents, and is associated with ECG manifestations of prolonged QT intervals and T wave abnormalities.[43-45] The prevalence of inherited LQTS is estimated to be 1 in 2500 live births.[18,26,28] However, reports have indicated that this number might be an underestimation since the likelihood for a misdiagnosis exists in approximately two-thirds of patients with LQTS due to the heterogeneity of the disease.[13,25,26,28] In addition, an estimated 10%–35% of patients present with a normal QT interval when measured on a resting 12-lead ECG.[44, 46] This further contributes to the underestimated prevalence of inherited LQTS in the general population.[28,42-44,46] The onset of symptoms usually occurs at a mean age of 12 years, with an earlier onset of symptoms typically associated with more severe outcomes.[42-44,47]

To date, a significant number of genetic variations have been associated with LQTS.[3,18,48] According to the Human Gene Mutation Database, more than 600 long QT variations have been identified in several ion-channel-related genes.[49] Three major genes are responsible for 75%–90% of these variants: the potassium voltage-gated channel subfamily Q member 1 (KCNQ1), the potassium voltage-gated channel subfamily H member 2 (KCNH2) and the sodium voltage-gated channel type V alpha (*SCN5A*) gene.[43,44,50] Loss-of-function variants in *KCNQ1*, encoding for the ion channel that mediates the slow delayed rectifying potassium current (IKs), cause long QT type 1 (LQT1) syndrome. Most arrhythmias experienced in LQT1 patients are triggered by exercise-related stress.[31,33,43,51] Loss of-function variants in *KCNH2*, encoding for the ion channel generating the rapid delayed rectifying potassium current (IKr)

during repolarisation, cause long QT type 2 (LQT2) syndrome.[44, 47] In LQT2 patients, the majority of events are triggered by emotional stress.[43,44,47] Gain-of-function variants in *SCN5A*, encoding for the sodium channel that generates the depolarising INa sodium current, cause long QT type 3 (LQT3) syndrome.[42,43,52-54] The cardiac events in LQT3 patients, which are considered the most lethal among LQTS, occur during a period of sleep/rest and have been reported in SUID cases.[20,26,44,53] The higher lethality rate can be best explained by the 20% increased risk of sudden death presenting as the first clinical manifestation in LQT3 patients versus the 4% risk among LQT1 and LQT2 patients.[20,43,44]

3.2.2 Long QT syndrome and SUID

Of all the channelopathies, LQTS is the most prevalent disorder associated with SUIDs[3,20,26,55,56] as well as sudden death in the young.[28,33,57,58] Postmortem genetic testing in SUID cases demonstrated that 13.9% of cases with identified variants in the LQTS genes have pathogenic clinical significance.[13,28]

A large population-based study conducted on the clinical association between a prolonged QT interval in ECGs and an increased risk of SUID analysed 33 034 ECGs of healthy Italian babies, which were taken on the third or fourth day of life.[31] In each case, the QT interval was measured and the infants were followed for 1 year. In total, 34 infants died, of which 24 deaths were attributed to SUID (incidence of 0.7 per 1000 live births).[31] A prolonged QT interval was recorded in 12 of the SUID cases (50%), whereas none of the survivors, or infants who died of other causes, demonstrated a prolonged QT interval.[31] As a result, Schwartz *et al*[31] calculated the OR for SUID in infants with a prolonged QT interval as 41, an OR significantly higher than that of prone posture and maternal smoking.[13]

A more recent follow-up study on the association of LQTS with an increased risk for SUID involved a comprehensive 19-year prospective review of ECGs, which were recorded between 15 and 28 days of life in more than 44 000 infants.[59] Molecular screening was performed in 28 infants who presented with a marked QT interval prolongation, which showed that 14 of these infants (50%) were carriers of potentially pathogenic LQTS-related variants.[59] All neonates who presented with a prolonged QT interval received successful treatment with a β-blocker (propranolol).[59]

An association between LQTS variations and SUID victims has been recognised by two well-known case studies:[56,60] one on a SUID case and the other on an infant with documented ventricular fibrillation who survived an ALTE. These two studies ultimately paved the way for

other cohort studies on SUIDs.[56,60] One study showed a 5.2% prevalence of LQTS causing variations in a study cohort of 68 SUID cases.[35] Another study, composed of 201 SUID cases and 187 controls, found that 9.5% (95% CI 5.8 to 14.4) of SUID cases carried functional LQTS pathogenic variations, whereas none of the controls did.[18] A third study, conducted by Wang *et al*, 25 identified variants of probable pathogenic significance in 19 of 141 SUID cases (13.5%).

Long QT type 3 syndrome seems particularly important in SUID cases as studies demonstrated a link between SUID and a predominance of *SCN5A* gene variants.[18,19,53,54,61,62] In three different studies, molecular screening identified pathogenic variants linked to LQTS in a number of SUID cases, where variations in the *SCN5A* gene comprised respectively 50%, 68.4% and 50% of all identified variants.[28,52,63] This could be ascribed to the known genotype–phenotype correlations that suggest patients with LQT3 (*SCN5A*) variants may experience a higher lethality rate, mostly occurring during sleep, compared with patients who have variants in other genes involved in LQTS.[18,20,64]

The *SCN5A* gene is a member of the voltage-gated sodium channel family, with at least nine sodium channel α-subunits in this family identified from various human tissues.[28,32,61] The genomic location of *SCN5A* is on the short arm of chromosome 3 at position 21 (3p21).[61] It consists of 28 exons with an approximate span of 80 000 base pairs (80 kb).[31,32,41,61] The *SCN5A* gene encodes for a protein (sodium (Nav1.5) ion channel poreforming α-subunit) of 2016 amino acids with a calculated molecular weight of 227 kDa.[62, 63] The voltage-gated Na+ channel α-subunit contains six transmembrane-spanning segments (S1–S6) found within each of four homologous domains (DI– DIV).[28,32,52,63] It is restrictively expressed in the myocardium and plays a critical role in heart excitability and conduction.[28,31,46] The integral membrane protein produces the fast inward Na+ current that is responsible for the depolarising phase of the cardiac action potential.[13,28,46] Variations of this gene cause a persistent Na+ current with a subsequent prolongation of the ventricular action potential, essentially resulting in an inherited predisposition to ventricular arrhythmias and sudden death, seen in several cardiac diseases, including LQT3.[29,54,65-67]

3.3 Postmortem genetic testing and SUID

Postmortem genetic testing is increasingly being recommended as a routine procedure in the investigation of any sudden unexpected death.[25,26,68,69] Sudden death is often the sentinel event of 10%–40% of LQTS, as most genetic variant carriers are unaware that the disease is

present.[26,69-71] The importance of postmortem genetic testing lies not only in determining the cause of death at autopsy but also serves as a diagnostic tool in identifying relatives (of the deceased) at risk for the same inherited genetic disorder.[26,29,69] Over 95% of cardiac genetic disorders (in the general population) are inherited as an autosomal-dominant trait.[69] Furthermore, the risk for subsequent siblings dying from SUID is reported to be between 3.7-fold and 10-fold (although this is regarded as controversial by some).[2]

Various treatment modalities for channelopathies are available, with the three most common/effective being that of β-adrenergic blockers, antiarrhythmic agents and the use of implanted device therapy.[13,28,63] Although β-adrenergic blockers are still considered the first line of therapy in LQTS, a lower efficacy in treatment for *SCN5A* variant-associated LQTS has been reported.[13,28] Evidence obtained from both clinical and *in vitro* settings suggests a successful counteraction of mexiletine against the aberrant persistent Na+ current, which ultimately shortens the QT interval in *SCN5A* pathogenic variation carriers.[28,63] In addition, flecainide also proves to shorten QT intervals in many *SCN5A* pathogenic variation carriers; however, concerns regarding the safety of this specific therapy have been raised.[13,28,63] Quinidine and sotalol, both class III-type antiarrhythmic agents, proved to be beneficial to patients diagnosed with BrS.[13,28] Patients with LQTS and BrS seem to benefit significantly from implantable defibrillators, whereas patients suffering from conduction disorders were managed successfully with pacemaker implantation as treatment option.[13,28,52,63]

The profound value of existing treatment for these arrhythmic diseases may be best portrayed by Wilders' comparison of two similar case studies and their associated clinical outcomes.[13] Both cases involved neonates with documented arrhythmias and a prolonged QT interval, though only one of the cases received treatment on presentation of clinical symptoms.[13,72] The first case was reported by Southall *et al*[72] on a neonate who presented with arrhythmias in utero and bradycardia for the first 9 days of life; however, on day 10, a normal heart rate was recorded and the baby was discharged from hospital. Unfortunately, the baby suffered a sudden and unexpected death 3 days later, which, after an autopsy investigation, remained unexplained. On retrospective analysis of the available ECG recordings, a substantial QT interval prolongation was observed.[13,72] In contrast, a second neonate who also presented with arrhythmias in utero and a 24-hour ECG illustrating a prolonged QT interval with frequent premature ventricular beats received a β-blocker (propranolol), which proved to be successful in treatment.[13,72] Since the disease is potentially treatable, the ability of molecular testing to identify these channelopathies as a cause of death in SUID cases will allow for testing and

initiation of preventive therapies not exclusively to just family members at risk but even in future pregnancies.[26,65,69] Unfortunately, as a consequence of the almost silent nature of the disorder (sudden death being the first 'symptom'),[26,69-71] genetic testing would be difficult to implement as a preventative measure before any SUID occurrence or without strong suspicion due to known family history. The role of postmortem genetic testing in this age group will be to establish the prevalence of these variations in the general population.

3.4 The role of molecular testing

Considering all the data, the question arises as to whether a routine postmortem genetic analysis should be implemented in all sudden infant deaths that remain unexplained after a thorough autopsy investigation.

First, as described by Skinner,[52] the identification of pathogenic variations in SUID victims does not necessarily prove causality even if their clinical significance has been proven to be disease causing in other families or by *in vitro* testing. This leads to the old dictum where the forensic pathologists need to decide if the person died with the disease or as a result thereof. However, evidence exists (referenced throughout this paper) that SUID may be due, in a minority, to cardiac channelopathies such as long QT syndrome.

Second, the question arises as to what extent forensic pathologists are legally and ethically bound to conduct these tests. It can be argued that the forensic pathologists need to determine the cause, and in some cases the manner, of death. The next-of-kin in these cases might benefit tremendously from testing, which in some instances could include ECG screening followed by genetic testing.[43,46,71] This would necessitate close working relationships between forensic pathologists and a team of other experts including molecular biologists, cardiologists and genetic counsellors. The importance of findings by forensic pathologists over the years has drastically led to the reduction of certain mortalities—for example, the implementation of restraint devices in road traffic accidents—and cannot thus be negated.

Third, in many instances, finances are not available to routinely conduct these tests. On average, screening only for variations in the *SCN5A* gene, which is reported to be found in 5.2% of SUID victims,[13,28,52,63] would cost approximately US $570 per case in South Africa (the cost of similar genetic testing may differ between countries). However, these costs will be dramatically reduced in the event of implementation of routine genetic testing in all unexplained SUID cases, as targeted genetic testing of known hotspot regions will be used instead of whole exome sequencing. Research should also focus on screening the general

population to determine which variations occur naturally in any given population. A recent molecular study conducted on South African SUIDs (unpublished data) revealed eight specific exons of the *SCN5A* gene as definite hotspot regions particular to this population. In effect, the costs of postmortem genetic testing, refined to those eight hotspot regions, in a single SUID case, would amount to approximately US $143. Considering the reduced costs, which should continue to decline due to advances in technology, one might argue that ethical issues far outweigh financial concerns with regard to targeted postmortem genetic testing in applicable SUID cases.

The question will always remain as to which genes should be tested for in each case. According to the Heart Rhythm Society/ European Heart Rhythm Association guidelines, targeted postmortem mutational analysis in all sudden unexpected deaths between 0 and 40 years of age is recommended.[30,73] In countries such as Australia and New Zealand, all sudden and unexpected deaths are mandated to undergo targeted postmortem genetic testing.[30,69] In 2015, the Swiss Society of Legal Medicine recommended that all sudden unexpected deaths under the age of 40 should be subjected to postmortem genetic testing.[73] In a recent study conducted by Sanchez *et al*, [73] next-generation sequencing (NGS) postmortem genetic analyses showed that in 13.4% of sudden unexplained death cases (between 0 and 10 years of age), a disease-causing variation linked to an inherited cardiac arrhythmic disorder (LQTS, BrS and CPVT) was identified and diagnosed as the cause of death.[73] In the remaining 31.9% cases, in which variants considered possibly pathogenic could not be fully defined as the cause of death, a necessity for family members to consider further genetic evaluation was established.[73] As a result of their findings, they recommend that NGS genetic analyses should be performed on all unexplained sudden deaths below the age of 40.[73]

In our opinion, interdisciplinary centres should conduct large studies in order to attempt identifying the true incidence of these cases. Prospective and retrospective studies could be undertaken. At most large medicolegal death investigation centres (which are often linked to tertiary academic institutions), forensic pathologists have established archives of formalin-fixed, paraffin-embedded (FFPE) tissue samples, which can serve as a (sometimes only) source of material that contains critical genetic information valuable to molecular testing.[74,75] Several studies have reported the successful, though not necessarily ideal, use of FFPE tissue samples in retrospective postmortem mutational analysis of previously admitted SUID cases.[53,57,74,75] This raises an important issue pertaining to a possible difference in cost between the usage of FFPE tissue samples versus more traditional samples such as DNA extracted from blood. From

experience working with FFPE tissue samples as a source of DNA for postmortem genetic testing, costs increase dramatically compared with using blood samples as the source of DNA. However, the rise in cost almost completely depended on factors associated with the incorrect conditions/circumstances surrounding the retention, fixation and storage of FFPE tissue samples. When prescribed guidelines were followed for the retention and fixation of FFPE tissue samples (fixed in formalin for a maximum of 24 hours, cleared in xylene and embedded in a paraffin block), DNA extraction and subsequent molecular applications were equal in quality, be it at lower concentrations, when compared with DNA extracted from blood. Thus, the difference in cost between using these two sources of genetic material for genetic testing may, in fact, be insignificant and therefore highlights the crucial importance of appropriate sampling/storage of all retained autopsy samples.

Combining resources and including all infants (regardless of the manner/cause of death) in testing for specific genetic variations could provide data on the most commonly encountered variations for each subset. Although this would most definitely be a very costly undertaking, identifying the specific genetic variations and their associated hotspot regions could prove cost-effective in the long term as more focused testing (which will be more affordable) could be undertaken.

Knowledge gained from the results of these tests could be imperative for adequate genetic counselling of parents of subsequent cases and provide closure to families who were previously informed that no cause of death was identified. This will assist in providing closure and planning options (such as genetic testing) for all siblings, adding significant value in the possible prevention of future similar cases to all individuals involved.[43,75]

Thus, ethical and reasonable justifications compel us to seek a molecular diagnosis of LQTS in an infant whose sudden death remains unexplained despite a thorough autopsy and ancillary investigations, and should therefore be considered in all medicolegal settings.[52]

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Chapter 4

Case report – The added value of molecular-based diagnostics in the African forensic medical setting

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This chapter followed the editorial style of the CVJA.

Abstract

Sudden unexpected infant death (SUDI) is reported to be an extraordinarily high burden in sub-Saharan Africa, with the incidence rate in South Africa among the highest in the world. Advances in molecular-based diagnostics allow researchers to identify numerous underlying inherited cardiac arrhythmogenic disorders in many SUDI cases, with a predominance of variants identified in the *SCN5A* gene. Such cardiac arrhythmogenic-related sudden deaths generally present with no structural alterations of the heart that are macroscopically identifiable at autopsy, therefore highlighting the importance of post mortem genetic testing. We report on a significant genetic finding that was made on a SUDI case in which the cause was ascribed to an acute bacterial pneumonia but it was still subjected to post mortem genetic testing of the *SCN5A* gene. The literature shows that many SUDI cases diagnosed with inherited cardiac arrhythmogenic disorders have demonstrated a viral prodrome within days of their death. It is therefore not uncommon for these cardiac disorders in infants to be mistaken for flu, viral upper respiratory tract infection or pneumonia, and without the incorporation of post mortem genetic testing, any other contributory causes of these deaths are often disregarded. This study highlights the need for research reporting on the genetics of inherited cardiac disorders in Africa.

Keywords: channelopathies, dilated cardiomyopathy (DCM), inherited cardiac disorders, post mortem genetic testing, *SCN5A*, sudden unexpected death in an infant (SUDI)

Sudden deaths in infants are still considered one of the leading causes of infant mortality worldwide and have also been reported to be an extraordinarily high burden in sub-Saharan Africa (SSA).[1-3] According to Duncan *et al*.,[4] for most countries, the rate of sudden unexplained infant deaths (SUIDs) [or the previously termed sudden infant death syndrome (SIDS) cases] is reported at approximately 0.2–0.5 per 1 000 live births. The most recent published incidence rate for South African SUID cases was 1.06 per 1 000 live births for the white population and 3.41 for infants from the mixed-ancestry population group, respectively.[2]

The investigation into SUDIs and child mortality remains a high-priority research area in South Africa.[2-3,5] It has universally been accepted that a SUID case can very rarely be explained by a convenient and simplistic 'single-cause' mechanism, but instead is attributed to a complex event with an increase in incidence when risk factors such as vulnerability, a critical period in development and exogenous stressors all intersect at the same time (triple-risk model proposed by Filiano and Kinney).[4,6,7]

One of these risk factors, and a possible preventable cause of SUIDs, which has received increased attention over the past few years is inheritable cardiac arrhythmogenic disorders.[1,3,4] Although these inherited cardiac disorders in SUID cases have primarily been associated with electrical conditions (channelopathies), recent studies have identified variants in genes encoding structural proteins, thereby suggesting a cardiomyopathy as a possible cause of death as well.[1,8-10]

Previous studies demonstrated a link between SUIDs and a predominance of *SCN5A* gene variants. This could be explained by the known genotype–phenotype correlations that suggest patients with *SCN5A* variants may experience a higher mortality rate, mostly occurring during sleep, compared to patients suffering from variants in other genes involved in inherited cardiac diseases.[1,8]

Advances in molecular-based diagnostics allow researchers to identify numerous underlying inherited cardiac arrhythmogenic disorders that have been misdiagnosed in many SUID cases.[1,11] In many developing countries, including Africa, there is still a significant lack as far as forensic molecular diagnostics is concerned, mainly due to financial and resource constraints.[12] As a result, the case in this study was subjected to retrospective post mortem molecular analysis of only the most prevalent gene (*SCN5A*) associated with SUID, in order to identify any possible pathogenic variations associated with an inherited cardiac disease, which may have predisposed this infant to a sudden death.

4.1 Case report

We report on a case of a two-month-old male infant of African ancestry whose mother found him unresponsive in his crib during a scheduled nap. Upon emergency medical services (EMS) arrival, the infant was declared dead at the scene without any medical care being administered. No written clinical history/records were available, however his mother reported him having a recent cold for which she administered cough medication. The mother also reported an increase in crying and that the infant struggled to feed.

Due to the sudden and unexpected nature surrounding the death, the body was admitted to the Pretoria Medico-Legal Laboratory for further medico-legal investigation, in accordance with the Inquests Act 58 of 1959. A complete macroscopic autopsy examination was conducted, which externally revealed the deceased to be of average physique and nutritional state. No injuries were noted on the body.

Upon internal examination, the intracranial examination showed no gross pathological changes. Examination of the heart revealed no abnormalities involving the epicardium. The myocardium and heart valves appeared normal. The lungs appeared congested and oedematous. On cut surfaces, the lungs showed sharply defined edges, had a friable texture and contained muco-purulent fluid. Examination of the stomach revealed contents of a milk-like residue and it was noted that the gastric mucosa appeared normal. As a result, no macroscopic cause of death could be identified at autopsy.

Toxicology results revealed only trace amounts of theophylline, a bronchodilator, which is in keeping with the history of cough medication administered to the infant. No sedatives could be detected in the blood specimen.

Histological examination of the thymus, brain and heart showed no obvious pathological changes. Sections of the heart showed no evidence of myocyte hypertrophy, nucleomegally or interstitial fibrosis. Sections of the lungs showed a mild mononuclear interstitial infiltrate with thickening, congestion and focal haemorrhage (Fig. 4.1). Focal intra-alveolar neutrophilic exudate was also noted in the lungs, as seen in Fig. 4.2. The features noted in the lungs were found to be in keeping with an acute bacterial pneumonia. Henceforth, the primary medical cause of death was acute bacterial pneumonia.

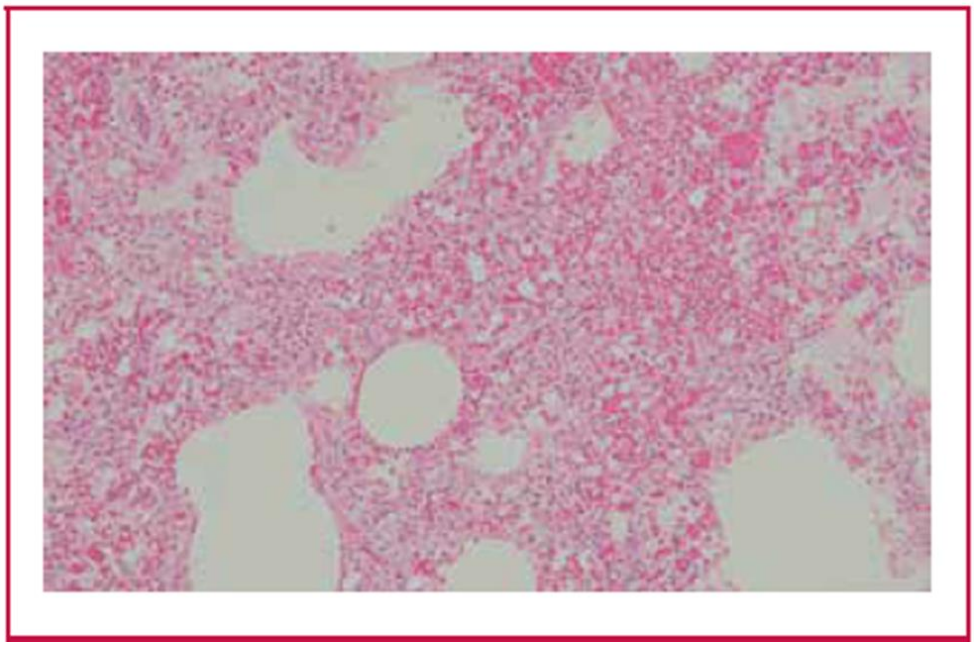

Figure 4.1 Haematoxylin and eosin stain of the lungs shows a mononuclear interstitial infiltrate with thickening, congestion and fresh focal haemorrhage

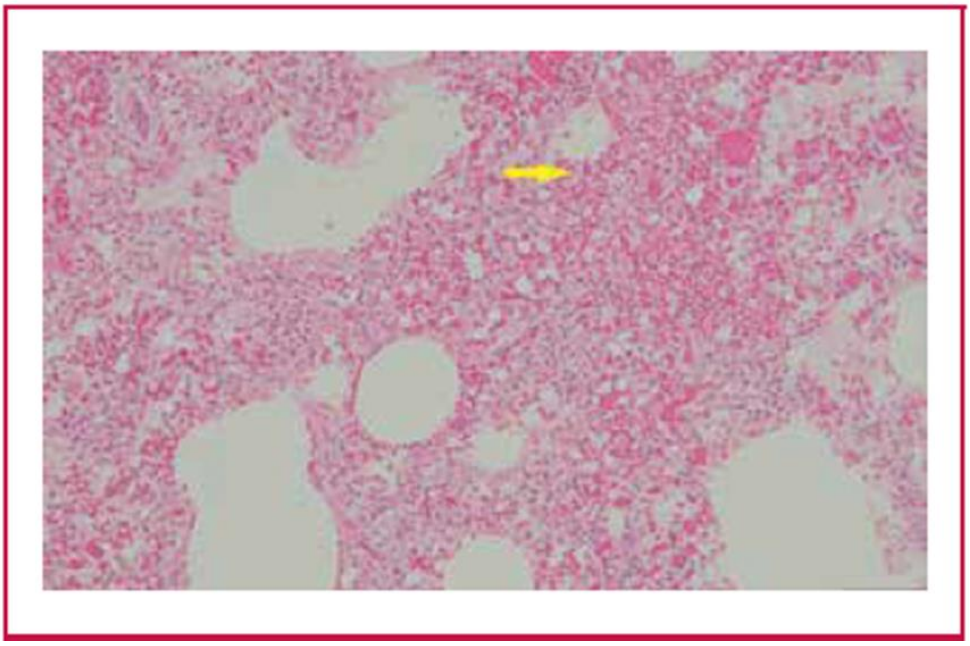

Figure 4.2 Haematoxylin and eosin-stained slide of the lungs showing a mixed intra-alveolar infiltrate chiefly composed of macrophages, neutrophils, fresh haemorrhage and oedema

4.2 Results

Genetic analysis revealed two different variations in exon 28 of the *SCN5A* gene.[13] The first was a novel heterozygous variation (c.5566G>A) in the coding DNA sequence. This missense variation leads to a G>A nucleotide change in codon 1856, with an amino acid change of alanine (Ala) to threonine (Thr) (p.A1856T) (Fig. 4.3). Due to the functional difference between these two amino acids, the possibility of this variation affecting the protein structure is high. The PolyPhen-2 online algorithm predicted this variant to be probably damaging with a score of 1.000.

The second heterozygous single-nucleotide variation, c.5668G>A, is registered on the Atlas of Genetic Cardiac Variation database, with an uncertain clinical significance, likely associated with dilated cardiomyopathy (DCM). The G>A nucleotide change in codon 1890 leads to an amino acid change of glutamic acid (Glu) to lysine (Lys) (p.E1890K) (Fig. 4.4). This variant is considered by 87.5% of algorithms to be likely damaging, predicting an adverse effect on the protein structure.

The E1890K variant is a non-conservative amino acid substitution, which would likely impact on the secondary protein structure as these residues differ in polarity, charge, size and/or other properties. This substitution occurs at a position that is conserved across species, and *in silico* analysis, predicts this variant to be probably damaging to the protein structure/ function. More recently, p.E1890K has been registered on the NCBI database under rs766875593, with an uncertain clinical significance associated with various channelopathies rather than a cardiomyopathy.

Recent studies reported on the identification of this variant in SUID cases associated with the long-QT syndrome (LQTS) as well as Brugada syndrome (BrS). The Genome Aggregation Database (gnomAD) and the Exome Aggregation Consortium (ExAC) reported on an allele frequency of 0.00001 and 0.00002, respectively. However, it is important to note that these allele frequencies are not representative of the African population since no studies have been done to provide statistics on this occurrence.

Wang *et al*.[14] reported on the identification of this variant in a two-month-old infant, whose cause of death could not be determined after a thorough autopsy, scene investigation as well as all ancillary investigations had been conducted. A review of the case history indicated a prone sleeping position at the time of death, with a history of a recent cold, similar to our case study.

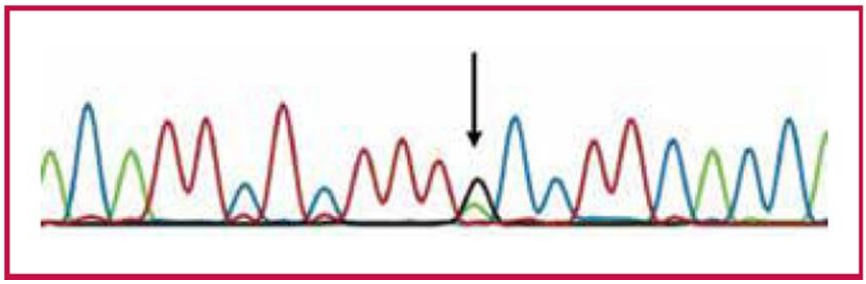

Figure 4.3 Heterozygous single-nucleotide variation p.A1856T (c.5566G>A) identified in exon 28 of the *SCN5A* gene

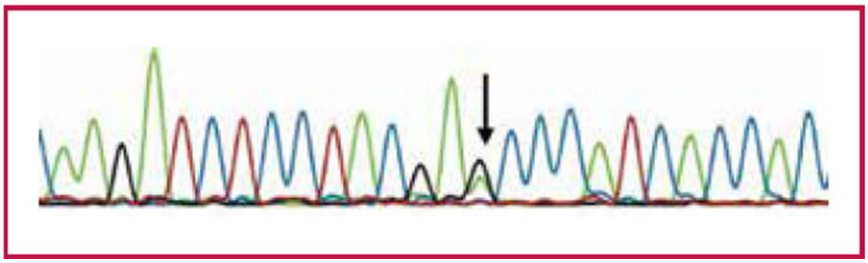

Figure 4.4 Heterozygous single-nucleotide variation p.E1890K (c.5668G>A) identified in exon 28 of the *SCN5A* gene

4.3 Discussion

Two heterozygous missense variations in the *SCN5A* gene were identified in this SUID. Bearing in mind that a cause of death (bacterial pneumonia) had been established for this case prior to genetic testing, the results were deemed significant, although unexpected, in explaining the full circumstances surrounding the death.

The *SCN5A* gene encodes a protein, sodium (Nav1.5) ion channel pore-forming α-subunit, that is expressed only in the myocardium and performs a critical role in heart excitability and conduction.[15-17] The integral membrane protein produces the fast-inward Na+ current that is responsible for the depolarising phase of the cardiac action potential. Variations in this gene cause an increased persistent Na^+ current, with a subsequent prolongation of the ventricular action potential, essentially resulting in an inherited predisposition to ventricular arrhythmias and sudden death, seen in several cardiac diseases.[4,18] Previous studies demonstrated a link between SUID and a predominance of *SCN5A* variants, more commonly associated with channelopathies.

Channelopathies are generally described as inherited cardiac arrhythmogenic disorders associated with isolated electric dysfunction caused by variants in genes encoding for cardiac ion channels and regulatory protein receptors, which are involved in the ionic control of the

cardiac action potential.[1,14] A link between many human diseases and the dysfunction of ion channels (channelopathies) has been established, either as a result of genetic variants or acquired malfunctions of ion channels.[13,17]

The three most common and epidemiologically relevant genetic heart channelopathies include LQTS, BrS and catecholaminergic polymorphic ventricular tachycardia (CPVT).[1,13,14] Although the involvement of numerous susceptibility genes has been identified, most of the variants (especially in SUID cases) have been located in the *SCN5A* gene, predominantly linked to LQTS and BrS.[13-15] Post mortem genetic studies have implicated channelopathy associated variants in 10 to 15% of SUID/SUDI cases.[1,8,14]

The most common clinical manifestations associated with LQTS and BrS are syncope, seizures and sudden death as a result of ventricular arrhythmias, usually occurring during a period of rest/sleep. Of particular note is that these channelopathy related sudden deaths generally present with no macroscopically identifiable structural alterations of the heart at autopsy.[4,8,15,16] Our case study was found to carry two variations, of which one has been documented with an uncertain clinical significance, although associated with LQTS, BrS and DCM.

Numerous studies have reported on the diversity of the phenotypic and genotypic expression of the *SCN5A* gene with variations linked to other arrhythmogenic disorders, including DCM, progressive familial heart block type 1 and sick sinus syndrome.[8,16,19] Reports of SUID cases where genetic variations associated with cardiomyopathies are increasingly identified in structurally normal hearts should prevent the tendency of eliminating associations between *SCN5A* variations and DCM.[1,8,20-22]

Cardiomyopathies can be described as a group of heart diseases that affect the structure and function of the myocardium, which can all lead to heart failure, arrhythmia and even sudden death.[9,12,23] The most common types of cardiomyopathies include hypertrophic cardiomyopathy, DCM, restrictive cardiomyopathy and arrhythmogenic cardiomyopathy.[12,21,23] Although it is generally associated with cardiac alterations macroscopically identifiable at autopsy, it is not uncommon for a cardiomyopathy to be inadvertently missed in SUID cases, which usually present with a macroscopically normal heart.[1,8,22,23]

Studies have been reported that genetic variations in cardiomyopathy-related genes, which may cause arrhythmia and sudden death, have been identified in SUID cases presenting without any cardiac changes. Research suggest that this might be explained by the progressive nature

of cardiomyopathy, whereby in the first stages of the disease the myocardial changes may be so incipient that it may not be visible at autopsy.[1,8,20,24]

The genetic basis of DCM in infants commonly demonstrates phenotypic overlap. Reported cases of DCM due to *SCN5A* variations identified in long-QT syndrome type 3 (LQT3), shows that not only can it result from structural changes in the myocytes, but also from altered calcium ion handling.[10,15,16,19-21] These inherited genetic susceptibilities in infant cases have been proven to play an important role in how the cardiac muscle responds to environmental and infectious factors.[11,22,24]

Researchers believe that variations in the *SCN5A* gene, with its associated higher risk of lethal arrhythmias, are linked to an increase in an infant's critical vulnerability to certain infections. Consequently, acute viral infections are regarded as one of the provocative factors associated with sudden death in infant channelopathy and/or DCM cases.[11,20,23,24] In fact, many of these SUID cases (diagnosed with inherited cardiac arrhythmogenic disorders) demonstrated a viral prodrome within days of their death. Such infants often present with respiratory signs, extreme sleepiness, difficulty in feeding and increased fussiness prior to death.[11,23,24]

It is not uncommon for inherited channelopathies and/ or cardiomyopathies in infants to be mistaken for flu, viral upper respiratory tract infection or pneumonia, and without the incorporation of post mortem genetic testing, any other contributory causes of these deaths are often disregarded.[1,8,11,20] Consequently, it is even of greater importance for countries with a high burden of infectious diseases to be especially aware of these findings, as there might be a reasonable tendency to overcall minor findings of viral infection in these SUID cases.[1,2,11,20]

Genetic testing is considered an ideal risk-assessment tool, not only for channelopathies, but for cardiomyopathies as well, due to its ability to identify patients at risk prior to overt disease development.[1,10,22-24] The use of post mortem genetic testing in SUID cases can benefit family members, especially those from poor communities, by providing the first indication of a familial cardiac arrhythmogenic disorder. Ultimately this will allow for the opportunity of preventative intervention, which can be used to avoid the progressive onset of the disease.[8,10,15,22,23]

For decades now, the undeniable benefit of post mortem genetic testing in SUID cases, especially those that remain unexplained, has been widely recognised worldwide.[1,4,8] The continued advancement in molecular diagnostics and its associated decrease in costs has allowed for expanded molecular testing using cardiac gene panels and next-generation sequencing.[1,4,9,24] Although this is not a novel concept to most first-world countries, it still eludes the radar of many medical professionals practicing in an economically and resource

strained country. These countries, including South Africa, have not yet been conducting post mortem genetic testing in unexplained SUID cases, at least not routinely.[2,3,7,12] The greatest benefit of such testing is not to define the cause of death, but rather the highly disease-specific diagnostic, therapeutic and prognostic benefit derived from subsequent genetic screening of family members of the deceased.[1,8,15,16]

In addition, disease-causing variants in the *SCN5A* gene have been reported as a possible predisposing factor of SUID, providing an apparent aetiology of arrhythmias due to secondary challenges/risk factors such as complicating lower respiratory infections, which are generally tolerated in infants not carrying such genetic variations. Considering South Africa's burden of infectious diseases coupled with a high infant survival rate in most of these cases, a more scrutinised and in-depth investigation into those SUID cases that typically present with no more than minimal findings such as the presence of a mild infection, should be considered.[2,12]

4.4 Conclusion

There is a lack of research reporting on the genetics of channelopathies and cardiomyopathies in Africa. The fact that cardiomyopathies are deemed an endemic form of non-communicable diseases, of high importance in the largely low-income communities in SSA, proves the need for local research on this topic. The results from this case study demonstrate the possible impact molecular diagnostics can have on identifying potential inherited cardiac disorders. Additionally, it highlights the occurrence of misdiagnosis of SUID cases in our population, or the possibility of an incomplete understanding pertaining to the circumstances surrounding these deaths. Further molecular testing may provide better knowledge as to why certain infants do not survive these viral and/or bacterial infections.

This case study aimed to create awareness on this subject among medical professionals, especially those practicing in resource-strained countries. Hopefully, this will motivate for more collaborative research and investigation to gain a better understanding of the unique genetic diversity and its associated inherited diseases in SSA.

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Chapter 5

Inherited cardiac arrhythmogenic disorders in a South African cohort of sudden unexplained deaths in the young

This chapter was written as a research article for submission to the journal of Forensic Science, Medicine and Pathology (Forensis Sci Med Pathol).

This chapter followed the editorial style of Forensic Sci Med Pathol.

Abstract

Sudden cardiac death is deemed a major global public health concern. In sub-Saharan Africa, including South Africa, there is a lack of reliable statistics on the incidence of SCD, even though a fourfold increase in noncommunicable diseases, largely due to cardiovascular diseases, has been reported. Considering that sudden cardiac deaths contribute to an estimated 50% of all cardiovascular deaths, it highlights South Africa's need for research into better detection, treatment and prevention of sudden cardiac deaths. The aim of this study was to identify an inherited cardiac arrhythmogenic disorder, caused by variants in cardiomyopathy and arrhythmia-related genes, as a possible contributing factor to the cause of sudden cardiac deaths.

Next generation sequencing identified a total of 178 different missense variants among the entire study population (n = 66);164 were known, documented variants whereas the remaining 14 were novel. A total of 127 variants were of like benign significance, 33 were variants of unknown significance, whereas the remaining six variants were of likely pathogenic significance.

Post mortem genetic testing provided evidence of a genetic arrhythmic/cardiac conduction disorder as the probable pathogenic basis for 9% of sudden unexpected death / sudden unexplained infant death cases. Targeted next generation sequencing of 16 prevalent genes are recommended for routine testing in all unexplained sudden unexpected death / sudden unexpected infant death cases in South Africa.

5.1 Introduction

In today's world of molecular genetics, numerous forensic medical institutions understand the concept - and perhaps more importantly - appreciate the value of post mortem genetic testing (or the so-called molecular autopsy).[1-3] Unfortunately, this mainly applies to first world countries and not economically - and resource - strained countries. The term 'molecular autopsy' can be described as the use of post mortem genetic testing to identify genetic variants associated with, or causative of, a disease, in order to help determine or better understand the cause of death (usually that of a sudden unexpected death).[4, 5] A sudden unexpected death (SUD) is defined as "a natural, unexpected fatal event that occurs within one hour of the beginning of symptoms, or 24 hours in cases where the death was unwitnessed, in an apparently healthy subject or in one whose disease was not so severe that such an abrupt outcome could have been predicted".[6, 7]

The majority of SUD's (60 – 90%) are cardiac-related, which is then termed a sudden cardiac death (SCD).[8, 9] Worldwide, SCD is deemed a major public health concern and receives great attention pertaining to the development of better detection, treatment, and ultimate prevention thereof.[8-10] The estimated global incidence of SCD ranges between 15 - 159 cases per 100, 000 inhabitants per year, with approximately four to five million global deaths per annum and constitutes 20% of all deaths in Western societies.[10-12]

The causes of SCD's are greatly dependent on the age of the deceased, with ischaemic heart disease (IHD) the most common cause of such a death in the older population.[13, 14] In the younger population, (≤ 45 years), up to 90% of cases are caused by inherited cardiac diseases of which the majority comprises of cardiomyopathies and arrhythmogenic disorders.[4, 15, 16] Unfortunately, microstructural cardiomyopathy changes can be overlooked at autopsy, whereas arrhythmogenic disorders cannot be macro - or - microscopically diagnosed after an autopsy investigation.[17, 18] Research has shown that the cause of up to 50% of these SCD's remains unexplained after an autopsy investigation, as these cases can only be diagnosed through the use of post mortem genetic testing.[15, 19, 20] Thus, many first world countries have implemented post mortem genetic testing as routine procedure when investigating SCD's in their young population.[9, 10]

In sub-Saharan Africa (SSA), including South Africa, there is a lack of reliable statistics on the incidence of SCD, even though it has been reported that SSA has seen a fourfold increase in noncommunicable diseases (NCD), largely due to cardiovascular diseases (CVD).[8, 21, 22] Apart

from being the second biggest killer in SSA, the mean age of CVD's has also been recorded as the youngest in the world.[21, 23] Considering that SCD's contribute to an estimated 50% of all CVD's, it highlights the importance of acknowledging SCD as a SSA (and South African) public health concern.[24] In fact, the incidence of sudden unexplained infant deaths (SUID) in SA, especially in Cape Town, has been reported amongst the highest in the world.[25] Also, an approximate 2,000 young South Africans die suddenly each year, in which most cases the cause of death remains unexplained.[23]

According to South African legislation all SUD's must be subjected to a full medico-legal death investigation in order to determine the cause and manner of death.[26] Unfortunately, post mortem genetic testing has not yet been incorporated into South African mortuaries, partly due to a lack of similar research conducted in SA. Recent international publications corroborate the underrepresentation of African cohorts in health research, especially pertaining to genetic aetiologies underlying SCD's.[27, 28] Apart from a study conducted on a small cohort of SUID's, no other study has yet been conducted in SA on the possibility of an inherited cardiac arrhythmogenic disorder as the cause of unexplained SCD's in the young.[25] This is the first local study to address the need for gaining a better understanding of the genetic aetiologies of SCD's in the South African young. Therefore, the aim of this study was to determine the prevalence of genetic variants in the major genes linked to inherited cardiac arrhythmogenic disorders in unexplained SUD cases in the young that were admitted and subjected to a medico-legal autopsy at the Pretoria Medico-Legal Laboratory (Pretoria MLL).

5.2 Methods

Ethics approval (Appendix A) for this study was obtained from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (495/2017).

5.2.1 Study cohort

A prospective genetic study was conducted on 66 SUD and SUID cases which were admitted to the Pretoria MLL for a full medico-legal death investigation. All SUD cases (age range between one and 45 years) and SUID cases (under one year of age) met the inclusion criteria for this study in which the cause of death could not be determined after a full medico-legal death investigation had been performed. These investigations comprised of a full autopsy, death scene investigation, review of available medical history as well as all appropriate ancillary investigations (e.g., virology, toxicology, histology, microbiology etc.) This study excluded all SUD and SUID cases where a cause of death was ascertained after the completion

of a full medico-legal death investigation. For each case, peripheral blood samples were prospectively collected at autopsy into two 5 mL EDTA tubes and immediately stored at -80°C until DNA extraction could be performed.

5.2.2 Genetic testing

DNA was extracted from post mortem blood samples using the QIAamp DNA Blood Mini Kit from Qiagen (Hilden, Germany). Following extraction, an initial concentration and purity ratio of each DNA sample was determined spectrophotometrically by using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, Massachusetts) and stored at -20°C until further use. Once all 66 case samples were collected, DNA samples were fluorometrically quantified and diluted to the required concentration of 10 ng per primer pool, using the Qubit dsDNA HS Assay kit on the Qubit 3.0 Fluorometer (ThermoFisher). For NGS, the AmpliSeq On-Demand DNA Panel**,** designed by DesignStudio^{TM} software (Illumina, San Diego, California), was used for the specific targeting of 49 genes linked to inherited cardiac arrhythmogenic disorders (see Appendix C). Libraries were prepared using the AmpliSeq Library Plus kit by following the prescribed instructions for the AmpliSeq^{TM} for Illumina, multiplex PCR-based, workflow. In order to confirm quality control and to ensure optimum cluster densities on the flow cell, the quality of the pooled libraries was analysed by using the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 kit (Agilent Technologies, Santa Clara, California), followed by the quantification thereof using the Qubit 3.0 Fluorometer with the Qubit DNA HS Assay kit. Pooled libraries were diluted to a final loading concentration of 1.5 pM, followed by sequencing using the v2.5 (300 cycles, 2 x 150 bp paired end reads) high output kit on the Illumina NextSeq 550 platform. For laboratory procedures, see Appendix B.

5.2.3 Bioinformatic analysis

Sequencing analysis was performed on the open-source Galaxy bioinformatics platform. The Galaxy web platform (public server at usegalaxy.com) was used to download all FASTQ sequencing files into dedicated data libraries, which only the researcher had access to. The quality of the raw sequencing data was assessed using the FastQC tool. To ensure that only sequences of good quality was subjected to further bioinformatics analysis, the Trimmomatic tool was used for trimming and filtering of sequences. A sliding window trimming was performed to cut sequences where the average quality within a four bp window fell below 20. Only reads with an average quality threshold of 30 (Q >= 30) and a minimum length of 80 bp were kept for further analysis. Following quality control, all post-trimming reads were aligned

to the reference human genome (CRCh37, hg19) using the Burrows-Wheeler Alignment – Maximal Exact Match (BWA-MEM) tool, which generated a binary alignment/map (BAM) file. In order to ensure that the identity of reads was maintained during the alignment process, all individual reads were labelled using the read group tag in the "sequence alignment map / binary alignment map (SAM / BAM) specification." A clean-up / grooming of BAM files was performed using the CleanSam tool from Picard. Duplicate reads were removed using the MarkDuplicates tool from Picard. Using SAMtools, reads were further filtered to only keep the reads with a mapping quality (MAPQ) >= 30 and mapped in a proper pair. Finally, all files were merged into one BAM file using Picard, and visualized using the JBrowse genome browser, as well as the University of California Santa Cruz (UCSC) genome browser. Variant calling was performed by using FreeBayes (a Bayesian genetic variant detector) to generate a variant call format (VCF) file. Variant representation was simplified by using the VCFAllelicPrimitives tool, followed by variant annotation using the SnpEff prediction tool. Variants were filtered by using the SnpSiftFilter tool, and all reads were kept which met the following criteria: mapping quality (MQ) >= 60, read depth (DP) >= 20, quality by depth (QD) > 2, genotype quality (GQ) >= 20 and for heterozygous variants an allele balance (AB) between 0.25 and 0.75. Finally, only missense, nonsense, insertion / deletion (INDEL), frameshift and / or splice site variants were retained, and again were visualized using the JBrowse genome browser.

The resulting VCF files (filtered variants) were uploaded to two different databases (Jpopgen – dbNSFP and Ensemble variant effect predictor) for further functional prediction and annotation. The Human Genome Variant Society (HGVS) guidelines were followed for variant nomenclature. Several different population databases were compared in relation to the allele frequency (AF) of identified variants, which can be seen in Table 5.1 below. A combination of eight different in-silico tools, based on different methodologies, were used for functional effect prediction (Table 5.1). UniProt was used for the location of the protein domain / region, and Blocks Substitution Matrix (BLOSUM) for the amino acid substitution conservation score. Sequence conservation was measured using the Genomic Evolutionary Rate Profiling (GERP) score as well as the PhyloP100way score. APPRIS was used to annotate alternative spliced transcripts, with splice site predictions scores generated by SpliceAI.

5.2.4 Variant classification

Variants were interpreted and classified, according to the American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) guidelines, into one of

five groups: (1) pathogenic, (2) likely pathogenic (LP), (3) variant of unknown significance (VUS), (4) likely benign (LB) or (5) benign. All novel variants as well as VUS's were further divided into one of three subcategories: (i) LP (all prediction tools in agreement of pathogenicity), (ii) LB (≤ 50% of algorithms predict a benign prediction tools), or (iii) remains VUS (> 50% algorithms predict a possible functional effect on the protein.). All variants were also subjected to a manual search across several variant databases as well as published literature.

Table 5.1. List of in-silico algorithms and population databases used in variant annotation and functional effect prediction

In-silico algorithm	Population database
FATHMM (http://fathmm.biocompute.org.uk) MutationAssessor (http://mutationassessor.org) MutationTaster (http://www.mutationtaster.org) PolyPhen-2 HDIV (http://genetics.bwh.harvard.edu/pph2) PolyPhen-2 HVAR (http://genetics.bwh.harvard.edu/pph2) PROVEAN (http://provean.jcvi.org/index.php) LRT (http://genome.cshlp.org/content/19/9/1553 SIFT (http://sift.jvci.org)	Exome Aggregation Consortium (ExAC) http://exac.broadinstitute.org/ Exome Variant Server (EVS) http://evs.gs.washington.edu/EVS 1000 Genomes http://browser.1000genomes.org Genome Aggregation Database (gnomAD) http://gnomad.broadinstitute.org dbSNP http://www.ncbi.nlm.nih.gov.snp dbVar http://www.ncbi.nlm.nih.gov/dbvar

5.3 Results

5.3.1 Demographics and history

The demographic details of all cases can be seen in Appendix D. The study cohort consisted of 66 unexplained sudden deaths with an average age of 21.5 years (ranging between one week and 45 years old). Most of the cases were between 19 and 45 years of age (n = 48; 73%), followed by 15 (23%) SUDI cases and eight cases (12%) between one and 18 years old. The majority of the cases were male (n = 46; 70%) vs. 20 (30%) females. Regarding their ethnicity, 48 (73%) of the cases were Black South Africans, 15 (23%) White South Africans, two (3%) Asian and one (1%) Mixed ancestry. Only three cases reported a personal medical history of seizures and palpitations. None of the cases had any documented family history of syncope or sudden death. In 12 of the cases (18.2%) the activity at time of death indicated exertion, of which three of them were recorded to occur on the sports field. Another 12 (18.2%) of the cases indicated a period of rest at time of death. The majority of cases (n = 26, 39.4%) were sleeping prior to being found dead/unresponsive. In the remaining 16 (24.2%) cases, the activity at time of death was unknown.

5.3.2 Genetic results

Next generation sequencing identified a total of 178 different missense variants among the entire study population (n = 66); 164 were known, documented variants whereas the remaining 14 were novel. Of the known variants, 49 were documented to be of benign significance, 78 of LB significance, 33 of unknown significance (VUS) and four to be of LP significance. Of the novel variants, 12 were predicted to be likely benign whereas the remaining two were predicted to be LP. Figure 5.1 shows an overview of the classification between documented and novel variants. In 20 of the 33 documented VUS's, in-silico algorithms predicted the functional effect to be likely benign, whereas 13 remained in the VUS category. All of the 66 cases were found to carry multiple missense variants, of which the majority were likely benign. In 48 (72.7%) of the cases, one or more VUS's were identified. A variant of LP significance was identified in six cases (9.1%). A more detailed description of the 178 missense variants is provided in Appendix E.

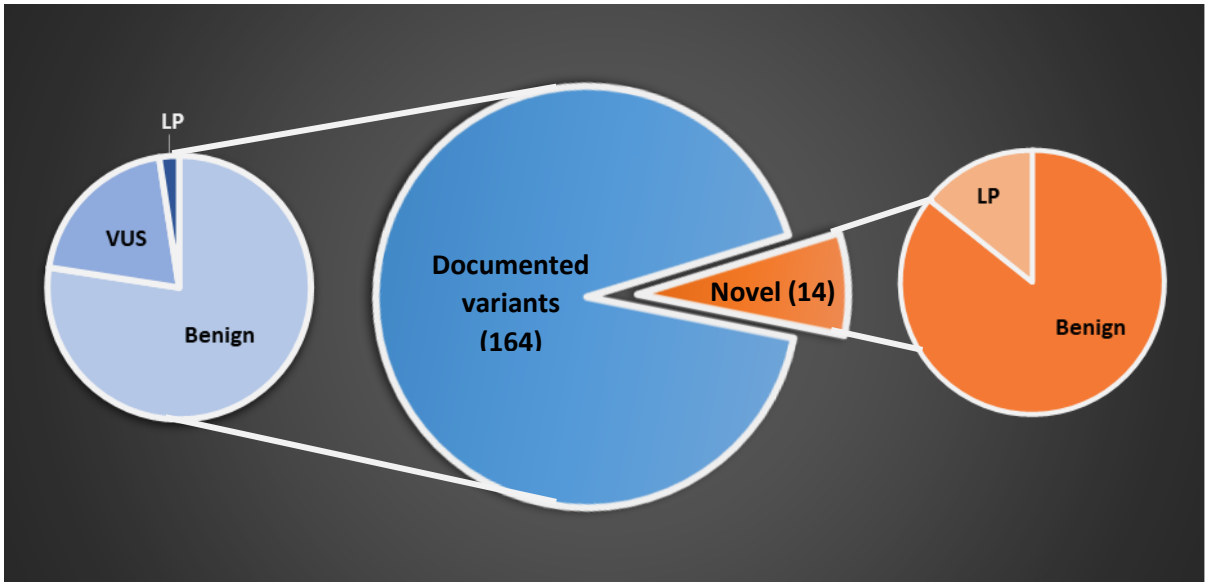

Figure 5.1 Classification off all documented and novel variants identified in this study. The majority of the 164 documented variants were of benign significance, followed by VUS's. The variants of LP significance were found to be the minority (shown on the left-hand side of the image). On the right-hand side of the image, it can be seen that the majority of novel variants were also of benign significance, followed by LP variants.

5.3.2.1 Gene profile

A total of 49 genes were subjected to NGS, of which 11 revealed to carry no missense variant at all (*CALM1*, *CALM2*, *CALM3*, *KCND3*, *KCNE2*, *KCNE5*, *KCNJ2*, *PLN*, *SCN2B*, *SCN3B* and *SCN4B*). The aforementioned 178 missense variants were identified in the remaining 38 genes, with a median of three variants, ranging between one and 18, per gene. In nine of the 38 genes, a novel variant was identified, of which only two had one LP variant each (see Figure 5.2) Figure 5.3 shows the distribution and classification of identified variants (documented and novel) among the 38 genes.

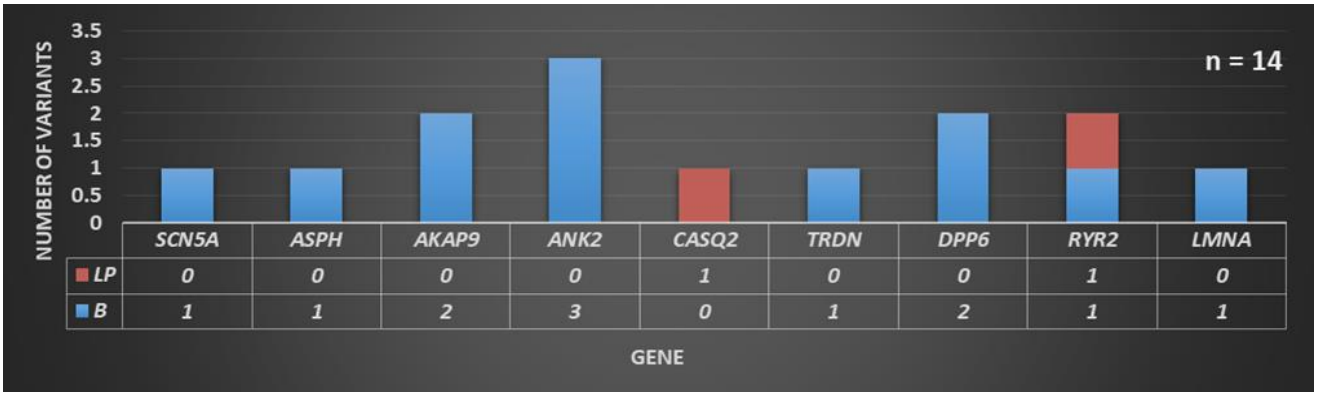

Figure 5.2 Classification of novel variants identified in nine different genes. Novel LP variants (shown in dark red) were identified in only two genes (*CASQ2* and *RYR2*); one variant each. The remaining blue, benign variants were identified in eight different genes.

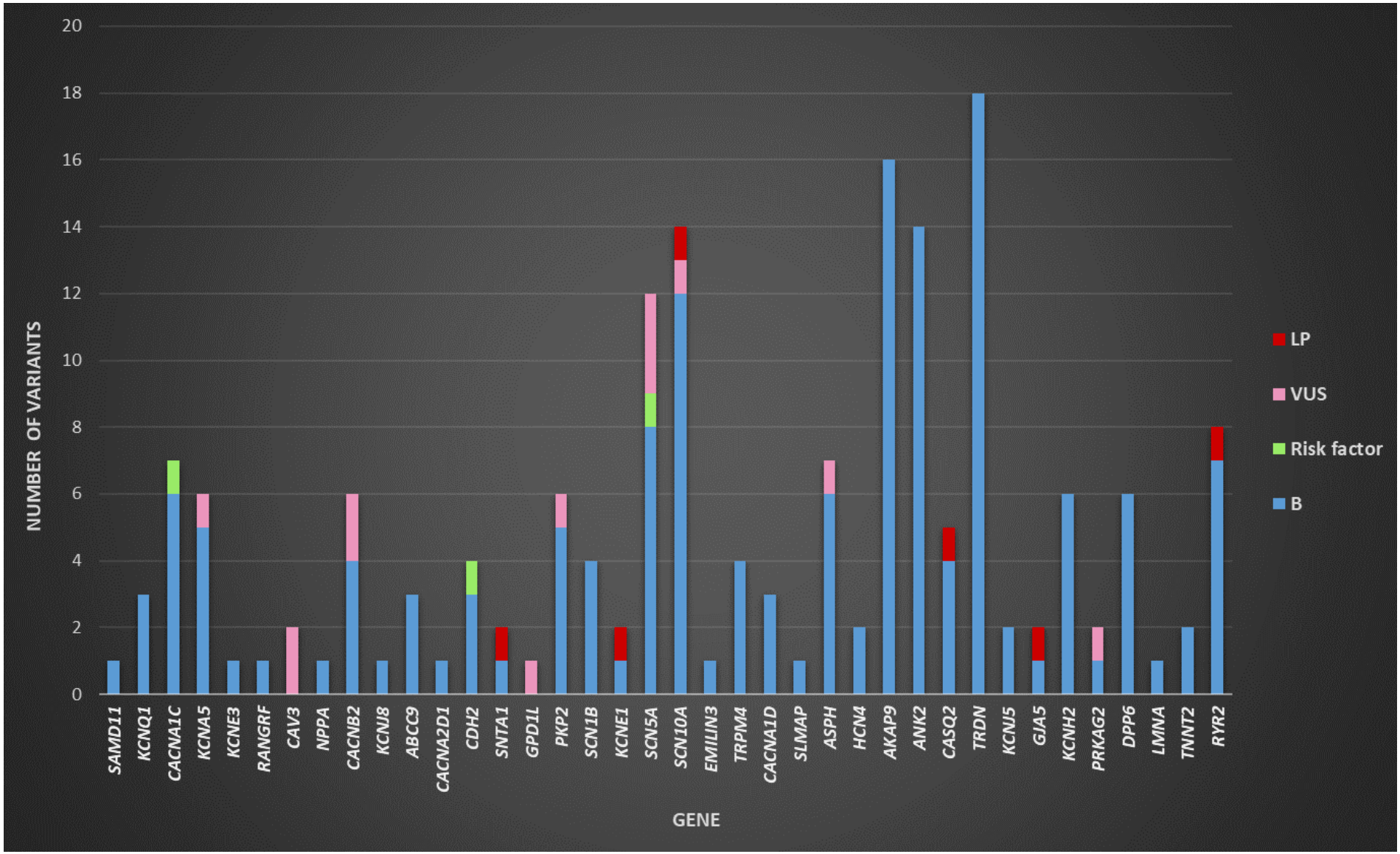

Figure 5.3 Classification of 178 identified variants among 38 genes. Variants of benign (B) significance are indicated in blue, whereas variants reported as risk factors are indicated in green. All VUS's are shown in pink and LP variants in dark red.

Only 16 genes were found to harbour 23 different VUS, 'risk factor' and / or LP variants (Appendix F). Figure 5.4 shows the proportion of these 23 variants identified in each of the 16 genes. The remaining 22 genes carried only variants of benign significance.

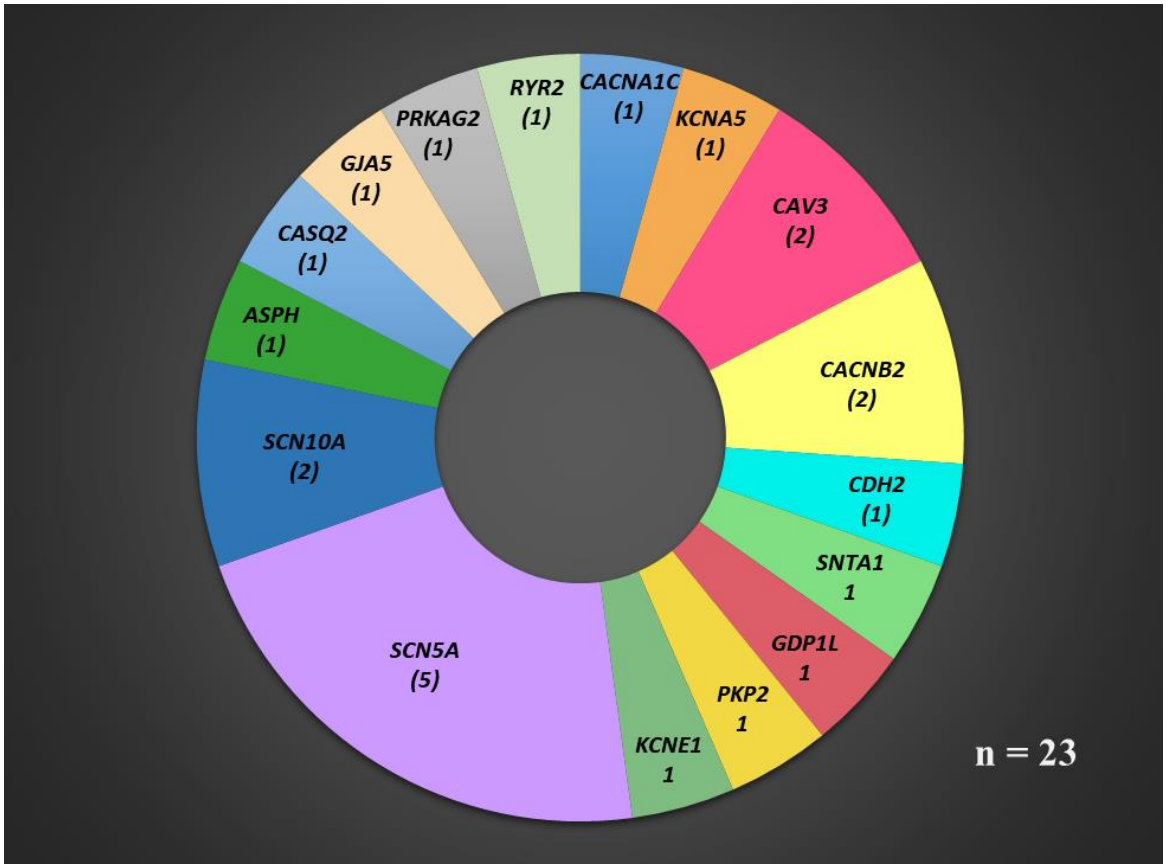

Figure 5.4 Distribution of 23 VUS, risk factor and / or likely pathogenic variants identified among 16 different genes. In 12 of the 16 genes, only one variant of either VUS, risk factor or LP significance were identified. Three of the genes (*SCN10A*, *CACNAB2* and *CAV3*) were identified with two VUS, risk factor and / or LP variants each. A total of five different VUS, risk factor and / or LP variants were identified in the *SCN5A* gene.

5.3.2.2 Demographics of cases identified with a VUS, risk factor and / or likely pathogenic variant

A total of 48 (73%) of the 66 cases carried at least one VUS, risk factor and / or LP variant. Only variants of benign significance were identified in the remaining 18 cases (27%). Of the 48 cases, eight (17%) were between the ages of zero and one year old (SUDI), five between one and 18 years old, with the remaining 35 cases (73%) between 19 and 45 years of age. Taking into consideration the total number of cases, included in this study, for each of the different age groups, 53% of SUDI cases ($8/_{15}$), 63% of the one – 18 year olds ($^{5}/_{8}$) and 81% of the 19 – 45

year olds ($^{35}/_{43}$) carried at least one VUS, risk factor and / or LP variant. Figure 5.5 below illustrates the proportion of cases, and their associated demographics, for each age group identified with VUS, risk factor and / or LP variants.

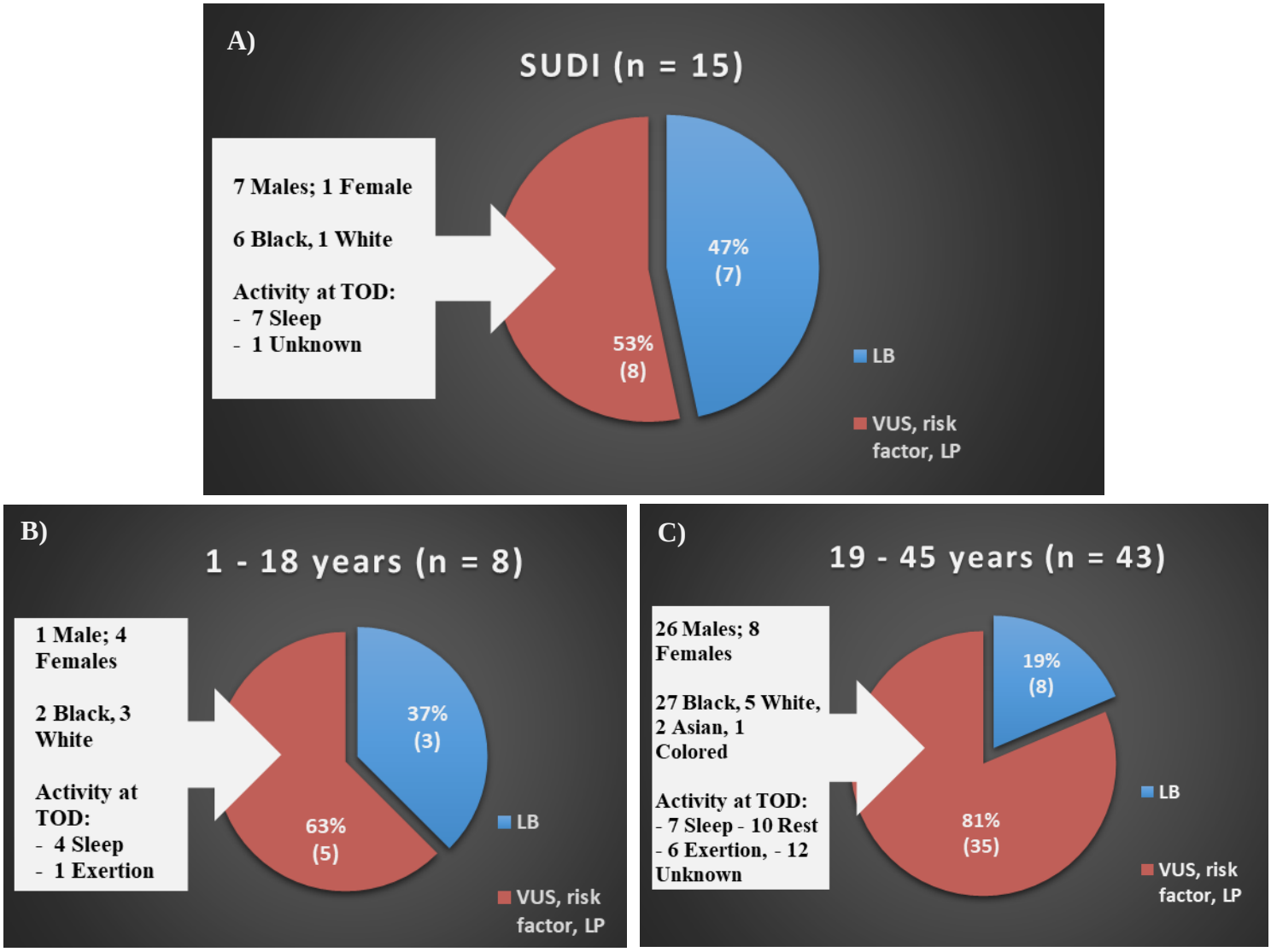

Figure 5.5 Percentage of cases for each of the three different age groups identified with VUS, Risk factor and / or LP variants. A) The percentage of SUDI cases identified with VUS, risk factor and / or LP variants were slightly higher (53%) in comparison to the remaining 47%, who only carried benign variants. Of the VUS- cases, most were Black males with a recorded period of sleeping prior to death. B) In comparison to the SUDI age group, the one – 18-year age group shows a slight increase in the percentage of cases who carried VUS, risk factor and / or LP variants. However, the majority of cases were White females, also indicating a period of sleep at time of death (TOD). C) The 19 – 45-year age group demonstrated the highest percentage of cases with VUS, risk factor and / or LP variants. Similar to the SUDI age group, the majority of cases were Black males, however with a period of rest and exertion surpassing that of sleep at TOD.

5.3.2.3 Profile of VUS, risk factor and / or likely pathogenic-harbouring genes between different age groups

The 16 different genes identified with a VUS, risk factor and / or LP variant were not equally represented / affected among the three different age groups. For each age group, the following genes were identified carrying such a variant (see Figure 5.6):

SUDI age group – among the cases between zero and one year of age, a total of six different variants were identified in four different genes. Both the *CAV3* and *SCN5A* genes carried two variants with an unknown, risk factor and / or LP significance. The *ASPH* and *GJA5* gene were identified with one such variant each.

One – 18-year age group – A total of eight different variants among six different genes were identified in cases between one and 18 years old. The *SCN5A* gene carried three different variants, whereas the remaining five genes (*CACNA1C*, *CAV3*, *GPD1L*, *PKP2* and *ASPH*) carried one variant each.

19 – 45-year age group – This age group had the highest number of genes carrying a VUS, risk factor and / LP variant, with 18 different variants identified among 13 different genes. The *SCN5A* gene had three different variants, followed by two variants each in *CAV3*, *CACNB2* and *SCN10A*. Each of the remaining nine genes (*CACNA1C*, *KCNA5*, *CDH2*, *SNTA1*, *PKP2*, *KCNE1*, *CASQ2*, *PRKAG2* and *RYR2*) carried one variant.

Of the 16 genes identified with VUS, risk factor and / or LP variants, only two (*SCN5A* and *CAV3*) were represented in all three different age groups. A variant in the *GJA5* gene was solely found in the SUDI age group, whereas a *GPD1L*-variant was exclusively identified in the one - 18-year age group. Variants identified in the *KCNA5*, *CACNB2*, *CDH2*, *SNTA1*, *KCNE1*, *SCN10A*, *CASQ2*, *PRKAG2* and *RYR2* genes exclusively represented the 19 – 45-year age group. For each of the 16 genes, Figure 5.7 shows a comparison between the percentage distribution of identified variants among the three different age groups.

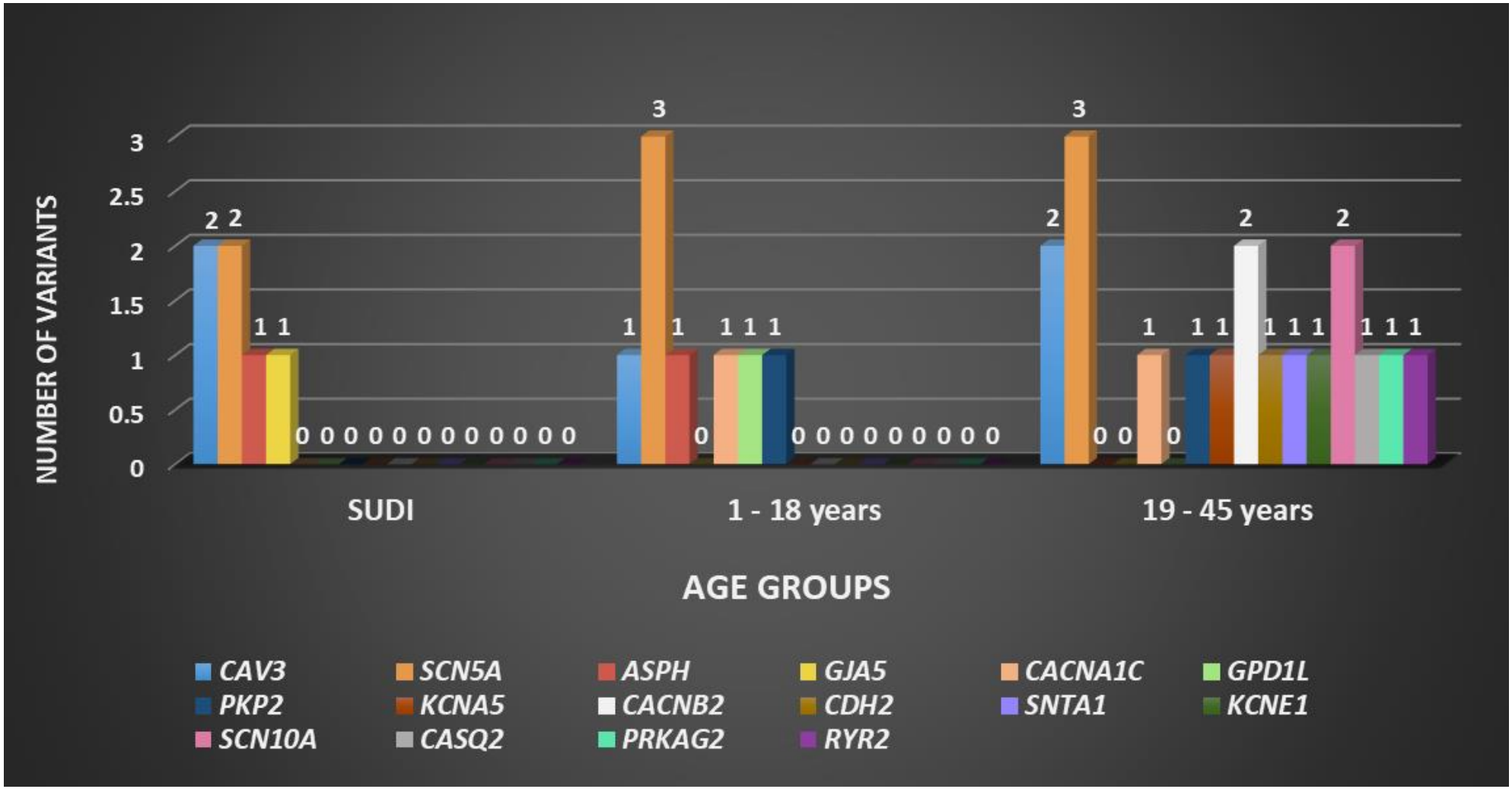

Figure 5.6 Profile of genes identified with VUS, risk factor and / likely pathogenic variants for each different age group. Cases in the SUDI age group showed only four genes identified with a variant of above-mentioned significance. In the one – 18-year and 19 – 45-year age groups, such variants were identified in six and thirteen different genes, respectively.

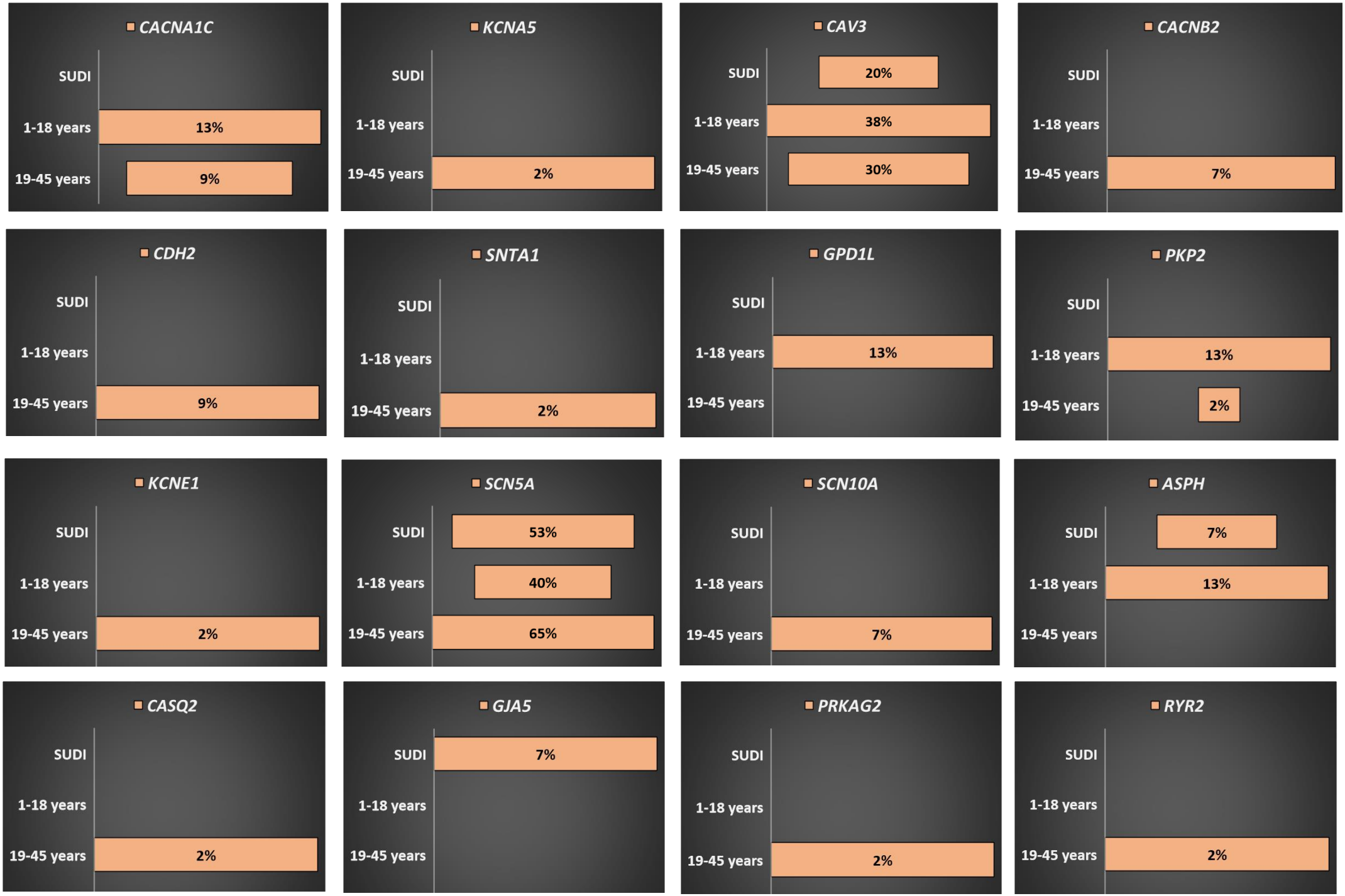

Figure 5.7 Comparison between variant distribution (in percentage) of three different age groups for each of the 16 genes.

5.3.2.4 Variants of likely pathogenic significance

A total of six LP variants were identified; each in a different case. Each of the six variants were identified in a different gene. Four of these variants were known, documented variants (*GJA5*, *SNTA1*, *KCNE1* and *SCN10A* gene), whereas the remaining two were novel (*CASQ2* and *RYR2* gene). A detailed description of each LP variant, along with its associated case demographics, can be seen in Table 5.2.

5.3.2.5 Demographics of cases identified with likely pathogenic variants

A total of six (9.1%) of the 66 cases carried a LP variant. All of the six cases were male, of which the majority were older than 19 years of age. One of the cases was between the ages of zero and one years old (SUDI), none of the cases between one and 18 years old, whereas the remaining five cases were between 19 and 45 years of age. Taking into consideration the total number of cases included for each of the three different age groups in this study, 6.7% of SUDI cases ($^{1}/_{15}$), 0% of the 1 – 18 year olds ($^{0}/_{8}$) and 11.7% of the 19 – 45 year olds ($^{5}/_{43}$) carried a variant of LP significance. Regarding their ethnicity, five of the cases were Black South Africans and one case was a White South African.

5.3.2.6 Profile of LP variant-harbouring genes between different age groups

Similar to the VUS-related genes, the six different genes identified with a LP variant were not equally represented / affected among the three different age groups. The one variant exclusively identified in the SUDI age group was in the *GJA5* gene. As mentioned before, none of the cases between one and 18 years of age carried a LP variant. The remaining five variants, exclusively identified in cases between 19 and 45 years old, included the *SNTA1*, *CASQ2*, *KCNE1*, *RYR2* and *SCN10A* gene, respectively (Figure 5.8). Figure 5.9 illustrates the relationship between variants in each of the six genes and their recorded activity at time of death.

Table 5.2 Summary of six likely pathogenic variants identified among entire study cohort

Case number	Age	Sex	Race	Activity at TOD	Variant	Gene	Exon	Reference number	Associated disease
Case 10	35 y	M	B	Exertion	c.8004G>C (p.Cys2668Trp)	*RYR2*	53	Novel	CPVT
Case 15	24 y	M	W	Unknown	c.500T>C (p.Lys167Arg)	*SNTA1*	3	rs932909554	LQTS
Case 24	38 y	M	B	Exertion	c.954C>A (p.Trp318Cys)	*CASQ2*	10	Novel	CPVT
Case 40	23 y	M	B	Rest	c.226C>T (p.Asp76Asn)	*KCNE1*	3	rs74315445	LQTS, BrS, ARVC
Case 55	4 m	M	B	Sleep	c.299A>C (p.Val100Gly)	*GJA5*	2	rs138375318	Atrial fibrillation, Atrial standstill
Case 65	28 y	M	B	Rest	c.40G>A (p.Arg14Cys)	*SCN10A*	1	rs750771811	BrS

Abbreviations: y = years; M = male; B = Black South African; W = White South African; CPVT = catecholaminergic polymorphic ventricular tachycardia; LQTS = long QT syndrome; ARVC = arrhythmogenic right ventricular dysplasia; BrS = Brugada syndrome

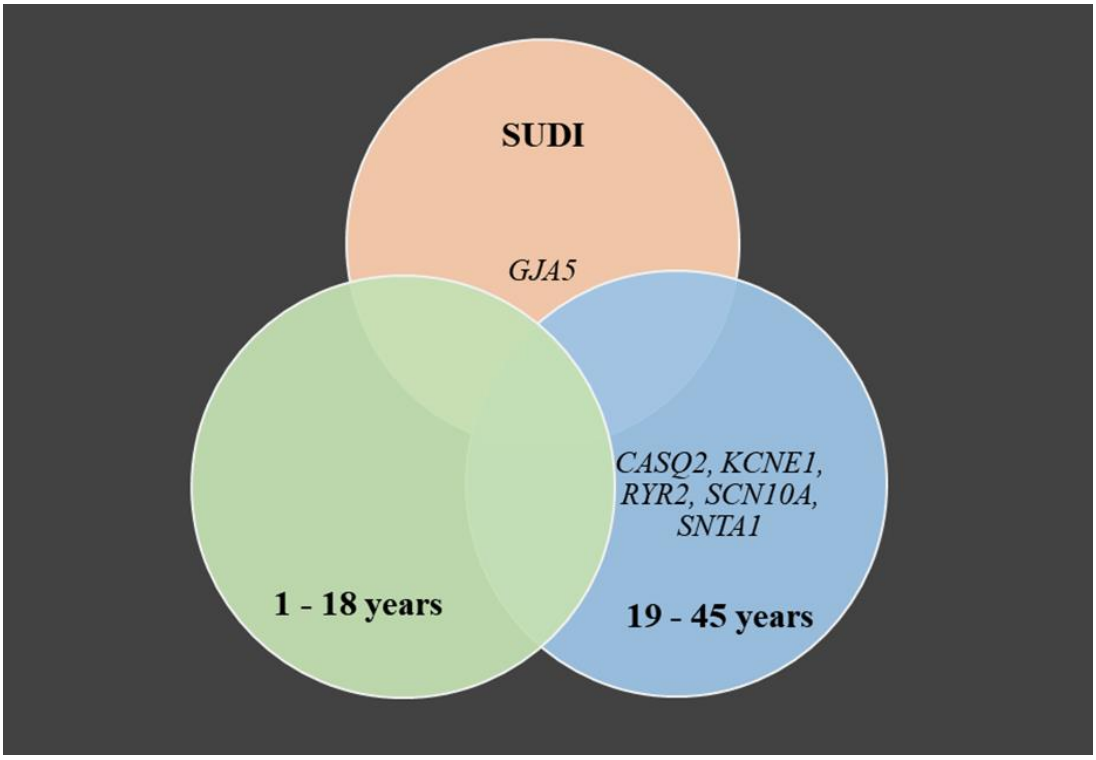

Figure 5.8 Likely pathogenic-harbouring genes for each of the three different age groups. Only one gene (*GJA5*) was identified with a LP variant in the SUDI age group (shown in the orange circle). The green circle indicates that none of the cases in the 1 – 18-year age group carried a LP variant. In the 19 – 45-year age group (shown in blue), LP variants were identified in five different genes.

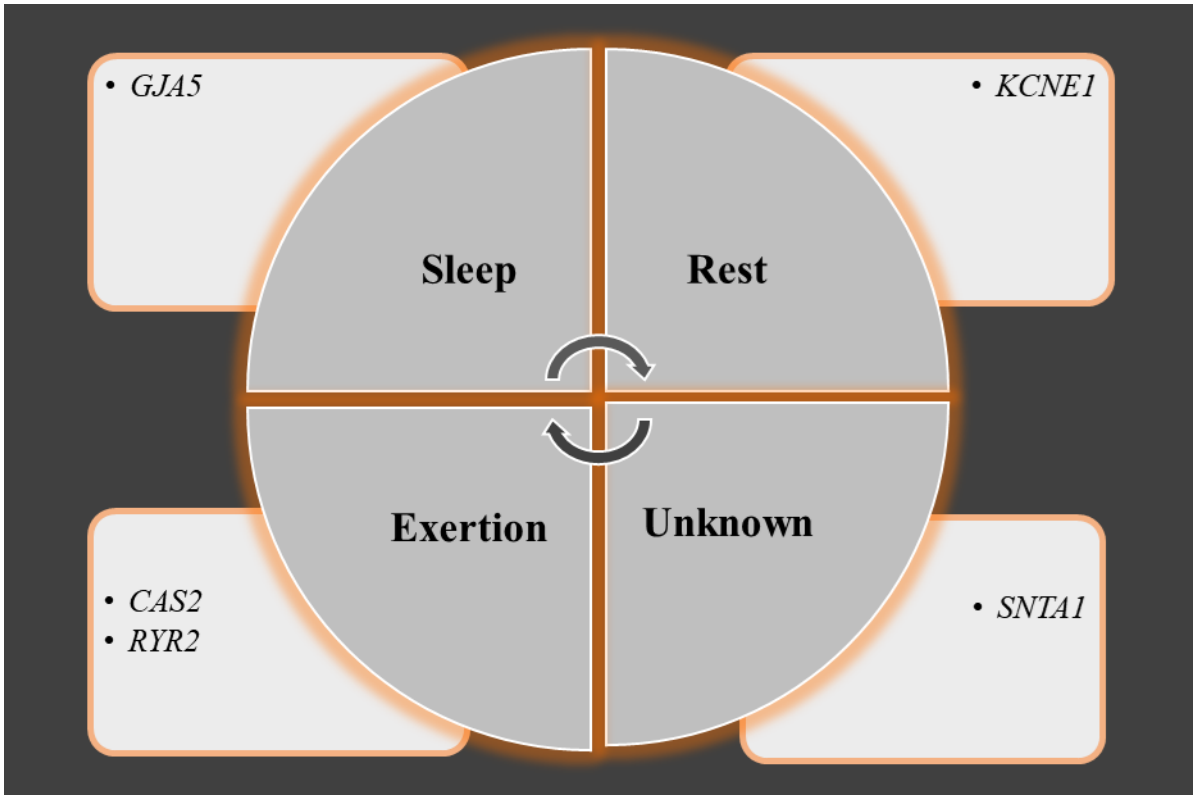

Figure 5.9 Association between genes and activity at time of death. Variants in the *GJA5* and *KCNE1* genes were associated with a period of sleep and / or rest at time of death, whereas the *CASQ2* and *RyR2* genes indicated some type of exertional activity at time of death. For the remaining case carrying a *SNTA1* variant, the activity at time of death was unknown.

5.3.3 Detailed analysis of cases identified with a likely pathogenic variant

For each of the six LP variants, eight different in-silico tools were all in agreement on predicting a deleterious functional effect on the protein. In five of the six cases carrying a LP variant, at least one VUS (where four or more (≥ 50%) of the functional prediction tools predicted a 'possible deleterious' effect), was also identified.

Case 10 was that of a 35-year-old Black South African male who suddenly collapsed while walking down the street. Upon arrival of the emergency medical services (EMS), he was declared dead on the scene. Apart from three small superficial abrasions on the left anterolateral aspect of the forehead and temporal region of the face, the autopsy revealed no other injuries or signs of pre-existing disease. All ancillary investigations were normal, and with a lack of any clinical history, the ultimate cause of death remained undetermined. Following NGS, a heterozygous likely pathogenic variant, c.8004C > G (p.Cys2668Trp), was identified in exon 53 of the *RyR2* gene. This variant was not found in any database or published literature and considered to be novel. Conservation scores determined it to be a non-conservative amino acid change in a conserved region of the protein. An additional 19 variants, all of which were predicted to be benign, were also identified in case 10 (Appendix F). These variants were identified in 11 different genes, of which *RyR2* was one.

Case 15 was a White South African male, 24 years of age, who similarly to case 10, suddenly collapsed while walking in a shopping mall. He was declared dead on the scene before any emergency care could have been administered. At autopsy, the external examination revealed no injuries to the body, with the internal examination revealing no more than non-specific congestion of the lungs and visceral organs. The results from ancillary investigations all returned normal, except for the toxicological report, which up to date, is still outstanding. However, with only a history of recreational marijuana use, the cause of death was not considered to be drug related. No family history of sudden death or cardiac-related disorders were known to any of the family members, and as a result, the cause of death remained undetermined.

Post mortem genetic testing revealed a likely pathogenic variant in exon three of the *SNTA1* gene (heterozygous c.500T > C; p.Lys167Arg; rs932909554). All eight in-silico tools predicted a deleterious functional effect, in addition to conservation scores indicating a non-conservative amino acid change in a conserved domain of the protein. This variant is located in the N-terminal PDZ domain of the protein, which interacts with other cardiac ion channels and

receptor proteins and reported to be associated with LQTS. An additional 25 missense variants were also identified in this case (Appendix G), of which one of them, c.116G > A; p.G56S; rs72546667 in the *CAV3* gene, was determined to be a VUS.

Case 24; a 38-year-old Black South African male, suddenly collapsed in a park while out with friends. Shortly after EMS arrival and numerous failed attempts at medical intervention, he was declared dead on the scene. At autopsy, no external injuries to the body were noted. An internal examination only revealed non-specific congestion of the brain, with the rest of the organs showing no signs of injury or underlying disease process. Toxicological analysis revealed the presence of cannabis; however, this was not considered to be the cause of death. All additional ancillary tests were normal, with no personal and / or family medical history of the deceased available. Following a thorough autopsy investigation, the cause of death remained unexplained.

Genetic analysis identified a novel, likely pathogenic variant (heterozygous c.954C > G; p.Trp318Cys) in exon 10 of the *CASQ2* gene. This non-conservative change from Trp, the largest aromatic amino acid, to the much smaller non-aromatic Cys, is located in the helix of the conserved domain III of the protein. This case also carried an additional 22 missense variants, identified in 12 different genes (Appendix H), of which the majority were LB. Only one of these variants (c.250G > A; p.Arg84Trp; rs61746358), identified in the *PRKAG2* gene, was deemed to be a VUS, where seven of the eight in-silico tools (88%) predicted a deleterious effect on the protein. This specific VUS was not identified in any of the other 65 cases.

Case 40 was that of a 23-year-old Black South African male who was found unresponsive in his home at approximately 21:15. Shortly after EMS arrival, he was declared dead on the scene. At autopsy, no injuries were noted upon external examination of the body. Blood-stained fluid was noted emanating out of the nostrils and mouth. An internal investigation revealed both lungs to be markedly oedematous and deeply congested with intra-pulmonary haemorrhage. Examination of the heart (weighing 400 g) showed an impression of dilated cardiomyopathy. Bilateral, biventricular dilatations and left ventricular hypertrophy were observed; however, no signs of ischaemic heart disease were present. Overall non-specific findings were noted at autopsy with no definitive cause of death ascertained.

Next generation sequencing revealed a LP variant (c.226C > T; p.Asp76Asn; rs74315445) in exon three of the *KCNE1* gene, most commonly associated with LQTS, BrS and ARVC. Not only did all eight in-silico tools agree on this variant having a deleterious functional effect, but

conservations scores also determined it to be a non-conservative amino acid change in a highly conserved domain [Carboxyl (C) - terminus] of the protein. A further 23 additional missense variants, in 12 different genes, were also identified in this case (see Appendix I). One of these variants (c.233C > T; p.Thr78Meth; rs72546668), identified in the *CAV3* gene, was deemed to be a VUS, where 6/8 (75%) of the in-silico tools predicted a deleterious functional effect. This specific VUS was only identified in three other cases.

Case 55, a four-month-old Black South African male, was found unresponsive during a scheduled period of sleep. Upon arrival at the hospital, the infant was declared deceased and subsequently subjected to a medico-legal death investigation. At autopsy, no external injuries were noted. Apart from non-specific organ congestion, the internal investigation revealed no signs of injury nor an underlying disease process. All the ancillary investigations yielded normal results. No known history of SCD or syncope have been recorded for any of the family members. The lack of any conclusive findings led this case to be classified a SUID.

Post mortem genetic findings in this case included 29 missense variants identified in 16 different genes, of which the majority were benign (Appendix J). One variant (c.299A > C; p. Val100Gly; rs138375318) in the *GJA5* gene, located in the conserved cytoplasmic loop of the protein, were found to be a non-conservative amino acid change and of LP significance.

Case 65 was a 28-year-old Black South African male who, as a passenger in his friend's car, suddenly started complaining of not feeling well, whereafter he rapidly lost consciousness. His friends rushed him to hospital, where he was declared dead upon arrival. Personal medical history indicated a visit to a general medical practitioner one week prior to his death, with complaints of flu-like symptoms. After receiving general medication for the relief of flu-like symptoms, family members reported him being well with no further complaints for the remainder of the week. No history or signs of drug use were reported. At autopsy, an external examination of the body revealed no injuries, with only signs of medical intervention recorded. Macroscopic and microscopic autopsy findings only included non-specific generalized organ congestion, with no injuries or comorbid illnesses noted. A full medico-legal death investigation failed to reveal a cause of death.

Following NGS, a LP variant (c.40G > A; p.Arg14Cys; rs750771811) was identified in exon one of the *SCN10A* gene. This variant, predicted by all eight in-silico tools to have a deleterious effect on the protein, was also found to be a non-conservative amino acid change in a conserved domain (N-terminus) of the protein. Of the 27 additional variants also identified in this case

(see Appendix K), one was determined to be a VUS. This variant (c.6007C > T; p.Asp2003Asn; rs376697724) in the *SCN5A* gene was predicted to have a deleterious effect by five of the eight (60%) in-silico tools.

5.4 Discussion

Next generation sequencing of 49 genes was performed in a South African cohort of SUID and SUD cases in the young, admitted to the Pretoria MLL for a full medico-legal death investigation. The aim of this study was to identify an inherited cardiac arrhythmogenic disorder, caused by variants in cardiomyopathy and arrhythmia-related genes, as a possible contributing factor to the cause of these sudden deaths. Owing to the continues development of molecular techniques and its associated decrease in cost (i.e., NGS technology), research has shown that in many as 40% of such cases, an inherited cardiac arrhythmogenic disorder could have been the cause of death.[1, 2] Due to a lack of studies conducted in SA, it cannot be assumed that sudden deaths in the South African population would reveal the same underlying genetic aetiology than cases from international populations. This is the first molecular screening study of 49 inherited cardiac arrhythmogenic-related genes in a South African cohort of SUD's in the young (0 – 45 years of age) to our knowledge.

A total of 66 cases, in which the cause of death remained unexplained following a full medico-legal death investigation, were included in this study. Although considered an integral part of the SUD investigation, the use of clinical records of the decedent and / or associated family members as an indication of a possible pre-existing arrhythmogenic disorder, proved to be challenging. Due to South Africa being an economically and resource-strained country, the majority of the population can't afford medical care, thus rarely visit any medical practitioner, let alone receive specialist care (e.g., ECG). In 60 of the 66 cases (91%), no medical history / clinical records were available, which is in keeping with similar findings in a Cape Town (South Africa) study.[3]

The ages of the study cohort ranged between one week and 45 years old, with an average age of 21.5 years; this seems to be in concordance with similar international studies.[4, 5] Of these 66 cases, 46 (70%) were male and 20 (30%) were female, which is consistent with the usual male to female ratio admitted to the Pretoria MLL, and also comparable to results from similar studies elsewhere.[4, 5] The majority of the study cohort were Black South Africans (73%), followed by 23% White South Africans, which accurately represents the South African population as well as the general admissions profile of the Pretoria MLL. Research has shown

that, depending on the specific gene and its functional role in a disease, certain variant-related arrhythmias are triggered by different activities / circumstances at, or just prior to, the time of death.[6, 7] In this study, the available history indicated that 26 of the 66 cases (39.4%) were asleep at time of death. For two different activities at time of death (exertion and rest), 12 cases (18.2%) were recorded each. In 16 cases (24.2%), no history was available and thus the activity at time of death remained unknown.

5.4.1 Subdivision of age groups

The ages of the cases were further divided into three categories in order to be consistent with the literature.

SUDI age group

Of the 66 cases, 15 (22.7%) were between the ages of zero and one years old. In comparison to the number of cases in the other two, very broad, age ranges, the number of SUDI cases is high. This, however, is in keeping with literature indicating that more infants die suddenly and unexpectedly than the older age groups.[8, 9] Anderson *et al.*[10] and Williams *et al*.[11] reported 31% and 25.6% SUDI cohorts, respectively.

In this study, the average age of SUDI deaths were 2.1 months, of which the majority were male (n = 11; 64%), which is in accordance with published literature.[12, 13] The majority of Black South African SUDI cases, (73.3%), also conformed to the admissions profile of the Pretoria MLL. Similar to what is reported on SUDI deaths, the majority of these cases (n = 13; 86.7%) were sleeping at the time of death.

One to 18-year age group

A total of eight cases (12.1% of this study cohort) were between the ages of one and 18 years old. Williams *et al.*[11] reported a similar case percentage in their American one - 18-year-olds. However, a study mostly representing the European population, reported different results, with 30% of their cases in the one - 18-year-age range.[5]

The ethnic distribution of these cases followed the general admission profile of the Pretoria MLL, with 63% and 37% recorded to be Black and White South Africans, respectively. Interestingly, the male to female ratio in this particular age group did not conform to either the admission profile of the Pretoria MLL nor to similar studies conducted on different population cohorts.[14, 15] The majority of these cases were female (n = 5; 63%), of which four (80%) were White South Africans. Regarding the activity at time of death, 50% of the cases were asleep,

12.5% of the cases recorded some type of exertional activity, another 12.5 % were at a period of rest, whereas the activity at time of death in the remaining 22.5% of the cases, remained unknown.

19 to 45-year age group

The majority of cases, 43 (65.2%), were between 19 and 45 years old, with an average age of 31 years, which correlates with international studies.[11, 16] Once again, the ethnic and sex profile of these cases, [Black South African males (72%)] followed the same profile of the South African population as well as that of the Pretoria MLL admissions.

For this specific age group, Shanks *et al.*[4] reported an unknown activity at time of death in 40% of their cases, similar to the 33% reported in this study. An exertional activity was recorded in a slightly higher number of cases in this study (21%), in comparison to the 12% reported by Shank *et al*.[4] and the 11% reported by Lahrouci *et al.*[16] The remaining 46% of the cases in this study, with a recorded period of sleep and / or rest at the time of death, agreed with other studies.[10, 11, 17]

5.4.2 Next generation sequence results

Although the size of gene panels in similar international studies vary greatly, more recent studies have increasingly reported on the use of larger gene panels or even whole exome sequencing (WES) analysis.[10, 18] Due to resource-and-budget constraints, a DNA panel targeting a maximum of 49 cardiac arrhythmogenic-related genes, (which have all been included in studies using larger gene panels), were used for NGS in this study.

Following NGS and bioinformatic analyses, a total of 178 different missense variants were identified among the entire study cohort. A similar study conducted on the Australian and New Zealand populations yielded a higher number of variants (n = 288), however in a larger case cohort (n = 302), using a larger gene panel (n = 77).[16] Heathfield *et al.*[3] reported a lower number of 123 variants among a smaller 43 gene panel in a smaller cohort, which only included 19 infant death cases.[3]

Heathfield *et al*.[3], Hertz *et al*.[19] and Ackerman *et al*.[10] each reported a respective average of 40, 69 and 67 variants identified per case, which seems high in comparison to our study's average of 17 variants per case. This, however, can be explained by this study's exclusion of reporting on synonymous variants. In fact, Nuebauher *et al*.[18] showed similar results with an average of 14.6 missense variants identified in their study.

No INDELS or frameshift variants were identified, which agreed with other studies that similarly reported a majority of missense, single-based variants.[15, 18] Although the majority of this study's variants were known and documented (n = 164; 92%), a higher number of novel variants were identified in comparison to other studies.[11, 15] Hertz *et al.*[19] reported only one novel missense variant among a total of 77 (1.3%), which is low in comparison to the 8% of novel variants identified in this study. This, however, should be foreseen, since South Africa is regarded to be one of the countries with the highest and most unique genetic diversity.[20, 21] Regarding ethnicity, our study mostly comprised of Black South Africans, whereas similar studies were mostly conducted on White populations, and where other study cohorts included Black / African cases, it was from an entire genetically different African population.[10, 11] A similar study has not been conducted on the South African population yet, which furthermore substantiates the number of novel variants identified in this study.

5.4.2.1 Significance of missense variants

The significance of variants identified in this study yielded comparative results, with the majority of variants being of benign significance (n = 127; 71.3%), followed by 33 (18.5%) VUS's and 6 (3.4%) LP variants. Additional filtering of all VUS's resulted in only 13 of them being ultrarare, and therefore remained to be classified as a VUS with a possible adverse functional effect on the protein. A total of 44 cases in this study (70%) carried such a VUS, agreeing with Shanks *et al.*[4], Lahrouci *et al.*[16] and Christiansen *et al.*[17] Also in agreement was that none of the demographical factors (age, sex and ethnicity) were associated with the presence of a VUS.

5.4.2.2 Variants of likely pathogenic significance

A total of six LP variants, each located in a different gene, were identified in six of the 66 cases (9%), which is low in comparison to the 28%, 27%, 50%, 34% and 20% yields reported by Shanks *et al.*[4], Bagnall *et al.*[14], Neubauher *et al.*[18], Christiansen *et al.*[17] and Hertz *et al.*[19], respectively. The differences in overall yield can be attributed to numerous factors, including the composition of study cohorts, the size of gene panels used for NGS or different criteria used for filtering and classification of variants. The aforementioned studies all included a significant larger study cohort as well as a larger gene panel used for NGS. It is also possible that the stringent criteria for variant filtering and classification utilised in this study, could have resulted in a lower percentage in overall yield, since different laboratories may use different software programs and different sets of filtering criteria. In fact, both Lahrouci *et al.*[16] and

Williams *et al*.[11] also reported on the application of stringent filtering criteria and its subsequent lower yield of LP variants in less than 13% of their study cohorts. The lack of personal and / or family medical history in the majority of our study cohort could also have contributed to the lower percentage in overall yield, since research has reported a higher yield of LP variants in cases with a positive personal and / or family history of syncope, SCA and / or SD.[10, 15]

Certain variants can have a modifying or potentiating effect in the co-occurrence of another rare variant. Research has shown that the identification of multiple VUS and / or LP variants, whether it be in a single gene or multiple genes, may result in a compound effect, with an increased risk of a fatal arrhythmia.[4, 22] In four of the six cases (67%), an additional rare VUS were also identified, which could have led to a more severe clinical phenotype / outcome.

5.4.3 Profile of cases identified with a likely pathogenic variant

A mean age at death of 24.7 years, ranging between four months and 38 years, was recorded for the six cases carrying a LP variant. All of these cases were male, of which the majority (83.3%) were Black South Africans, and the remaining one case (16.7%), a White South African. Due to our study's limited sample size and the general higher male population, no statistical significance could be ascertained between the sex of our cases and the identification of a LP variant. This however, should not be of great concern, since other studies, conducted on different populations (and larger study cohorts), could also not confirm a definite link between the sex of the deceased and an increase or decrease in the identification of LP variants.[10, 11] In comparison to similar studies conducted elsewhere, a markedly different ethnic profile (Black South African vs. White Europeans) was observed for cases carrying a LP variant in this study.[10, 15, 19] This finding, however, was anticipated because of the unique, genetically diverse population of South Africa, which yet again, reiterates the need for local research into the underlying genetic aetiology of inherited disorders.

When taking into consideration the three different age groups representing the study cohort, the results pertaining to the identification of LP variants differ from what was found in other studies.[11, 16] In this study, one of the six LP variants (16.7%) was identified in the zero to one-year age category, whereas the remaining five (83.3%) were in the 19-45-year age group. Out of all SUDI cases included in this study, 7% were found to carry LP variants. None of the cases between the ages of one and 18 years old carried a LP variant, whereas all the cases in the 19-45-year-age group, a total of 12%, carried a LP variant. These findings are in contrast with what Ackerman *et al*.[10], Williams *et al*.[11] and Lahrouci *et al*.[16] all reported on; a lower number

of SUDI cases, and a much larger number of children and young adult cases, carrying a LP variant. Lahrouci *et al.*[16], who conducted a similar study on the New Zealand, Australian, United Kingdom (UK) and Netherland populations, found 20% of their cases in the one – 18-year-age group carrying a LP variant, whereas only 9% of their 19-35-year-olds were identified with a LP variant. None of their SUDI cases demonstrated any LP variant. Very similarly, Williams *et al.*[11] recorded that none of their (American) SUDI cases carried a LP variant, whereas 21% of their children and young adult cases were found to carry a LP variant. Furthermore, Ackerman *et al.*[10] found the overall yield of LP variants to be higher in their cases below the ages of 10 years old, whereas our study indicated a clearly higher yield in the 19 – 45 -year age group. This study's different profile of the overall LP-variant yield per age category furthermore supports the argument for more similar research to be conducted on the African genetic population.

5.4.4 Significance of likely pathogenic variants identified in six cases

All eight in-silico prediction tools that were used in this study supported the likely pathogenic significance of the six different variants identified. Furthermore, these variants were only identified in one different case each, with no publications on being found in a control cohort of any other study.

The location of an identified variant in a specific gene is one of the most well-known determining factors regarding the effect and clinical significance thereof.[23, 24] The novel **p.Cys2668Trp variant (case 10)**, in exon 53 of the *RyR2* gene, falls just outside one of the four previously-defined *RyR2*-hotspots (exons 44 – 50), however still located in the bridging solenoid (BSol), one of the so-called "major structural domains", of the protein.[25, 26] A link between *RyR2*-variant location and disease phenotype has been reported, with variants in the BSol and N-terminal domains (forming the RyR2 interprotomer contact domain) affecting the opening of the channel pore.[27, 28] Of importance is the stabilization of the RyR2 by the binding of FK506 binding protein (FKBP12.6) to the BSol domain.[29] Variants in this domain can affect the affinity of FKBP12.6 for RYR2, disrupting the interaction between the cytoplasmic region and the pore of the channel.[29, 30] These variants, typically in combination with some form of exercise and / or stressful situations, result in an increase in SR Ca^{2+} release, with an increased risk of delayed onset depolarization (DOD) and the triggering of fatal ventricular arrythmias.[25, 30] Numerous gain of function (GoF) variants, linked to CPVT, have been located in the BSol domain, similar to our novel variant.[26, 31] The fact that BSol has been determined to be one of

the five structural domains within the *RyR2* gene with a higher LP-variant frequency (11.9%; Figure 5.10), supports the finding of this LP variant in one of our cases.[32] Records of this case show a period of light exercise just prior to the time of death, which furthermore supports the finding of a LP variant in a gene notorious for its exercise-induced ventricular tachycardias.[31] Figure 5.10 illustrates the organisation of all the structural domains of the *RyR2* gene, and its associated variant frequency.[26] Following the central solenoid [(CSol) 12.5%] and the transmembrane domain [(TMD) 13.7%], the BSol domain has the third highest frequency of variants associated with disease (11.9%). Considering the location and the LP significance of the novel variant identified in our case, it once again highlights the importance and need for genetic research in the South African population.

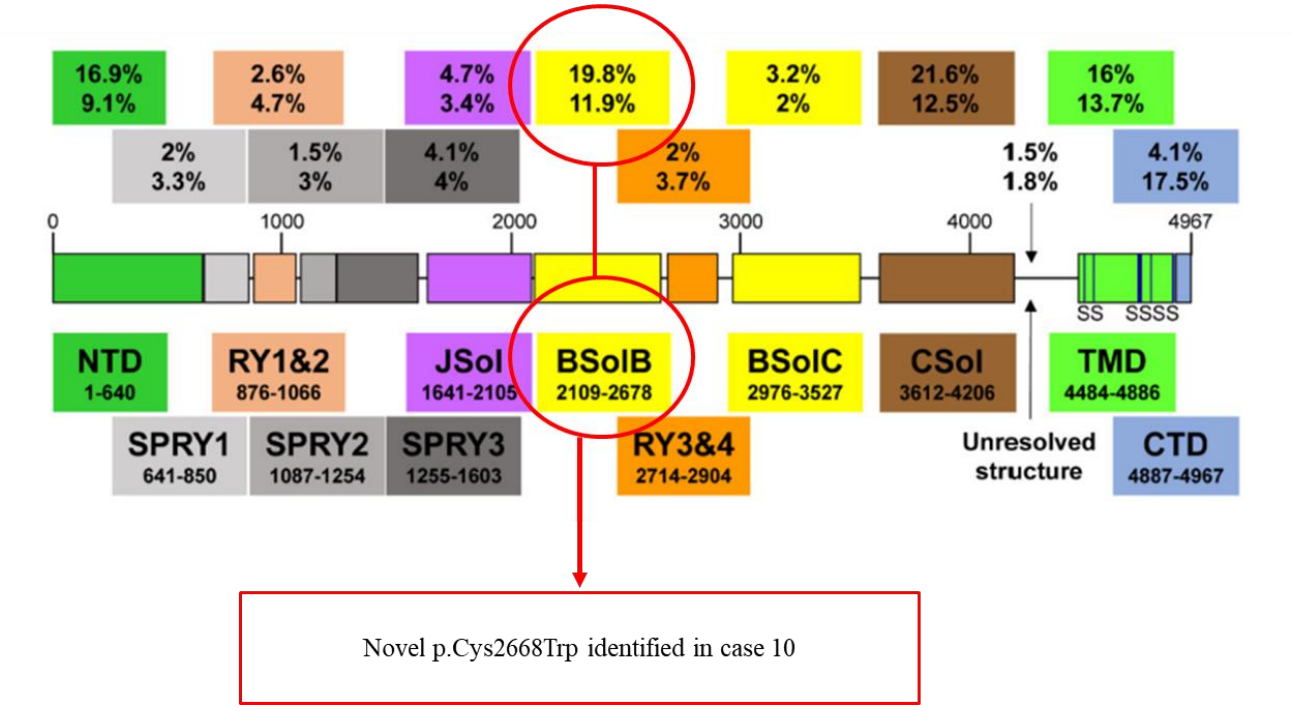

Figure 5.10 Schematic illustration of structural domain organisation of the *RyR2* gene. In the middle of the image, the six transmembrane segments (S) of the RyR2 are indicated by six horizontal lines (from amino acid zero to amino acid 4967). Furthermore, structural domains of the RyR2 are represented by a different colour box each, with its frequency of variants associated with disease indicated in each corroborating box at the top of the image. Indicated in the red circles are the BSol domain, where the novel variant p.Cys2668Trp was identified, as well as the variant frequency of 11.9% for this specific domain.[26]

An additional 19 variants were also identified in this case, of which all of them were classified as LB. These variants were identified in 11 different genes, including *RyR2*, *CASQ2*, *KCNJ5*,

CACNA1C, *SCN1B*, *KCNE1*, *SCN10A*, *ANK2*, *TRDN*, *AKAP9* and *NPPA*, all of which play an important role in the cardiac conduction pathway.[33] Although the additional p.Ser1400Gly variant in the *RyR2* gene was determined to be LB, the possibility of a compounding effect in the co-occurrence of the novel, LP *RYR2* variant, cannot be excluded. Furthermore, the *CASQ2* gene, in which an additional variant was also identified, plays an integral role in the regulation of the *RyR2* gene.[34] The *CASQ2* gene encodes a binding protein within the SR lumen, where it buffers the free Ca^{2+} ion concentration. By binding to the integral triadin and junction proteins, CASQ2 also interacts with the S1S2 luminal loop of the RyR2, which results in an inhibitory effect on Ca^{2+} release.[34, 35] Any variants in CASQ2 may impair RyR2 regulation, which should be considered in this specific case of ours.[34, 35]

The **p.Lys167Arg variant; rs932909554** in exon three of the *SNTA1* gene, and of LP significance, was identified in case 15 of this study. The *SNTA1* gene encodes the syntrophin isoform expressed in the heart.[36] Syntrophins are cytoplasmic submembranous scaffold proteins and form part of the dystrophin-associated glycoprotein complex (DGC), that plays an important role in the binding and organisation of the subcellular localisation of numerous proteins.[37, 38] Described as a channel-interacting protein, SNTA1 regulates the gating kinetics of ion channels, especially that of the SCN5A channel, through its linkage to various intracellular pathways.[36, 37] Syntrophin consists of two pleckstrin homology (PH) domains at the N-terminus of the protein, the internal PDZ domain and the SU domain at the C-terminus. The PDZ domain, also where our p.Lys167Arg variant was located, plays a critical role in the regulation of the cardiac sodium (SCN5A) channel, due to its interaction and binding to the C-terminus of the SCN5A.[36, 37] As a result, reports have shown SNTA1 to play an integral role in the genetic pathway regulating the membrane repolarisation of the ventricular cardiac muscle cells (see Figure 5.11), with variants in this gene linked to atrial fibrillation and LQTS.[36-38]

This same individual (case 15) carried an additional 23 missense variants, of which the majority were benign. Interestingly, of the 12 major genes included in the above-mentioned pathway (see Figure 5.11), three others also presented with missense variant/s. Three different missense variants were identified in the *RyR2* gene, one in the *SCN5A* gene and one in the *CAV3* gene.

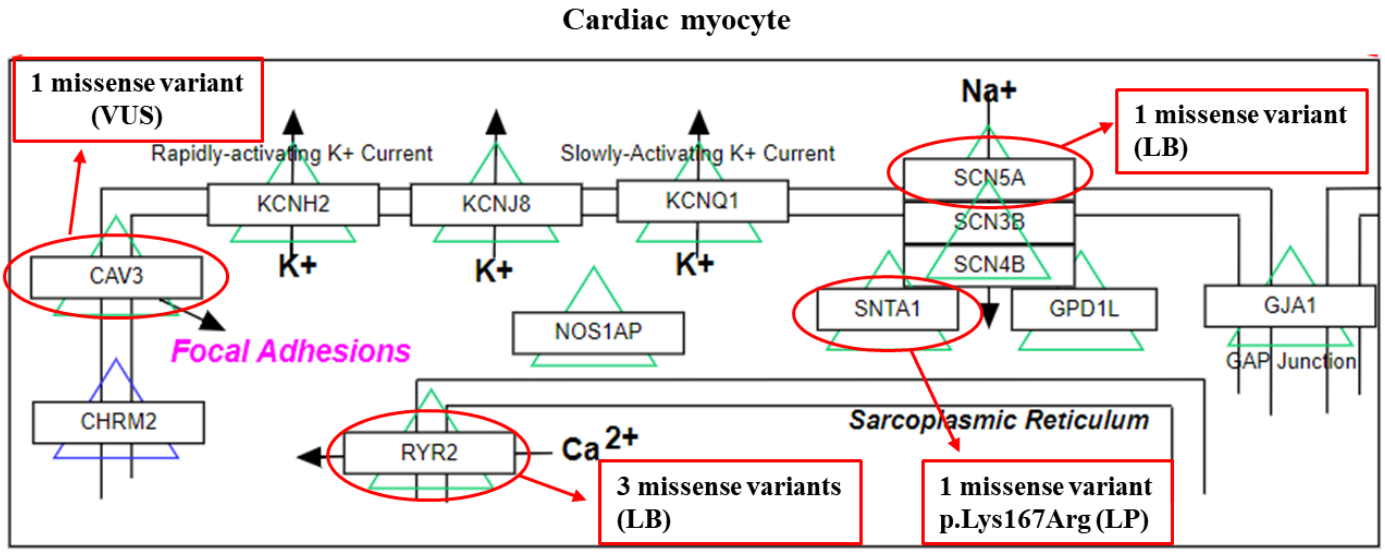

Figure 5.11 Illustration of the genetic pathway involved in the ventricular cardiac myocyte cell membrane. This specific genetic pathway consists of 13 major genes, all linked in terms of their function, in regulating signalling proteins as well as ion channel gating kinetics in the cardiomyocyte. The four genes encircled in red all carried missense variant/s: 1 LP variant in *SNTA1*, 1 LB variant in *SCN5A*, 3 LB variants in *RyR2* and 1 VUS in *CAV3*.[37]

Of note, the *CAV3* gene is also a component of the DGC complex, which contributes to its structural and signal transduction properties in the cardiac myocyte.[38] The CAV3 acts as docking sites for signalling proteins, and disruptions in this complex may affect the gating kinetics of ion channels. Variants in *CAV3* have been associated with LQTS and SIDS.[39, 40] The *CAV3* variant identified in this case, and published as a VUS, was predicted to have a deleterious effect on the protein by six of the eight in-silico tools used in this study. The possibility of all these combined variants leading to an increased risk of atrial fibrillation, LQTS and SD, especially in an apparently healthy 24-year-old with no established cause of death, should be considered.

The second novel, LP variant was found in case 24, a young 38-year-old Black South African male who collapsed in a park while playing sports with friends. The **p.Trp318Cys variant** was identified in exon 10 of the exertional-triggered *CASQ2* gene. As mentioned previously, CASQ2 is an important Ca^{2+} binding protein, located in the SR, with a main function of protein buffering and regulation of Ca^{2+} release channels in cardiac muscle cells.[34, 35] CASQ2 is comprised of three thioredoxin-like domains, enclosing a highly negatively charged hydrophilic structure, with an N-terminal and C-terminal (tail) domain as well (Figure 5.12).[41] The p.Trp318Cys variant is located in the highly conserved, helical part of domain III, closer to the C-tail. Domain III plays an important role in junctional SR targeting and CASQ2 polymerization, with variants in this location reportedly associated with CPVT.[35, 41] Lahat *et*

al.[35] reported the p.Asp307His missense variant in an Israeli family diagnosed with CPVT, with the variant location also in Domain III of the *QASQ2* gene. The location of p.Asp307His in relation to this study's p.Trp318Cys are indicated in Figure 5.12 below.

The variant identified in our case resulted in a structural amino acid change, from Trp, the largest amino acid with two aromatic rings, to the much smaller Cys, which contains no aromatic ring. This structural change in the amino acid sequence in Domain III of the gene could have resulted in an impaired Ca^{2+} release process, ultimately increasing the individual's susceptibility to exercise-induced ventricular tachycardias and SD.

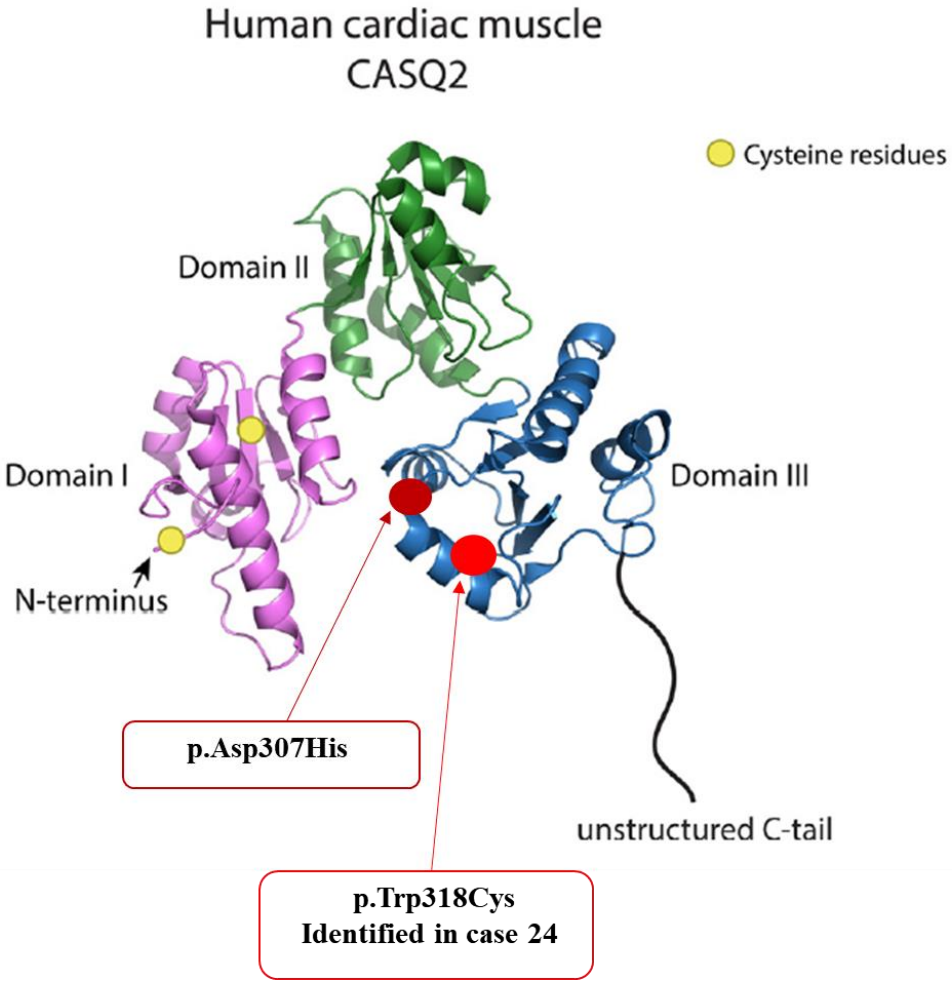

Figure 5.12 Location of two identified variants in the CASQ2 protein structure. The structure consists of six different domains: the N-terminus domain, domain I, domain II, domain III and the unstructured C-tail. Both variants are located in the same functional domain III, the pAsp307His variant that was found in an Israeli family (indicated in maroon) and the p.Trp318Cys variant that was identified in our case 24.[41]

Another 22 missense variants were also identified in this case, of which 21 were LB and one remained a VUS. All 22 variants were identified in 12 different genes, including *CASQ2*, *RyR2*, *KCNJ5*, *PRKAG2*, *CACNA1C*, *SCN1B*, *KCNE1*, *SCN10A*, *ANK2*, *TRDN*, *AKAP9* and *DPP6*. All 12 genes are interlinked in forming part of the cardiac conduction and ion channel transport pathways.[33] Of note, two of the additional missense variants were also identified in the *CASQ2* gene, although each one in isolation, were deemed to be of LB significance. One of these two variants was in Domain I of the protein, whereas the other was located in the conserved, functionally relevant Domain III of the protein. Research has shown that multiple missense variants in one gene may have a compounding effect on each other, possibly leading to an earlier onset of symptoms.[23, 42, 43] In this case, the finding of three missense variants in one gene, of which one was deemed LP, and two located in a critical domain of the protein, might have contributed to a possible increase in the severity of the phenotype / outcome. Furthermore, of relevance is the fact that another one of the additional missense variants was identified in the *RyR2* gene, which as mentioned before, forms an important complex by the binding of triadin, junction and CASQ2 to RyR2.[35] Any variants in one of these genes may negatively affect this binding complex and lead to an impairment in the regulation and transport of Ca^{2+} through appropriate channels, leading to ventricular tachycardia, arrhythmia, and SD. Another factor to consider in this case, is the co-occurrence of VUS's and LP variants, which has also been reported to result in a possible compound effect and increase in disease severity, whether it be in the same gene or multiple genes.[42, 43] This case also carried a VUS identified in the *PRKAG2* gene. This variant, p.Arg84Trp; rs61746358, has been documented in public databases to have an unknown clinical significance, however seven of the eight in-silico tools used in this study, predicted a deleterious effect on the protein.[44] The variant is located in exon three of 16, which according to literature, is one of the hotspot regions for missense variants and VUS's.[45, 46] The *PRKAG2* gene encodes the adenosine monophosphate–activated protein kinase gamma 2 regulatory subunit, which in the heart plays an important role in the cellular energetic homeostasis control. Variants in this gene can modify the enzyme activity in the cardiomyocytes, which in turns affects the cell's glucidic uptake and metabolism, resulting in the deposition of glycogen.[47-49] Previous variants identified in this gene, especially in the younger population, have been associated with high rates of atrial fibrillation, conduction disease and life-threatening arrhythmias, leading to an increased risk of premature SCD.[45, 46, 49] The finding of this specific VUS in case 24, in the co-occurrence of the LP variant, might have increased the individual's susceptibility to life-threatening arrhythmias and SD.

The fourth LP variant, **p.Asp76Asn in the *KCNE1* gene**, was identified in a 23-year-old Black South African male. The *KCNE1* gene encodes for a small transmembrane modulatory subunit, which binds to the KCNQ1 protein to form a voltage-gated ion channel complex and expressed in the cardiac ventricular myocytes.[50, 51] This complex forms a slowly activating and slowly deactivating cardiac delayed rectifier current (I_{Ks}) that is critical in regulating the cardiac action potential.[51, 52] The KCNE1 protein consists of an extracellular N-terminus, a single helical transmembrane domain, and a highly conserved intracellular C-terminus. It is the C-terminus of the KCNE1, specifically the region between amino acid 70 and acid 81, that binds to the C-terminus of the KCNQ1 protein to form the potassium channel complex.[53, 54] The p.Asp76Asn variant identified in this study was located in this critical region of the KCNE1 C-terminus (see Figure 5.13). When bound to the KCNQ1 protein, KCNE1 modulates the physical properties of the channel, resulting in an increase in the outward current amplitude and thereby slowing its ultimate activation.[51, 53, 54] Variants located in the C-terminus of the KCNE1 gene, especially the LP p.Asp76Asn, have been linked to LQTS-diagnosed patients and SCD's.[53]

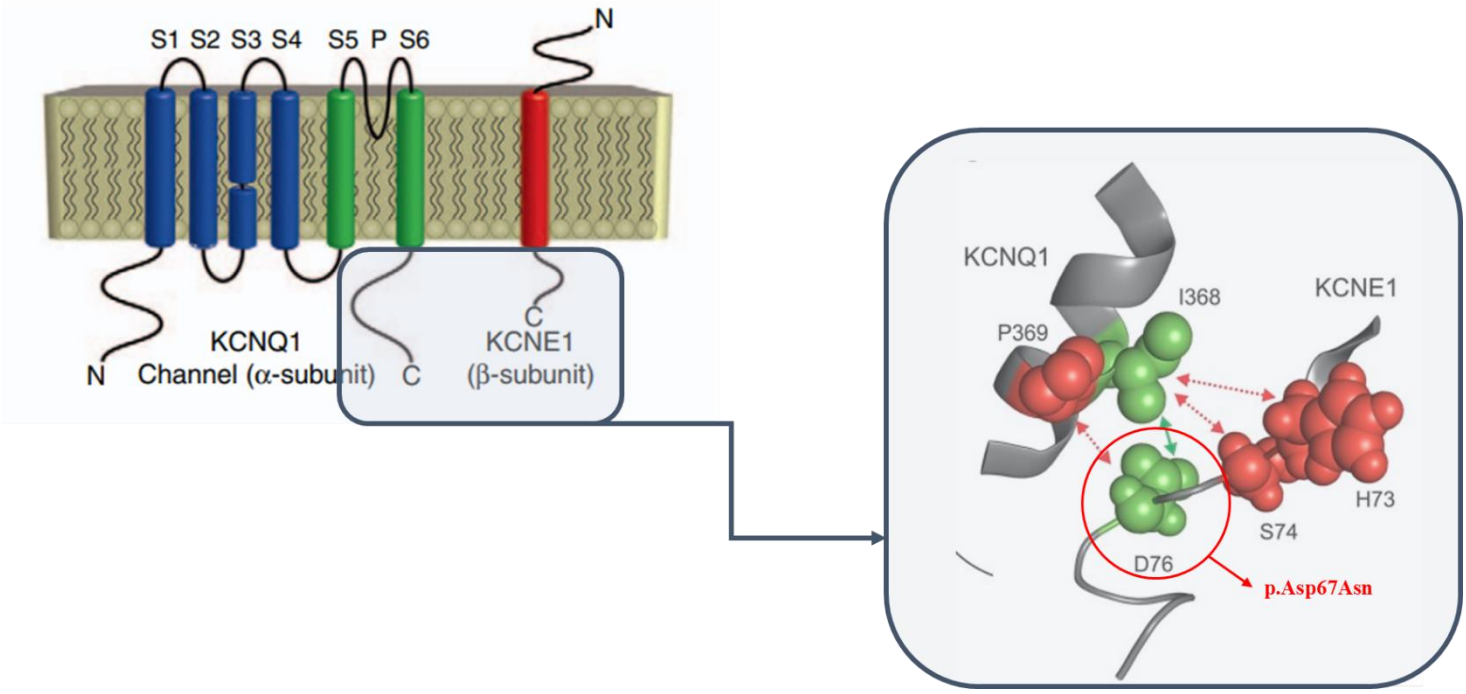

Figure 5.13 Schematic illustration of the potassium channel complex formed by a KCNQ1 α-subunit bound to a KCNE1 β-subunit. The small grey box on the left-hand side of the image illustrates the intracellular binding site between the C-terminus of the KCNQ1 α-subunit and the C-terminus of the KCNE1 β-subunit. The grey box on the right-hand side of the image is an enlargement of the binding site between the C-termini of the two subunits (KCNQ1 and (KCNE1), specifically indicating the important role of residue D76 in KCNE1. Indicated in the red circle is the p.Asp76Asn variant identified in this case.[54]

Studies have shown that variants in the critical region of the KCNE1 C-terminus, shift the KCNQ1's voltage dependence of activation to more depolarising (positive) voltages, causing a reduction in potassium channel conductance. The p.Asp76Asn variant has specifically been shown to reduce the outward I_{Ks} current density, causing a delay in repolarisation and ultimately prolonging the cardiac action potential, leading to an increase in susceptibility to cardiac arrhythmias and SCD.[51, 52, 54]

An additional 23 missense variants were also identified in this case; most of which were of LB significance. These variants were identified in 13 different genes (*CASQ2*, *TNNT2*, *KCNJ5*, *CACNA1C*, *SCN1B*, *TRPM4*, *KCNE1*, *CAV3*, *SCN10A*, *ANK2*, *TRDN*, *AKAP9* and *DPP6*), which all play a role in the cardiac conduction pathway.[33] One of these variants (p.Ser38Gly) was identified in the *KCNE1* gene, the same gene in which this case's LP variant was found. Although databases reported this KCNE1 variant as LB, Wu *et al*.[52] reported it to be a risk factor for atrial fibrillation in a Chinese cohort. Furthermore, one VUS was also identified in this case. The *CAV3* gene carried the missense p.Thr78Met (rs72546668) variant, which was reported in public databases and literature to be a VUS.[39, 55] Of the eight in-silico tools used in this study, six predicted this variant to have a deleterious effect on the protein. Keeping in mind the role CAV3 plays in the gating kinetics of ion channels (as discussed previously), as well as its link to LQTS, the possible compounding effect of this variant with the LQTS-associated p.Asp76Asn (LP) variant could have led to an increased risk factor for cardiac arrhythmias and SD.

The LP **p.Val100Gly (rs138375318) variant in the *GJA5* gene,** was identified in a case of a four-month-old infant boy, who very typical of a SUID case, was found deceased during a scheduled period of sleep. The *GJA5* gene encodes for the gap junction α-5 protein, also known as connexin 40 (Cx40), which is expressed in both the atria and ventricles of the heart.[56, 57] Connexin 40 forms part of the myocardial gap junctions; transmembrane channels that allows for the passive diffusion of ions between cells.[57, 58] Transmembrane channels consists of two connexons, one provided by each cell, which each comprises of six transmembrane connexin isoforms (Figure 5.14).[59, 60] In cardiomyocytes, these connexins are mainly isoforms 40, 43 and 45, in which the ion exchange between these channels is crucial in maintaining the electrical synchronisation of the atrium as well as the rapid conduction of impulses.[59-61] Variants in the *GJA5* gene (Cx40) have been associated with atrial fibrillations and atrial standstills caused by abnormal electrical coupling.[57, 62, 63]

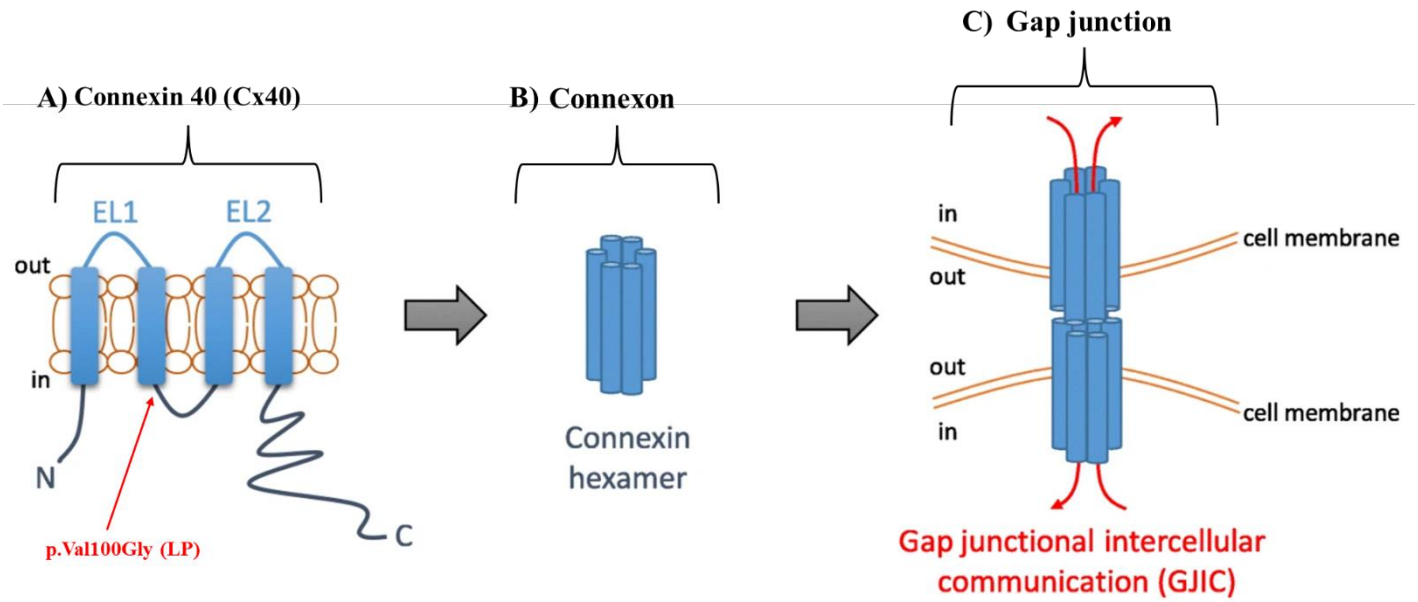

Figure 5.14 Schematic illustration of the structural components of the cardiomyocyte gap junction. A) Protein structure of the Cx40 protein encoded by the *GJA5* gene. B) A cluster of six connexin isoforms (hexamer) forming one connexon. The red arrow indicates the position of the p.Val100Gly variant identified in this study. C) Two connexons (one per cell) forming a transmembrane channel, called a gap junction, which allows for the passive diffusion of ions between two cells.[64]

The GJA5 protein, or Cx40, consists of four transmembrane domains, two extracellular loops, one cytoplasmic loop (CL), a cytoplasmic N-terminus and a cytoplasmic C-terminus.[63] As shown in Figure 5.14 above, the p.Val100Gly variant identified in this study was located in the CL region, closer to the second transmembrane domain, which in-silico tools predicted to be a conserved domain in the protein. Public databases assign this variant with conflicting levels of pathogenicity, whereas all eight in-silico tools used in this study predicted a deleterious effect on the protein. No published case reports specific to the p.Val100Gly variant could be found, with the variant most similar to its protein location, reported by Shi *et al*.[65] They identified the p.Lys107Arg variant, also located in the CL region of GJA5, in a family diagnosed with lone atrial fibrillation, and was associated with impaired intercellular electrical coupling and enhanced susceptibility to cardiac arrhythmias.[65]

An additional 28 missense variants, in 14 different genes, were also identified in this case. These genes included *RyR2*, *KCNQ1*, *KCNJ5*, *KCNA5*, *SCN5A*, *SCN10A*, *SCN1B*, *PKP2*, *CACNA1C*, *TNNT2*, *ANK2*, *TRDN*, *AKAP9* and *DPP6*, of which the majority play a critical role in the genetic pathway of controlling the cardiac action potential.[33] Although of LB significance, studies have reported on numerous of these variants having an enhanced

pathogenic effect in individuals carrying multiple variants in genes linked to the same genetic pathway.[42, 43]

The sixth, and last, LP variant in this study, **p.Arg14Cys (rs750771811) in the *SCN10A* gene**, was identified in a 28-year-old Black South African male, who's cause of death remained unexplained following a thorough medico-legal autopsy investigation. The gene in which this variant was found encodes for the tetrodotoxin-resistant voltage-gated sodium channel α-subunit 10, which is expressed in the atrial end ventricular myocytes and is located next to the *SCN5A* gene on chromosome three, sharing very similar amino acid sequences (70.4%).[66, 67] The SCN10A channel plays an important role in the electrical function of the heart. Dependent on the voltage difference across the cardiomyocyte's membrane, the SCN10A channel mediates its sodium ion permeability, and is responsible for the initiation and propagation of the cardiac action potential.[68, 69] The SCN10A channel comprises of a cytoplasmic N-terminus, four homologous domains (each consisting of six α-helical transmembrane segments) and a cytoplasmic C-terminus.[68, 69] Figure 5.15 illustrates the linear structure of the SCN10A channel, with its most prevalent variants mostly located in the transmembrane spanning regions and the cytoplasmic loops. Very few variants are found in the N-terminus and C-terminus of the channel.[68-70] Numerous variants in the *SCN10A* gene have been associated with BrS, characterised by a reduction in the late sodium current and a slowing in the action potential firing, resulting in cardiac arrhythmias and SCD.[67, 69, 71] In this study, the p.Arg14Cys variant was identified in exon one and located in the cytoplasmic N-terminus of the protein. Regarding the Arg amino acid at position 14 (p.Arg14) of the *SCN10A* gene, three different variants, all linked to BrS and / or SCD, have been reported.[3, 67, 68] Zhang *et al*.[67] identified the p.Arg14His variant in a case of SUD in the Chinese population, which they considered to be LP and the genetic cause of death. Hu *et al*.[68] identified the p.Arg14Leu variant in an American family whose diagnosis of BrS was made during a bout of fever. Functional expression studies showed that this variant causes a significant reduction in sodium channel availability, leading to a positive shift of half-activation voltage, ultimately reducing the cardiomyocyte's excitability, and initiating cardiac arrhythmias.[68] The third variant, p.Arg14Cys (also identified in our case), was reported by Heathfield *et al*.[3] in a Black South African, two-month old male SUID case. No cause of death could be established, with only a history of flu-like symptoms reported. As a result, they considered the p.Arg14Cys variant to be pathogenic and the probable cause of the SUID. Considering that our case shared the same ethnicity, together with similar reports of flu-like symptoms in the absence of any significant autopsy findings, this variant identified in

our study was determined to be of likely pathogenic significance and the probable cause of SUD. Furthermore, three additional missense variants (p.Val1073Ala; rs , p.Val1141Met; rs and p.Met1713Val), also in the *SCN10A* gene, were identified in our case, of which all three are linked to BrS and an increase in the susceptibility to atrial fibrillation.[71] Both p.Val1073Ala (homozygotic) and p.Val1141Met (heterozygotic) were located in the 'hotspot' cytoplasmic loop, linking transmembrane domain II with transmembrane domain III. The homozygotic p.Met1713Val variant was located in the transmembrane spanning S6 region of domain IV of the protein (see figure 5.15).

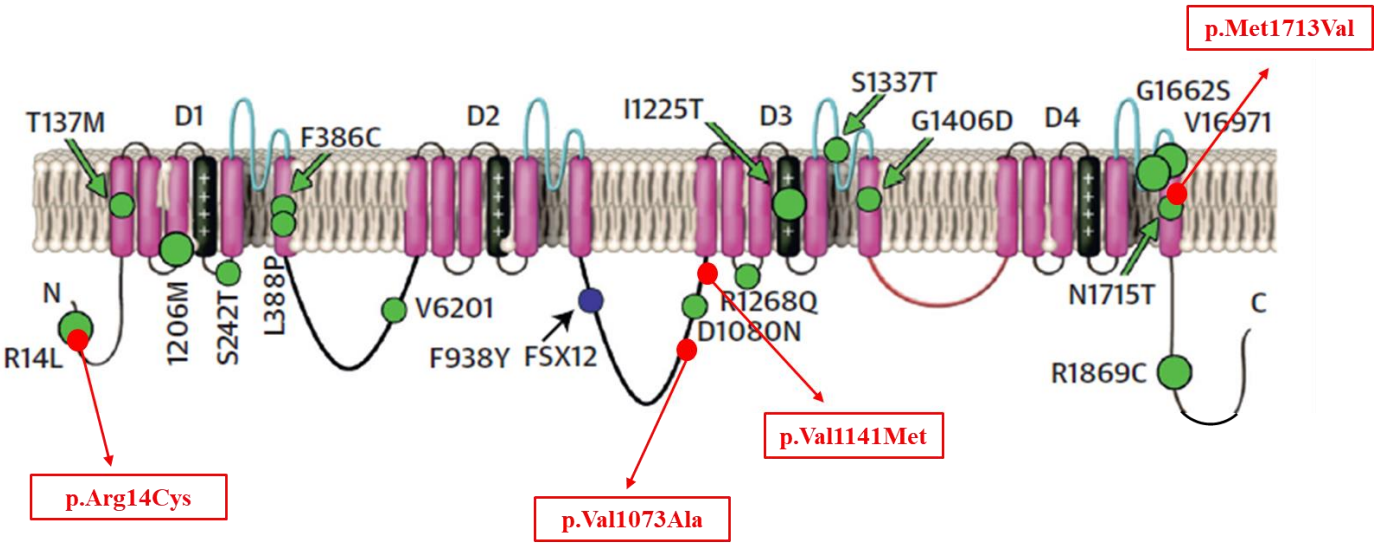

Figure 5.15 Illustration of the linear structure of the voltage-gated sodium channel α-subunit 10, encoded by the *SCN10A* gene. The location, within the protein structure, of the most prevalent BrS-associated variants in the *SCN10A* gene are indicated by green dots. The four red dots show the four missense variants, and their locations, that were identified in case 65 of this study.[68]

The SCN10A channel has also been associated with the functioning of the SCN5A channel during the depolarisation phase of the cardiac action potential. Due to its shared enhancer binding site with the *SCN5A* gene, certain *SCN10A*-variants may result in a decrease in *SCN5A* gene expression.[67-69] In our case, two additional missense variants (of which one was a VUS) have been identified in the *SCN5A* gene. In agreement with published literature, five of the eight in-silico tools used in this study predicted the p.Asp2003Asn variant to have a deleterious effect on the protein. Another missense variant in this case, and of relevance to the *SCN10A* gene, was identified in the *SCN1B* gene. Although this variant, in its single entity, was determined to be LB, the possibility of an increased risk to cardiac arrythmias, in the co-

occurrence with the four *SCN10A*- variants, should be considered.[67] The *SCN1B* gene encodes for the regulating β-subunit of the sodium (SCN10A) channel, which plays an important role in the regulation of SCN10A-surface expression and the trafficking thereof.[67] This case carried a further 24 LB missense variants in 11 different genes (*CASQ2*, *TNNT2*, *KCNJ5*, *CACNA1C*, *CACNA2D1*, *TRPM4*, *KCNE1*, *CACNA1D*, *TRDN* and *AKAP9*), of which most play an integral role in the cardiac action potential genetic pathway.[33]

5.4.5 Study implications towards South Africa

The clinical effect of variants associated with SCD should always be interpreted with caution, especially when no clinical symptoms are available in the setting of post mortem genetic testing.[72, 73] Fortunately, due to the rapidly developing technology, the prediction of a variant's clinical significance, based on its location in the protein, whether it is a non-conservative amino acid change in a conserved region of the protein, its predicted functional effect on the protein as well as its frequency of detection in different populations, can nowadays be achieved with much greater certainty.[74, 75] In this study, post mortem genetic testing provided evidence of a genetic arrhythmic/cardiac conduction disorder as the probable pathogenic basis for 9% of SUD / SUID cases. Since no cause of death could be established for these cases, even after conducting a thorough autopsy and extensive ancillary investigations, an inherited cardiac arrhythmogenic disorder was implicated in the cause of death for these cases.

Considering the high prevalence of a genetic background in cardiac disorders, the genetic analyses in SUD / SUID cases provides significant clinical implications regarding the possible diagnosis and treatment of family members at risk for the same disease.[22] Bearing in mind the reported high incidence of a SUD being the sentinel event without any clinical symptoms or warning signs, coupled with the evidence of a marked reduction in mortality associated with proper treatment, the critical importance of genetic testing cannot be overstated.[7, 22, 76] Furthermore, the advancements in technology and the associated decrease in cost, have led the forensic medical profession to increasingly become aware of the dangerous implications that an unidentified aetiology of a possible inherited disorder can have on family members at risk. As a result, most first-world countries have adopted molecular testing as part of their routine standard investigation into all unexplained SD's suspected of having an underlying inherited cardiac arrhythmogenic disorder.[77, 78] The results obtained in our study provides evidence that South Africa cannot afford to not implement the same routine into their forensic medical practice.

Unfortunately, by no means is South Africa currently able to implement routine genetic tests on the same comprehensive scale than that of developed countries. Not only has there never been a genetic research study conducted on South African SUD's (between one and 45 years old) but also the use of technology and reagents are still limited due to a lack of adequate resources and funding. The findings of this study present the first basic concept and understanding of the genetics underlying South African SUD's in the young, and aim to provide the framework for an initial, more cost-effective implementation thereof.

Current internationally published guidelines recommend targeted genetic testing of the major cardiomyopathy and arrhythmic-related genes as the minimum standard that is required in the routine autopsy practice for adequate investigation of a SCD.[77, 78] The use of commercial panels consisting of a combination of up to 100 cardiomyopathy and channelopathy genes is also becoming more common.[4, 11] Although the findings of this study currently do not support the implementation of such a broad genetic panel, we are of the opinion that the targeted testing of only major cardiomyopathy and arrhythmic genes, linked to other population groups, would be insufficient. Given the uniqueness of South Africa's genetic diversity, which was supported by the identification of novel variants in this study, coupled with the lack of genetic research specific to the South African population, we would like to make the following recommendations.

Of the 49 different genes included for NGS, only 16 were identified with VUS, risk factor and / or LP variants. All VUS's were filtered to only include those with a high suspicion of pathogenicity and an estimated predictive value (EPV) above 90%. We recommend the inclusion of these 16 genes in the initial routine testing for possible cardiomyopathies and / or arrhythmogenic disorders in all SD's (between zero and 45 years of age) which remained unexplained after a full medico-legal death investigation. Furthermore, the inclusion of two additional genes (*KCNH2* and *KCNQ1*) are also recommended, due to its reported high clinical significance in similar international studies. It is of the opinion that this study's cohort is too small to exclude these two relevant genes. The few cardiomyopathy-related genes represented in the results of this study highlights the importance for their inclusion in routine testing, even in a cohort of cases which showed no and / or minimal CM findings at autopsy. Table 5.3 lists the 18 genes, along with their associated number of exons, recommend for inclusion in testing.

Table 5.3 List of 16 different genes recommended for inclusion in routine post mortem genetic testing

Gene	Total number of exons
ASPH	20
CACNA1C	47
CACNB2	20
CDH2	17
CASQ2	11
CAV3	3
GPD1L	8
GJA5	2
KCNA5	1
KCNE1	7
KCNH2	15
KCNQ1	19
PRKAG2	16
PKP2	14
RYR2	105
SCN5A	28
SCN10A	27
SNTA1	8

In terms of the genetic sequencing method to use in this routine testing, an argument can be made for both Sanger sequencing and NGS. Sanger sequencing is generally more targeted towards "hotspot" regions / exons of a gene, which is more cost-effective, without the burden of producing massive data sets, however it is very time consuming and labour-intensive.[15] Next generation sequencing techniques are typically faster and more comprehensive in terms of the number and range of genes being targeted for testing, however, is associated with producing massive datasets to be analysed and remains expensive for technological and financial constrained departments.[4, 15] Due to our limited sample size (n = 66) and the lack of comparable results for the South African population, it is our recommendation to not utilise the Sanger

sequencing method for routine testing of these 16 genes. At this point in time, we are unable to accurately determine the specific "hotspot" exons for each of these genes, thus Sanger sequencing of the entire 18 genes will be too labour-intensive and time consuming. As of now, it is our recommendation to implement NGS of all 18 implicated genes for routine testing in unexplained SD cases. The inclusion of only these genes identified with VUS, risk factor and / or LP variants, and not all 49, will be much more cost-effective and realistic considering the resources in SA. However, the ultimate aim is to develop and implement, via Sanger sequencing, a more accurate exon-targeted genetic test of all major genes associated with South African unexplained SD's.

In the event of genetic testing revealing results that affect the stated cause of death, therefore affecting the autopsy report and / or death certificate, an important question arises as to the required ethical / legal steps that need to follow.[79] Since the establishment of the cause of death forms part of a formal legal investigation, in association with several other judicial authorities, it should only be logical that an amendment on the cause of death, and the notification of family members thereof, is the most ethical and legal step to take. However, although the improvements in technology have led to a faster, more accurate variant identification process, this has inadvertently led to the identification of variants with an uncertain clinical significance. Due to its far-reaching implications to at-risk family members, one should always practice great caution in classifying a VUS's predicted pathogenicity. Only P, LP and VUS's with a high suspicion of pathogenicity should be reported to at-risk family members, and only in consultation with genetic experts and counsellors.[79]

5.5 Limitations of this study

The size of this study cohort might have limited the true nature and scope of the genetic basis underlying inherited cardiac arrhythmogenic disorders in the South African population. More genetic research on South African SD cases, is recommended. Another aspect of this study which deserves greater attention, is the lack of a standerdised protocol for the investigation of SUID and SUD cases, which might have led to some cases not being included in this study. Another limitation was the lack of available personal and / or family medical history. A cohort selection more targeted towards an existing phenotype or positive family history of syncope and SD, may increase the overall yield of LP variants. Although the forensic pathologist observed no obvious signs at autopsy to suspect a fatal overdose, the outstanding toxicology results for most of our cases is problematic. Studies have shown the presence of certain drugs may cause an increased susceptibility to cardiac arrhythmias, and the status thereof in our cases

could have led to a better understanding of the genetic results. Unfortunately, the toxicological services in South Africa have reached a dire state, with the urgent need for intervention currently being realised. In terms of variant classification and interpretation of its clinical significance, this study could have benefitted with co-segregation studies in family members, as well as functional expression studies. The majority of the South African population are still wary of the concept of genetic testing, with their availability and co-operation in these investigations still limited. More community awareness, education and guidance on this topic is much needed. Finally, more resources need to be allocated towards the Forensic Pathology Service in South Africa, in order to ensure maximum public health benefits to the community.

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Chapter 6

Conclusion

This chapter was published as a continuing medical education (CME) article, entitled "Post mortem genetic testing in young individuals: What clinical medical practitioners need to know in the South African Medical Journal (SAMJ), December 2022;112(12):886-889.htpps://doi.org/10.7196/SAMJ.2022.v112i12.16800. A PDF copy of the published article is available as Appendix O.

The editorial style of the SAMJ was followed in this chapter.

Abstract

The death of a young person is most often a tragic occurrence, more so when this death was unexpected. Forensic pathologists are mandated to investigate such deaths, and there has been a strong move internationally towards genetic testing as an additional investigative tool. The aim of our article is to bring the advantage of implementing the so-called molecular autopsy in a local setting to the attention of medical practitioners. When a multidisciplinary approach is taken in cases of sudden unexpected death, the benefits to family members, and society as a whole, are irrefutable.

6.1 Concluding remarks

A general misconception regarding the field of forensic medicine seems to be that the purpose thereof is only to conduct medico-legal autopsies on cases that are criminal in nature, and to deal with the subsequent judicial matters resulting from such cases. However, forensic medical practitioners are in fact in a most fortunate and unique position, as they observe the exact pathology of various diseases (whether or not these are attributed as the cause of death) in thousands of autopsies performed each year. In most cases these medicolegal autopsies reveal an underlying, natural disease as the cause of death. All information pertaining to the cause and mechanism of these deaths are relayed to several entities, including the Department of Home Affairs and Statistics South Africa. This, perhaps, can be considered to be one of the most valuable contributions from the field of forensic medicine to society. Such data, relayed to the various stakeholders in the public health sector (including clinical medical practitioners), contribute to determining population health status as well as identifying and dealing with priority areas. Currently, one of the most important public health concerns in Africa and considered to be among the priority areas that should receive greater attention is that of the continuous increase in cardiovascular diseases (CVD). A particular subset of CVD in South Africa (SA) that is of great concern is sudden unexpected deaths (SUDs) in the young population.

It is a common occurrence to read poignant media headlines in SA of young, seemingly healthy, individuals who suddenly and unexpectedly die, often with family members in the dark as to the cause of the death. Fortunately, research on these SUDs has shown that postmortem genetic testing can detect if an inherited cardiac arrhythmogenic disease is the cause of death in many (up to 40%) of these cases.[1] Such cardiac diseases often present with no clinical symptoms or warning signs prior to a sudden death (SD), showing the added benefit of conveying autopsy findings to clinicians. The societal benefits from clinicians receiving such evidence-based findings associated with the cause of a patient’s SD are currently underutilised in SA. Such practical recommendations are an added benefit for clinicians when evaluating patients presenting with a sudden cardiac arrest. It could even be said that of greater importance is the benefit to patients with a family history of SUD. This matter urgently requires the attention it deserves.

6.1.1 The role of genetic testing in SUDs

Rapid and continuing development in molecular techniques allows the field of forensic molecular biology, also known as forensic molecular pathology, to use a molecular approach in not only studying but also diagnosing the underlying genetic basis of human disease and death processes.[2-4] International reports show that no other scientific discipline has embraced the application of molecular biology techniques for diagnostic purposes more than the field of forensic science and pathology, particularly in the medicolegal investigation of SDs in the young.[2,5]

Approximately 70 - 85% of SDs in young populations are cardiac related (termed sudden cardiac death (SCD)), and up to 90% of these SCD cases are potentially caused by inherited cardiac diseases, making this an important global topic in molecular research.[6-8]Molecular screening in cases of SCD has provided an explanation to the aetiology underlying the inherited cardiac disease, ultimately leading to an increased understanding of critical conditions and the clinical management thereof.[2,9,10] Most international medical associations deem SCD a global public health issue, and are currently advocating for prioritising and aligning their research plans and healthcare delivery objectives to utilise the diagnostic benefits that molecular analysis can provide.[8,11,12]

The growing global burden of non-communicable diseases, of which CVD is the predominant cause, is one of the United Nations' health priorities.[13] A published editorial in SA Heart addresses the topic of global health and, more specifically, the CVD burden in Africa.[14] Not only is CVD one of the leading causes of death worldwide, but it has also been reported that ~85% of these deaths occur in low- and middle-income countries.[6,13,14] Apart from CVD being the second biggest killer in Africa, the mean age of these deaths has also been recorded as the youngest in the world.[13,15]

One of the greater health priorities in sub-Saharan Africa should be a focus on preventing and treating cardiac diseases. There are key gaps in knowledge, and especially on research priorities on genetic causes of cardiac diseases specific to the African population.[13,16,17]

6.1.2 The SD conundrum

SD is a leading cause of mortality in the young, and considered a public health problem worldwide owing to its prevalence and significant impact on society.[18-20] The global incidence of SD in the young ranges between 1.3 and 8.5 per 100 000 person years.[6]

In SA, a SUD of a person is classified as an unnatural death, and therefore mandated in terms of the Inquests Act No. 58 of 1959 to be investigated in the medicolegal environment, where the forensic medical practitioner will conduct a thorough autopsy of the case in order to determine a cause and mechanism of death. However, in many instances, a clinician has previously known a patient who presented with features of a heart disease, and consequently classifies these cases as natural, attributing the sudden death to ischaemic heart disease (acute myocardial infarction). In decedents who have documented clinically investigated coronary artery disease, this would be the reasonable cause of death. However, in a subset of individuals, ischaemic heart disease might not have been the most accurate cause of death to explain the SCD.[12,21]

It has long been assumed that ischaemic heart disease is responsible for most SDs. However, data from the young population obtained in the last decade have refuted this assumption.[22,23] Fortunately, with the rapid development of technology and our increase in genetic knowledge, postmortem genetic testing (the so-called molecular autopsy) has been an invaluable tool in identifying inherited cardiomyopathies and arrhythmogenic disorders as the cause of death in many SDs, including infant cases.[23-26] The American Heart Association, European Heart Rhythm Association and the Royal College of Pathologists of Australasia have published recommended guidelines that they consider the minimum standard required in the routine autopsy practice for adequate investigation of a SCD.[7,8,11,12]

6.1.3 SCD

Approximately 5 000 000 lives per year are lost to SCD globally, with an annual incidence rate that ranges between 50 and 100 per 100 000 in the general population.[27,28] It has been reported that SCD accounts for 15 - 20% of all international deaths. Although the true incidence remains unknown, the Heart and Stroke Foundation estimated that approximately 2 000 young and healthy South Africans die suddenly each year as a result of SCD.[29] An increase in the incidence of SCD has been observed worldwide, regardless of socioeconomic status and ethnicity, and this creates a public health burden. In fact, the impact of SCD in the young has created a premature death burden exceeding any other cause of death, except those attributed to all types of cancers combined.[8,13]

The causes of SCD are greatly dependent on the age of the deceased. Although the incidence of cardiac-related death increases with age, the proportion of SDs is much higher in the young population.[6,23,30] Ischaemic heart disease is the most common cause of SCD in individuals

>40 years old, but inherited cardiomyopathies and arrhythmogenic disorders rank higher than ischaemic heart and valvular disease in individuals <40 years of age, with up to 75% of SCD in the young a result of the former.[6,24,25,28,31]

6.1.4 Using the molecular autopsy to identify causes of SUD

Although the molecular autopsy is not a novel concept to most First-World countries, it still eludes the radar of many clinicians practising in an economically and resource-strained country. The term 'molecular autopsy' can be described as the use of postmortem genetic testing to identify genetic variants associated with, or causative of, a lethal disease, in order to help determine or better understand the cause of death (usually that of a SD).[28,32] Although its causes may vary, it has been determined that ~85% of all SDs are of cardiac origin.[6-8]

6.1.5 Inherited cardiomyopathies

The most prevalent cardiomyopathies implicated in SCD can, in most cases, be macroscopically identified at autopsy.[33] Every so often, these cardiomyopathies will be described as idiopathic, postpartum or a consequence of chronic alcohol abuse, only to be recognised, after examining the relatives, to be familial.[34,35] A further challenge often experienced by forensic pathologists is the fact that these cardiomyopathies may at times present with very subtle or even absent cardiac alterations at autopsy, especially in infant cases.[33,36]This, in combination with sometimes minor, potentially misleading findings, substantiates the need for postmortem genetic testing.[33,36,37]

6.1.7 Channelopathies

Alarmingly, not all SCDs in children, adolescents and young adults have an obvious cause of death that can be determined at autopsy. Research has estimated that between 3% and 53% of SCD cases have no identifiable abnormal morphological findings at autopsy and remain unexplained, whereas the number of unexplained sudden deaths in infants (SUDI) may rise to 80%.[24,38,39] Only through postmortem genetic testing has it been shown that inherited cardiac arrhythmogenic disorders, commonly referred to as channelopathies, are the cause of a large number of these unexplained cases.[28,36,38]

6.1.8 The benefit of molecular autopsy

It could be argued that the greatest benefit of such testing is not to define the cause of death, but rather the highly disease-specific diagnostic, therapeutic and prognostic benefit derived from subsequent genetic screening of family members of the deceased.[40-42] Considering the

high heritability of cardiac disorders and the fact that they are often treatable, genetic analysis of SUD/SUDI victims provides significant clinical benefit with regard to the diagnosis and treatment of family members at risk for the same disease. Over 95% of these genetic cardiac disorders are inherited in an autosomal dominant manner, leading to a 50% chance for first-degree relatives to inherit the same genetic variant.[41] Several authors have reported on studies that evaluated family members of SUD cases, and found that up to 53% of family members tested positive for an inherited cardiac disease.[30,42,43] In the majority of those affected family members, considerable lifesaving interventions such as β-blockers and implantable cardioverter-defibrillators proved to be highly beneficial.[40,41] Family members of SUD/SUDI cases are usually unaware of carrying a disease-causing variant associated with an arrhythmogenic disorder. With few, if any, clinical symptoms or warning signs (family history of syncope, SD, epilepsy, deafness or early pacemaker implantation) before a SUD, the critical importance of postmortem genetic testing cannot be overstated.[22,38,41] The confirmed marked reduction in mortality associated with the administration of proper treatment in such cases leaves no ethically arguable justification for allowing family members at potential risk to remain undiagnosed and untreated.[41,44]

Postmortem genetic testing is recommended (in published guidelines) in all SUDs in the young (0 - 40 years of age) and in all cases suggestive of cardiomyopathy.[7,8,11,12] The minimum requirements involve only targeted genetic testing of the major genes. However, the use of commercial panels consisting of a combination of up to 100 cardiomyopathy and channelopathy genes is becoming more common.[8, 23] These minimum guidelines aim to prevent criticism of case analysis in the medico-legal setting and protect surviving family members with possible genetic disorders.[7,8,23]

With advancements in technology and the associated decrease in cost, the forensic medical profession is increasingly becoming aware of the dangerous implications that an unidentified aetiology of a possible inherited disorder can have on family members at risk.[45,46] The ethical duty and legal liability pertaining to the ‘failure to diagnose’ and ‘duty to warn’ in forensic pathology is currently debated on an international level. According to the Code of Medical Ethics of the American Medical Association, the implications of genetic information for the biological relatives of a patient must be included in the pre- and post-genetic counselling process.[47] Consequently, it is argued that the legal precedent laid down by two primary American court cases (Pate v Threlkel and Safer v Estate of Park) should be, and most probably will be, applied to the forensic pathology profession. The court held that the physician has a

duty to warn biological relatives of an inheritable genetic condition, if the standard of care available will be to their benefit, and if the physician is aware of the existence of these biological relatives.[47] Forensic pathologists in the USA already recognise their duty to warn, and are currently in the process of drafting a standard national set of guidelines for the notification of family members of all cases where a possible genetically heritable aetiology is found.

6.1.9 The way forward

Internationally, the application of postmortem genetic testing as routine investigation in all unexplained SUD/SUDI cases has been adopted.[7,8] The results produced by these molecular autopsies have successfully contributed to the public health sector in improving the population's health status by diagnosing and treating at-risk family members. Inevitably, it raises the question as to the current stance on the implementation of the molecular autopsy in African forensic medical institutions.

In SA, there is no medicolegal mortuary that offers targeted genetic testing in SUDs in the young, even though the departments of forensic medicine at the University of Pretoria and University of Cape Town both conduct valuable research on this topic.[48,49] Our pilot study, conducted on one gene linked to unexplained SUDI, yielded interesting results, with 22.5% of cases having possibly pathogenic *SCN5A* variants considered to be associated with the cause of death.[49]

SA needs to realise that SCDs, especially in the young, should be deemed a clinical health priority, and urgently treated as such. It can be inferred that a high proportion of these SD victims were actively occupying the workforce, and thus contributing to the national economy, emphasising the impact on society. Tackling this health concern can only be successful through a multidisciplinary approach, where all relevant stakeholders, including forensic medical practitioners, clinicians, governmental agencies and funding bodies, to name a few, accept their responsibilities and play their part. With adequate funding and resources, and stricter referral, according to legislation, of SUDs for medicolegal death investigation, a significant increase in molecular research can be conducted into these deaths. A direct result of this will be to enable researchers to detect with greater certainty the most prevalent genes associated with inherited cardiac diseases specifically targeted towards the SA population. The ultimate aim, through adequate research, is to reach that point of targeted genetic testing that can be used as affordable

point-of-care testing, which will be of immense value to clinicians, forensic medical practitioners and society as a whole.

African medical professionals have often been at the forefront when it comes to innovative and ground-breaking medical procedures. Therefore, there is no excuse not to excel at the implementation of the molecular autopsy and reap the clinical benefits it has to offer.

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Appendix A – Ethics approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

20 May 2022

Approval Certificate
Annual Renewal

Dear Mrs BS van Deventer,

Ethics Reference No.: 495/2017 – Line 6
Title: Development of a cardiac channel molecular autopsy in a South African cohort of sudden unexplained deaths in the young

The **Annual Renewal** as supported by documents received between 2022-04-26 and 2022-05-20 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-05-20 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2023-05-20.
- Please remember to use your protocol number (495/2017) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
***Deputy Chairperson** of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix B - Laboratory procedure

DNA quantification and dilution

- Following DNA extraction, the initial concentration and purity ratio of all DNA samples were determined spectrophotometrically, by using the NanoDrop spectrophotometer (Thermo Scientific, Waltham, Massachusetts), and stored at -20°C until further use.

- Once all 66 case samples were collected, each DNA sample was fluorometrically quantified and diluted, (by using Low TE), to the required concentration of 4 ng/µl in a total diluted DNA volume of 5 µl (10 ng per primer pool), using the Qubit dsDNA HS Assay kit on the Qubit® 3.0 Fluorometer (ThermoFisher).

Amplification of DNA targets

The following reagents were used for PCR amplification of two primer pools:

20X AmpliSeq Sample ID Panel for Illumina
2X AmpliSeq DNA Panel Pool 1
2X AmpliSeq DNA Panel Pool 2
5X AmpliSeq HiFi Mix
DNA
Nuclease-free water

- To account for all 66 samples, a mastermix 1, containing 33 µl 20X AmpliSeq Sample ID Panel for Illumina and 330 µl of 2X AmpliSeq DNA Panel Pool 1 was prepared and mixed in a 1.5 ml tube. (Extra volume was prepared to account for small pipetting errors).

- An additional mastermix 2, containing 330 µl 5X AmpliSeq HiFi Mix and 165 µl nuclease-free water was prepared and mixed in a second 1.5 ml microcentrifuge tube.

- For each sample, a volume of 2.5 µl mastermix 2 was transferred to two wells of a new 96-well PCR plate. Next, 5.5 µl of mastermix 1 was added to the one well and 5 µl of 2X AmpliSeq DNA Panel Pool 2 was added to the second well. This procedure was followed for all case samples. Lastly, 2.5 µl (4 ng/µl) of DNA was added to each sample's two prepared wells and pipetted to mix. The plate was sealed and briefly centrifuged.

- The plate was transferred to the Bio-Rad C1000 Touch PCR Thermal Cycler (Bio-Rad, Hercules, California) and ran on an AMP_DNA program according to the following settings:
  - Preheated lid, set to 105ºC with a reaction volume of 10 µl for two pools
  - Denaturation at 99ºC for two minutes
  - Sixteen cycles of:
    - 99ºC for 15 seconds
    - 60ºC for four minutes
  - Hold at 10ºC for 24 hours

Partial digestion of amplicons

Following PCR amplification, primer dimers and amplicons were partially digested by using FuPa reagent.

- The PCR plate (used in the previous step) was briefly centrifuged and unsealed.

- For each sample, the 10 µl of target amplification reactions in the two wells (prepared and amplified in the previous steps) were combined into the one well containing Pool 1.

- A total of 2 µl FuPa reagent was added to each 20 µl target amplification reaction for each sample.

- The plate was sealed, vortexed and briefly centrifuged before placing it in the thermal cycler, with the following conditions set:
  - Preheated lid, set to 105ºC and a set reaction volume of 22 µl
  - 50ºC for 10 minutes
  - 55ºC for 10 minutes
  - 62ºC for 20 minutes
  - Hold at 10ºC for one hour

Index ligation

The following reagents were used for ligating Index 1 (i7) and Index 2 (i5) adapters to each sample:

AmpliSeq CD Indexes for Illumina
DNA Ligase
Switch solution

- The library (PCR plate) was briefly centrifuged and unsealed, whereafter 4 µl of Switch Solution was added to each well containing digested amplicons.

- A volume of 2 µl AmpliSeq CD Indexes were added to each well, followed by the addition of 2 µl of DNA Ligase, also to each well.

- The library plate was sealed, vortexed and briefly centrifuged, whereafter it was placed on the thermocycler with the following preprogramed settings:
  - Preheated and set at 105ºC
  - 22ºC for 30 minutes
  - 68ºC for five minutes
  - 72ºC for five minutes
  - Hold at 10ºC for 24 hours

Library clean-up

Agencourt AMPure XP beads and freshly prepared 70% ethanol were used to clean up the library.

- Following brief centrifugation, the library plate was unsealed and 30 µl of AMPure XP beads was added to each well.

- The plate was briefly vortexed and centrifuged and incubated at room temperature for five minutes.

- The library plate was placed onto a magnetic stand until the mixture was clear, whereafter the plate was unsealed, and the entire supernatant removed and discarded from each well.

- The beads were washed by adding 150 µl of 70% ethanol to each well, incubated at room temperature until the solution was clear, followed by removing and discarding the supernatant from each well. This step was repeated once.

- After the washing step, the plate was sealed, vortexed and again placed on the magnetic stand and unsealed.

- All residual ethanol was removed from each well and air-dried on the magnetic stand for at least 10 minutes.

Second library amplification

For amplification, the following reagents were used to prepare an amplification mastermix:

Reagent	Volume (µl)
1X Lib Amp Mix	45
10X Library Amp Primers	5
Total volume per reaction	**50**

- The mastermix was briefly vortexed and centrifuged, whereafter the plate was removed from the magnetic stand and 50 µl of amplification mastermix was added to each library well.

- The plate was sealed, vortexed and briefly centrifuged, and then place onto the preprogramed C1000 Touch PCR Thermal Cycler (Bio-Rad) according to the following settings:
  - Preheated and set at 105ºC
  - 98ºC for two minutes
  - Seven cycles of:
    - 98ºC for 15 seconds
    - 64ºC for one minute
  - Hold at 10ºC for 24 hours

Second library clean-up

AMPure XP beads were also used for the second clean-up of the library:

- Following brief centrifugation, the library plate was unsealed and 25 µl of AMPure XP beads was added to each library-containing well.

- The library was once again quickly vortexed and centrifuged, followed by incubation at room temperature for five minutes.

- The plate was placed on a magnetic stand for at least five minutes, or until the liquid was clear.

- Next, the entire supernatant, containing the amplicon library, was transferred to a new plate.

- A volume of 60 µl of AMPure XP beads was added to each well containing the supernatant. The plate was sealed, vortexed briefly, followed by centrifugation.

- Following incubation at room temperature for five minutes, the plate was placed on the magnetic stand, again for five minutes, or until the liquid was clear.

- The plate was unsealed and the supernatant from each well was removed and discarded.

- The beads were washed by adding 150 µl of 70% ethanol to each well, incubated at room temperature until the solution is clear, followed by the removal of the supernatant from each well. This step was repeated once.

- All residual 70% ethanol was removed from each well and discarded, whereafter the plate was airdried on the magneticsstand for at least five minutes.

- The plate was removed from the magnetic stand, and 30 µl of Low TE was added to each well.

- The plate was vortexed and centrifuged, and then placed on a magnetic stand for at least five minutes.

- A total of 27 µl amplicon library-containing supernatant was transferred to a new LoBind PCR plate.

Assess library quality

To assess the quality of the library, the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 Kit was used. This allowed for 12 samples to be processed, simultaneously, per DNA chip.

The following reagents were used for this procedure:

DNA ladder (yellow)
DNA markers (green)
DNA dye concentrate (blue)
DNA gel matrix (red)
Amplified library

- A volume of 25 µl DNA dye concentrate was added to a DNA gel matrix vial, vortexed and briefly centrifuged to spin it down.

- The solution was transferred to a spin filter, whereafter it was centrifuged at 2240 g for 15 minutes.

- A new DNA chip was placed in the chip priming station, and 9 µl of the prepared gel-dye mix was pipetted into the allocated, marked well.

- Next, the plunger, positioned at 1 ml, was pressed for 60 seconds before the clip was released and pulled back to its original position.

- Another 9 µl of gel-dye mixed was added to the other two allocated, marked wells.

- Next, 5 µl of DNA marker was added to all 12 sample well, as well as the ladder well.

- A total of 1 µl DNA ladder was added to its allocated, marked well, whereafter 1 µl of sample was added to its 12 allocated wells.

- The chip was horizontally placed in the adapter and vortexed for one minute at a speed of 650 x g, and finally run on the Bioanalyzer for measurements.

Library quantification

The following reagents were used for the quantification of the library:

Qubit® dsDNA HS reagent
Qubit® dsDNA HS buffer
Qubit® ds DNA HS Standard #1
Qubit® ds DNA HS Standard #2

- A Qubit working solution was prepared by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer. Enough working solution was prepared to reach the required 198 µl and190 µl for each sample and standard tube.

- Next, 190 µl of Qubit working solution was added to two tubes, followed by the addition of 10 µl of each DNA standard #1 and #2 to its corresponding tube.

- For library-containing samples, 198 µl of the Qubit working solution was added to each tube, followed by 2 µl of library-containing sample to each corresponding tube.

- With a total volume of 200 µl, each tube was quickly vortexed and incubated at room temperature for two minutes.

- Following incubation, sample concentration was measured using the Qubit® 3.0 Fluorometer. DNA standard #1 and #2 were used for calibration, whereafter the DNA concentration of all library-containing samples was measured.

Library dilution to starting concentration

The following formula was used to determine the molarity value of the pooled libraries:

$$\textbf{Molarity (nM)} = \frac{ng/\mu l \times 10^6}{660\ ^{g}/_{mol} \times \text{average library size (bp)}}$$

- Using Low TE, each library pool was diluted (in a new LoBind PCR plate), to a starting concentration of 2 nM.

Library denaturation and dilution to final loading concentration

The following reagents were used to denature and dilute the libraries:

Fresh prepared 0.2 N NaOH dilution
HT1 buffer
Low TE buffer
200 mM Tris-HCl (pH 7.0)

- Equal volumes of each library were transferred to a 1.5 ml LoBind tube, whereafter it was vortexed and briefly centrifuged.

- A total of 10 µl of each library pool was added to a new tube, followed by 10 µl of 0.2 N NaOH. The samples were briefly vortexed and centrifuged, and incubated at room temperature for five minutes.

- Next, 10 µl of 200 mM Tris HCl was added to the tube containing pooled libraries, and vortexed, followed by brief centrifugation.

- A volume of 970 µl prechilled HT1 buffer was added, which resulted in a 20 pM denatured library.

- After vortex and brief centrifugation, prechilled HT1 buffer was used to further dilute the pooled libraries to a final loading concentration of 1.5 pM, up to a final volume of 1.3 ml. The tube was inverted to mix, briefly vortexed and then centrifuged.

Next generation sequencing

Pooled libraries were sequenced using the Illumina NextSeq Reagent kit (v2.5 300 cycles) on the Illumina NextSeq 550 platform. The Reagent kit contained the following:

Reagent cartridge
Buffer cartridge
Flow cell
HT1 buffer

- The reagent cartridge was thawed in a room temperature water bath for at least one hour, or until completely thawed.

- The flow cell package was unwrapped and set aside at room temperature for 30 minutes.

- Once removed from the plastic package, the surface of the flow cell was cleaned with a lint-free alcohol wipe, whereafter the glass was dried with lint-free lab tissue.

- A volume of 1.3 ml of prepared libraries (1.5 pM) was loaded into reservoir #10 on the Reagent cartridge.

- Finally, the Reagent cartridge, clean flow cell and buffer cartridge were loaded into the NextSeq system (each into their allocated compartments), whereafter cluster and sequencing was performed.

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Appendix C - List of 49 genes included in the Ampliseq On-Demand DNA panel

Gene	Protein name	Chromosome	Accession number
ABCC9	ATP Binding Cassette Subfamily C Member 9	12	NM_020297.3
AKAP9	A-Kinase Anchoring Protein 9	7	NM_00575.4
ANK2	Ankyrin 2	4	NM_020977.3
ASPH	Aspartate Beta-Hydroxylase	8	NM_001164751.1
CACNA1C	Calcium Voltage-Gated Channel Subunit Alpha1 C	12	NM_199460.2
CACNA1D	Calcium Voltage-Gated Channel Subunit Alpha1 D	3	NM_000720.3
CACNA2D1	Calcium Voltage-Gated Channel Auxiliary Subunit Alpha2delta 1	7	NM_000722.2
CACNB2	Calcium Voltage-Gated Channel Auxiliary Subunit Beta 2	10	NM_201596.2
CALM1	Calmodulin 1	14	NM_006888
CALM2	Calmodulin 2	2	NM_001743
CALM3	Calmodulin 3	19	NM_005184.2
CASQ2	Calsequestrin 2	1	NM_001232.3
CAV3	Caveolin 3	3	NM_001234.4
CDH2	Cadherin 2	18	NM_001792.5
DPP6	Dipeptidyl Peptidase Like 6	7	NM_130797
EMILIN3	Elastin Microfibril Interfacer 3	20	NM_052846.2
GJA5	Gap Junction Protein Alpha 5	1	NM_181703.4
GPD1L	Glycerol-3-Phosphate Dehydrogenase 1 Like	3	NM_015141.3
HCN4	Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 4	15	NM_005477.2
KCNA5	Potassium Voltage-Gated Channel Subfamily A Member 5	12	NM_002234.4
KCND3	Potassium Voltage-Gated Channel Subfamily D Member 3	1	NM_004980.4
KCNE1	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1	21	NM_001127670.1
KCNE2	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 2	21	NM_172201.2
KCNE3	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 3	11	NM_005472.5
KCNE5	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 5	X	NM_012282.4
KCNH2	Potassium Voltage-Gated Channel Subfamily H Member 2	7	NM_000238.3
KCNJ2	Potassium Inwardly Rectifying Channel Subfamily J Member 2	17	NM_000891.2
KCNJ5	Potassium Inwardly Rectifying Channel Subfamily J Member 5	11	NM_000890.3

Gene	Protein name	Chromosome	Accession number
KCNJ8	Potassium Inwardly Rectifying Channel Subfamily J Member 8	12	NM_004982.3
KCNQ1	Potassium Voltage-Gated Channel Subfamily Q Member 1	11	NM_000218.2
LMNA	Lamin A/C	1	NM_170707.2
NPPA	Natriuretic Peptide A	1	NM_012612.2
PKP2	Plakophilin 2	12	NM_004572.3
PLN	Phospholamban	6	NM_002667.3
PRKAG2	Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma 2	7	NM_016203
RANGRF	RAN Guanine Nucleotide Release Factor	17	NM_016492.5
RYR2	Ryanodine Receptor 2	1	NM_001035.2
SAMD11	Sterile Alpha Motif Domain Containing 11	1	NM_152486.4
SCN10A	Sodium Voltage-Gated Channel Alpha Subunit 10	3	NM_006514.2
SCN1B	Sodium Voltage-Gated Channel Beta Subunit 1	19	NM_001321605
SCN2B	Sodium Voltage-Gated Channel Beta Subunit 2	11	NM_004588.5
SCN3B	Sodium Voltage-Gated Channel Beta Subunit 3	11	NM_018400.3
SCN4B	Sodium Voltage-Gated Channel Beta Subunit 4	11	NM_174934.3
SCN5A	Sodium Voltage-Gated Channel Alpha Subunit 5	3	NM_198056.2
SLMAP	Sarcolemma Associated Protein	3	NM_007159.2
SNTA1	Syntrophin Alpha 1	20	NM_003098.2
TNNT2	Troponin T2, Cardiac Type	1	NM_000364.4
TRDN	Triadin	6	NM_006073.3
TRPM4	Transient Receptor Potential Cation Channel Subfamily M Member 4	19	NM_017636.3

Appendix D – Case sample data sheet

Case number	Age	Ethnicity	Sex	Cause of death	Activity at time of death
1	35y	White	Male	U/I	Exertion
2	19y	Black	Male	U/I	Unknown
3	5m	Black	Male	U/I	Sleep
4	1w	White	Male	U/I	Rest
5	35y	Black	Female	U/I	Unknown
6	1m	Black	Female	U/I	Sleep
7	20y	Asian	Female	U/I	Sleep
8	41y	Black	Male	U/I	Unknown
9	5m	Black	Male	Unascertained at autopsy alone	Unknown
10	35y	Black	Male	U/I	Exertion
11	29y	Black	Male	U/I	Sleep
12	31y	Black	Male	U/I	Under severe stress
13	4m	Black	Male	U/I	Sleep
14	27y	White	Male	U/I	Exertion
15	24y	White	Male	U/I	Unknown
16	25y	Black	Female	U/I	Rest
17	30y	Black	Male	U/I	Rest
18	2m	White	Male	U/I	Sleep
19	32y	White	Male	U/I	Sleep
20	16y	Black	Male	U/I	Exertion
21	35y	Black	Male	U/I	Sleep
22	18y	White	Female	U/I	Sleep

Case number	Age	Ethnicity	Sex	Cause of death	Activity at time of death
23	4y	Black	Male	U/I	Unknown
24	38y	Black	Male	U/I	Exertion
25	7m	White	Male	U/I	Sleep
26	9m	Black	Female	U/I	Sleep
27	36y	Asian	Male	U/I	Rest
28	27y	White	Male	U/I	Unknown
29	26y	Black	Male	U/I	Exertion
30	4y	White	Female	U/I	Sleep
31	23y	White	Female	U/I	Sleep
32	23y	Black	Male	U/I	Exertion
33	27y	Black	Male	U/I	Rest
34	40y	White	Male	U/I (Possible HCM)	Rest
35	17y	Black	Female	U/I	Rest
36	30y	Black	Male	U/I	Unknown
37	33y	Black	Male	U/I	Unknown
38	41y	Black	Male	U/I Possible HCM / Arrhythmia	Sleep
39	21	Black	Female	U/I	Unknown
40	23	Black	Male	U/I	Rest
41	7w	White	Female	U/I	Sleep
42	5m	Black	Male	U/I	Sleep
43	28y	Black	Male	Unascertained at autopsy alone	Unknown
44	2m	Black	Male	U/I	Sleep
45	30y	Black	Male	U/I	Rest
46	2m	Black	Male	U/I	Sleep
47	29	Black	Female	SCD (Possible DCM)	Rest

Case number	Age	Ethnicity	Sex	Cause of death	Activity at time of death
48	35y	Black	Female	U/I	Rest
49	17y	Black	Male	U/I	Exertion
50	4w	Black	Female	U/I	Sleep
51	21y	Black	Female	U/I	Sleep
52	39y	Black	Female	U/I	Sleep
53	32y	Colored	Male	U/I	Exertion
54	38y	Black	Female	U/I	Exertion
55	4m	Black	Male	U/I	Sleep
56	35y	Black	Male	U/I	Unknown
57	14y	Black	Female	U/I	Sleep
58	35y	Black	Male	U/I	Rest
59	32y	Black	Female	U/I	Sleep
60	45y	Black	Male	U/I	Unknown
61	13y	White	Female	U/I	Sleep
62	44y	White	Male	U/I	Unknown
63	29y	Black	Male	U/I	Exertion
64	29y	Black	Male	U/I	Unknown
65	28y	Black	Male	U/I	Unknown
66	1w	Black	Male	U/I	Sleep

Appendix E – List of 178 missense variants identified among the total study cohort

Gene	Chromosome	Variation g.	Variation c.	Variation p.	rs number	Predicted effect
SAMD11	chr1	865694	c.232C>T	p.H78Y	rs9988179	LB
KCNQ1	chr11	2610079	c.1388G>C	p.S463T	rs184636161	LB
CACNA1C	chr12	2742849	c.3883A>G	p.I1295V	rs114851656	LB
CACNA1C	chr12	2788878	c.5360C>T	p.T1787M	rs192749597	LB-Risk factor
CACNA1C	chr12	2788901	c.5383G>A	p.G1795R	rs111298509	B
CACNA1C	chr12	2791130	c.5459C>T	p.P1820L	rs10848683	B
CACNA1C	chr12	2791132	c.5461A>G	p.M1821V	rs10774053	B
CACNA1C	chr12	2791205	c.5534A>G	p.K1854R	rs10774054	B
CACNA1C	chr12	2794937	c.5666C>T	p.T1889M	rs201777030	B
KCNQ1	chr11	2869129	c.1546G>A	p.G516S	rs1800172	LB
KCNQ1	chr11	2869144	c.1561G>A	p.V521I	rs34150427	B
KCNA5	chr12	5153947	c.634C>T	p.R212C	rs77281462	VUS (75%)
KCNA5	chr12	5154173	c.860C>A	p.A287E	rs144246051	LB
KCNA5	chr12	5154431	c.1118G>C	p.G373A	rs1219418345	VUS (LB)
KCNA5	chr12	5154463	c.1150G>A	p.G384R	rs76709779	LB
KCNA5	chr12	5154925	c.1612G>C	p.E538Q	rs528221767	LB
KCNA5	chr12	5155046	c.1733G>A	p.R578K	rs12720445	B
KCNE3	chr11	6525592	c.68C>T	p.R23Q	rs35771371	LB
RANGRF	chr17	8192742	c.361C>G	p.R121G	rs773051695	LB
CAV3	chr3	8787263	c.166G>A	p.G56S	rs72546667	VUS (80%)
CAV3	chr3	8787330	c.233C>T	p.T78M	rs72546668	VUS (75%)
NPPA	chr1	11907648	c.94C>T	p.V32M	rs5063	B
CACNB2	chr10	18803444	c.510G>T	p.K170N	rs199539261	VUS (LB)
CACNB2	chr10	18803951	c.713G>A	p.G238D	rs142899184	LB
CACNB2	chr10	18827163	c.1357C>T	p.L453F	rs145638628	VUS (88%)
CACNB2	chr10	18828223	c.1553A>C	p.E518A	rs138060429	LB

Gene	Chromosome	Variation g.	Variation c.	Variation p.	rs number	Predicted effect
CACNB2	chr10	18828606	c.1936C>T	p.R646W	rs546669133	VUS (75%)
CACNB2	chr10	18828635	c.1803T>G	p.D601E	rs58225473	B
KCNJ8	chr12	21918931	c.1347A>G	p.V334A	rs34811413	B
ABCC9	chr12	21995312	c.3409C>T	p.V1137I	rs147895473	B
ABCC9	chr12	22017410	c.2200C>T	p.V734I	rs61688134	VUS (LB)
ABCC9	chr12	22040762	c.1909C>T	p.V637I	rs113542001	VUS (LB)
CACNA2D1	chr7	24890157	c.16C>T	p.L6F	rs1974332	LB
CDH2	chr18	25532304	c.2441T>C	p.N814S	rs2289664	LB
CDH2	chr18	25593655	c.298A>G	p.S100P	rs183606230	VUS (LB)
CDH2	chr18	25593694	c.352C>T	p.A118T	rs17445840	B
CDH2	chr18	25727748	c.61C>T	p.A21T	rs25727748	LB Splice
SNTA1	chr20	32005726	c.500T>C	p.K167R	rs932909554	LP
SNTA1	chr20	32026826	c.317C>T	p.R106Q	rs75025585	LB
GPD1L	chr3	32207381	c.1035G>T	p.Q345H	rs780760018	VUS (85%)
PKP2	chr12	32949140	c.2392T>C	p.T798A	rs112592855	LB
PKP2	chr12	32977026	c.1759C>T	p.V587I	rs146102241	VUS (LB)
PKP2	chr12	32996206	c.1420C>T	p.A474T	rs138538072	LB
PKP2	chr12	33003888	c.1190A>T	p.I397N	rs772334698	VUS (88%)
PKP2	chr12	33021934	c.1097A>G	p.L336P	rs1046116	B
PKP2	chr12	33049457	c.324C>A	p.S70I	rs75909145	LB
SCN1B	chr19	35524607	c.412G>A	p.V138I	rs72558029	LB
SCN1B	chr19	35524824	c.629T>C	p.L210P	rs55742440	B
SCN1B	chr19	35524939	c.744C>A	p.S248R	rs67701503	B
SCN1B	chr19	35524944	c.749G>C	p.R250T	rs67486287	LB
KCNE1	chr21	35821707	c.226C>T	p.D76N	rs74315445	LP
KCNE1	chr21	35821821	c.112T>C	p.S38G	rs1805127	B
SCN5A	chr3	38591856	c.6007C>T	p.D2003N	rs376697724	VUS (60%)
SCN5A	chr3	38603947	c.3922G>A	p.L1308F	rs41313031	VUS (88%)

Gene	Chromosome	Variation g.	Variation c.	Variation p.	rs number	Predicted effect
SCN5A	chr3	38620907	c.3308G>T	p.S1103Y	rs7626962	LB Riskt factor
SCN5A	chr3	38620953	c.3262C>T	p.A1088T	rs369704754	VUS (LB)
SCN5A	chr3	38645378	c.1715G>A	p.A572L	rs36210423	LB
SCN5A	chr3	38645379	c.1714C>A	p.A572L	rs36210423	LB
SCN5A	chr3	38645420	c.1673T>C	p.H558R	rs1805124	LB genetic modulator
SCN5A	chr3	38645522	c.1571G>T	p.S524Y	rs41313691	LB
SCN5A	chr3	38651303	c.856C>A	p.A286S	rs61746118	LB
SCN5A	chr3	38651342	c.817T>C	p.M273V	Novel	LB
SCN5A	chr3	38662385	c.560G>C	p.T187S	rs199473558	VUS (75%)
SCN5A	chr3	38674699	c.152G>A	p.R34C	rs6791924	LB
SCN10A	chr3	38739054	c.5657G>A	p.A1886	rs142653846	LB
SCN10A	chr3	38739574	c.5137T>C	p.M1713V	rs6599241	B
SCN10A	chr3	38739622	c.5089C>T	p.V1697I	rs77804526	B
SCN10A	chr3	38743380	c.4607G>C	p.T1536R	rs778754301	VUS (75%)
SCN10A	chr3	38760281	c3544C>A	p.V1182L	rs1396134485	LB
SCN10A	chr3	38763835	c.3427C>T	p.V1141M	rs112412281	VUS (LB)
SCN10A	chr3	38764998	c.3275A>G	p.L1092P	rs12632942	B
SCN10A	chr3	38766675	c.3218A>G	p.V1073A	rs6795970	B
SCN10A	chr3	38768300	c.2884T>C	p.I962V	rs57326399	B
SCN10A	chr3	38770193	c.2480C>G	p.W827S	rs1273210195	VUS (LB)
SCN10A	chr3	38793940	c.1525A>G	p.S509P	rs7630989	B
SCN10A	chr3	38793943	c.1522G>A	p.R508W	rs112774699	VUS (LB)
SCN10A	chr3	38812827	c.542T>G	p.E181A	rs142203439	VUS (LB)
SCN10A	chr3	38835462	c.40G>A	p.R14C	rs750771811	VUS-LP
EMILIN3	chr20	39990711	c.1498G>A	p.R500W	rs61739310	LB
TRPM4	chr19	49671207	c.301G>A	p.A101T	rs113984787	B
TRPM4	chr19	49671228	c.322C>T	p.R108C	rs115335683	LB
TRPM4	chr19	49685947	c.1376G>A	p.R459H	rs142312281	LB

Gene	Chromosome	Variation g.	Variation c.	Variation p.	rs number	Predicted effect
TRPM4	chr19	49693976	c.2156G>A	p.R719Q	rs78381230	LB
CACNA1D	chr3	53834369	c.5077G>A	p.E1693K	rs147973409	LB
CACNA1D	chr3	53835422	c.5438G>A	p.R1813Q	rs143003364	LB
CACNA1D	chr3	53839116	c.5752G>A	p.V1918Q	rs142184099	LB
SLMAP	chr3	57911641	c.943G>A	p.G315S	rs757046462	VUS (LB)
ASPH	chr8	62475338	c.1402C>T	p.G468R	rs61731238	LB
ASPH	chr8	62489332	c.1061T>C	p.Q354R	Novel	LB
ASPH	chr8	62496504	c.974C>A	p.R325M	rs6995412	VUS (60%)
ASPH	chr8	62546280	c.809C>T	p.S270N	rs111708484	LB
ASPH	chr8	62555970	c.645T>A	p.E215D	rs138586020	LB
ASPH	chr8	62577854	c.634A>T	p.Y212N	rs377016597	LB
ASPH	chr8	62577914	c.574A>G	p.S192P	rs762260016	LB
HCN4	chr15	73615097	c3337T>C	p.M1113V	rs142735148	B
HCN4	chr15	73615603	c.2831G>A	p.A994V	rs144450232	B
AKAP9	chr7	91603115	c.139C>T	p.H47Y	rs35669569	LB
AKAP9	chr7	91630532	c.1337G>A	p.R446Q	rs60031334	LB
AKAP9	chr7	91630603	c.1372G>C	p.A458P	rs143894795	B
AKAP9	chr7	91630620	c.1389G>T	p.M463I	rs6964587	B
AKAP9	chr7	91659259	c.4199T>C	p.M1400T	rs73407505	LB
AKAP9	chr7	91670136	c.4841G>A	p.R1614Q	rs2230768	B
AKAP9	chr7	91670172	c.4913T>A	p.L1638Q	Novel	LB
AKAP9	chr7	91708472	c.7025A>G	p.K2342R	Novel	LB
AKAP9	chr7	91708898	c.7451A>G	p.K2484R	rs35759833	LB
AKAP9	chr7	91712698	c.8375A>G	p.N2792S	rs6960867	B
AKAP9	chr7	91712808	c.8485G>A	p.E2829K	rs149946443	B
AKAP9	chr7	91714911	c.8935C>T	p.P2979S	rs1063242	LB
AKAP9	chr7	91726202	c.9929G>A	p.R3310Q	rs78351282	LB
AKAP9	chr7	91726522	c.10225C>T	p.R3409C	rs146495719	LB

Gene	Chromosome	Variation g.	Variation c.	Variation p.	rs number	Predicted effect
AKAP9	chr7	91726604	c.10331A>G	p.Q3444R	rs34956633	LB
AKAP9	chr7	91729127	c.10840A>G	p.M3614V	rs34327395	B
ANK2	chr4	114274492	c.4763	p.R1588K	Novel	LB
ANK2	chr4	114276255	c.6481A>C	p.K2161E	Novel	LB
ANK2	chr4	114276408	c.6679GA	p.G2227S	rs61734478	B
ANK2	chr4	114276781	c.7052C>T	p.A2351V	rs61734477	B
ANK2	chr4	114276880	c.7151T>C	p.V2369A	rs28377576	LB
ANK2	chr4	114277605	c.7831	p.Y2611H	rs35338364	B
ANK2	chr4	114277689	c.7915C>G	p.H2639D	rs529384341	LB
ANK2	chr4	114277870	c.8096T>C	p.M2699T	Novel	LB
ANK2	chr4	114278277	c.8503C>T	p.P2835S	rs3733617	B
ANK2	chr4	114278835	c.9061G>A	p.A3021T	rs74348333	LB
ANK2	chr4	114279228	c.9454A>G	p.T3152A	rs61741040	LB
ANK2	chr4	114288907	c.11218C>A	p.L3740I	rs35530544	LB
ANK2	chr4	114290816	c.11465G>C	p.G3822A	rs79577190	LB
ANK2	chr4	114294251	c.11616C>G	p.D3872E	rs768755447	LB
CASQ2	chr1	116243925	924A>T	p.D308E	rs776130201	VUS (LB)
CASQ2	chr1	116245602	c.954C>G	p.W318C	Novel	LP
CASQ2	chr1	116247854	c.898C>T	p.D300N	rs376147306	VUS (LB)
CASQ2	chr1	116269619	c.731T>C	p.H244R	rs28730716	LB
CASQ2	chr1	116310967	c.196T>C	p.T66A	rs4074536	B
TRDN	chr6	123539749	c.2187C>A	p.Q729H	rs373439044	VUS (LB)
TRDN	chr6	123580777	c.1862T>G	p.E621A	rs1211286909	LB
TRDN	chr6	123594486	c.1620T>C	p.I540M	rs7771303	B
TRDN	chr6	123637602	c.1510C>T	p.G504S	rs150531306	LB
TRDN	chr6	123658776	c.1408G>T	p.L470M	rs6569336	B
TRDN	chr6	123687288	c.1313A>C	p.I438S	rs2873479	B
TRDN	chr6	123696766	c.1257G>T	p.D419E	rs17737379	B

Gene	Chromosome	Variation g.	Variation c.	Variation p.	rs number	Predicted effect
TRDN	chr6	123699019	c.1211A>C	p.V404G	rs28494009	B
TRDN	chr6	123714778	c.1096C>T	p.A366T	rs35047281	B
TRDN	chr6	123759243	c.1016C>T	p.S339N	rs35766971	B
TRDN	chr6	123786108	c.814G>A	p.P272S	rs549030753	LB
TRDN	chr6	123824902	c.755T>C	p.D252G	rs969285752	LB
TRDN	chr6	123824915	c.742T>C	p.K248E	Novel	LB
TRDN	chr6	123824918	c739G>C	p.P247A	rs1340220194	LB
TRDN	chr6	123833457	c.601G>C	p.L201V	rs6902416	B
TRDN	chr6	123868506	c.403C>T	p.E135K	rs192289289	B
TRDN	chr6	123869607	c.383G>C	p.T128S	rs9490809	B
TRDN	chr6	123869716	c.274C>T	p.V92I	rs34808221	B
KCNJ5	chr11	128781893	c.725G>A	p.R242Q	rs746240972	VUS (LB)
KCNJ5	chr11	128782012	c.844C>G	p.Q282E	rs7102584	B
GJA5	chr1	147230352	c.995C>T	p.R332H	rs116551187	VUS (LB)
GJA5	chr1	147231048	c.299A>C	p.V100G	rs138375318	LP
KCNH2	chr7	150644005	c.3290A>G	p.V1097A	rs1484012284	LB
KCNH2	chr7	150644066	c.2209C>T	p.A737T	rs201382073	VUS (LB)
KCNH2	chr7	150644883	c.2488G>A	p.P830S	rs899224669	LB
KCNH2	chr7	150652572	c.20T>C	p.K7R	rs145819084	LB
KCNH2	chr7	150655521	c.542C>T	p.R181Q	rs41308954	LB
KCNH2	chr7	150671921	c.185C>T	p.R62Q	rs199473664	LB
PRKAG2	chr7	151478454	c.250G>A	p.R84W	rs61746358	VUS (75%)
PRKAG2	chr7	151573647	c.59C>A	p.S20I	rs116605521	LB
DPP6	chr7	154379517	c.785C>T	p.S262L	rs35392762	B
DPP6	chr7	154598774	c.1426A>G	p.S476G	Novel	LB
DPP6	chr7	154645534	c.1519A>C	p.K507Q	rs140460765	VUS (LB)
DPP6	chr7	154667632	c.1900A>G	p.S634G	Novel	LB
DPP6	chr7	154667692	c.1768G>A	p.G590S	rs150218787	LB

Gene	Chromosome	Variation g.	Variation c.	Variation p.	rs number	Predicted effect
DPP6	chr7	154684153	c.2369T>C	p.L790P	rs3734960	LB
LMNA	chr1	156106187	c.1340A>G	p.E447G	Novel	LB
TNNT2	chr1	201330429	c.749T>C	p.K250R	rs3730238	LB
TNNT2	chr1	201331240	c.604G>A	p.A206T	rs150008205	LB
RYR2	chr1	237619942	c.1519G>A	p.V507I	rs16835270	LB
RYR2	chr1	237711759	c.2935G>T	p.A979S	rs202015519	VUS (LB)
RYR2	chr1	237755076	c.4198A>G	p.S1400G	rs56229512	LB
RYR2	chr1	237778084	c.5656G>A	p.G1886S	rs3766871	LB
RYR2	chr1	237780752	c.5882A>G	p.K1961R	rs772508255	LB
RYR2	chr1	237813249	c.7585A>G	p.T2529A	Novel	LB
RYR2	chr1	237819159	c.8004G>C	p.C2668W	Novel	LP
RYR2	chr1	237841390	c.8873A>G	p.Q2985R	rs34967813	LB

Appendix F – Variations identified in case 10

Variation (g. number)	Gene	Variation (p. number)
11907648	*NPPA*	p.V32M
237755076	*RyR2*	p.S1400G
237819159	*RyR2*	p.C2668W
128782012	*KCNJ5*	p.Q282E
2788901	*CACNA1C*	p.G1795R
2791130	*CACNA1C*	p.P1820L
2791132	*CACNA1C*	p.M1821V
2791205	*CACNA1C*	p.K1854R
35524824	*SCN1B*	p.L210P
35821821	*KCNE1*	p.S38G
38645420	*SCN5A*	p.H558R
38739574	*SCN10A*	p.M1713V
38766675	*SCN10A*	p.V1073A
114276255	*ANK2*	p.K2161E
114278277	*ANK2*	p.P2835S
123687288	*TRDN*	p.I438S
123833457	*TRDN*	p.L201V
91630620	*AKAP9*	p.M463I
91714911	*AKAP9*	p.P2979S
62555970	*ASPH*	p.E215D

Appendix G – Variations identified in case 15

Variation (g. number)	Gene	Variation (p. number)
116269619	*CASQ2*	p.H244R
237711759	*RyR2*	p.A979S
237778084	*RyR2*	p.G1886S
237813249	*RyR2*	p.T2529A
128782012	*KCNJ5*	p.Q282E
2791130	*CACNA1C*	p.P1820L
2791132	*CACNA1C*	p.M1821V
2791205	*CACNA1C*	p.K1854R
35524824	*SCN1B*	p.L210P
35524939	*SCN1B*	p.S248R
35524944	*SCN1B*	p.R250T
32005726	*SNTA1*	p.K167R
35821821	*KCNE1*	p.S38G
8787263	*CAV3*	p.G56S
38645420	*SCN5A*	p.H558R
38739574	*SCN10A*	p.M1713V
38760281	*SCN10A*	p.V1182L
38766675	*SCN10A*	p.V1073A
38793940	*SCN10A*	p.S509P
123658776	*TRDN*	p.L470M
123687288	*TRDN*	p.I438S
123696766	*TRDN*	p.D419E
123833457	*TRDN*	p.L201V
123869607	*TRDN*	p.T128S
91630620	*AKAP9*	p.M463I

Appendix H – Variations identified in case 24

Variation (g. number)	Gene	Variation (p. number)
116245602	*CASQ2*	p.W318C
116247854	*CASQ2*	p.D300N
116310967	*CASQ2*	p.T66A
237778084	*RyR2*	p.G1886S
128782012	*KCNJ5*	p.Q282E
2791132	*CACNA1C*	p.M1821V
2791205	*CACNA1C*	p.K1854R
35524824	*SCN1B*	p.L210P
35821821	*KCNE1*	p.S38G
38739574	*SCN10A*	p.M1713V
38766675	*SCN10A*	p.V1073A
38793940	*SCN10A*	p.S509P
114276255	*ANK2*	p.K2161E
114276408	*ANK2*	p.G2227S
114276880	*ANK2*	p.V2369A
114288907	*ANK2*	p.L3740I
123687288	*TRDN*	p.I438S
123833457	*TRDN*	p.L201V
123869607	*TRDN*	p.T128S
91708472	*AKAP9*	p.K2342R
91714911	*AKAP9*	p.P2979S
151478454	*PRKAG2*	p.R84W
154684153	*DPP6*	p.L790P

Appendix I – Variations identified in case 40

Variation (g. number)	Gene	Variation (p. number)
116310967	*CASQ2*	p.T66A
201331240	*TNNT2*	p.A206T
128782012	*KCNJ5*	p.Q282E
2791130	*CACNA1C*	p.P1820L
2791132	*CACNA1C*	p.M1821V
2791205	*CACNA1C*	p.K1854R
35524824	*SCN1B*	p.L210P
49671228	*TRPM4*	p.R108C
35821707	*KCNE1*	p.D76N
35821821	*KCNE1*	p.S38G
8787330	*CAV3*	p.T78M
38739574	*SCN10A*	p.M1713V
38766675	*SCN10A*	p.V1073A
38793940	*SCN10A*	p.S509P
114277870	*ANK2*	p.M2699T
114278277	*ANK2*	p.P2835S
123687288	*TRDN*	p.I438S
123833457	*TRDN*	p.L201V
123869607	*TRDN*	p.T128S
91630620	*AKAP9*	p.M463I
91712698	*AKAP9*	p.N2792S
91714911	*AKAP9*	p.P2979S
154684153	*DPP6*	p.L790P

Appendix J – Variations identified in case 55

Variation (g. number)	Gene	Variation (p. number)
147231048	*GJA5*	p.V100G
201331240	*TNNT2*	p.A206T
237778084	*RyR2*	p.G1886S
2869144	*KCNQ1*	p.V521I
128782012	*KCNJ5*	p.Q282E
2791130	*CACNA1C*	p.P1820L
2791132	*CACNA1C*	p.M1821V
2791205	*CACNA1C*	p.K1854R
5154431	*KCNA5*	p.G373A
33021934	*PKP2*	p.L336P
35524824	*SCN1B*	p.L210P
35821821	*KCNE1*	p.S38G
38651303	*SCN5A*	p.A286S
38739574	*SCN10A*	p.M1713V
38766675	*SCN10A*	p.V1073A
38793940	*SCN10A*	p.S509P
114276255	*ANK2*	p.K2161E
114276880	*ANK2*	p.V2369A
114278277	*ANK2*	p.P2835S
114279228	*ANK2*	p.T3152A
123658776	*TRDN*	p.L470M
123687288	*TRDN*	p.I438S
123696766	*TRDN*	p.D419E
123833457	*TRDN*	p.L201V
91630532	*AKAP9*	p.R446Q
91659259	*AKAP9*	p.M1400T
91708472	*AKAP9*	p.K2342R
91714911	*AKAP9*	p.P2979S
154684153	*DPP6*	p.L790P

Appendix K – Variations identified in case 65

Variation (g. number)	Gene	Variation (p. number)
116310967	*CASQ2*	p.T66A
201331240	*TNNT2*	p.A206T
128782012	*KCNJ5*	p.Q282E
2791130	*CACNA1C*	p.P1820L
2791132	*CACNA1C*	p.M1821V
2791205	*CACNA1C*	p.K1854R
24890157	*CACNA2D1*	p.L6F
35524824	*SCN1B*	p.L210P
49671228	*TRPM4*	p.R108C
35821821	*KCNE1*	p.S38G
38591856	*SCN5A*	p.D2003N
38645420	*SCN5A*	p.H558R
38739574	*SCN10A*	p.M1713V
38763835	*SCN10A*	p.V1141M
38766675	*SCN10A*	p.V1073A
38835462	*SCN10A*	p.R14C
53835422	*CACNA1D*	p.R1813Q
123658776	*TRDN*	p.L470M
123687288	*TRDN*	p.I438S
123696766	*TRDN*	p.D419E
123699019	*TRDN*	p.V404G
123833457	*TRDN*	p.L201V
123869607	*TRDN*	p.T128S
91630620	*AKAP9*	p.M463I
91714911	*AKAP9*	p.P2979S
150671921	*KCNH2*	p.R62Q
62546280	*ASPH*	p.S270N
62577854	*ASPH*	p.Y212N

Appendix L

Cardiovascular deaths: What do the genes say?

Cardiovascular diseases (CVDs) are ever-increasing, and as such are considered to be one of the most concerning public health burdens worldwide. They remain the leading cause of death across the world (~17.7 million deaths were reported in 2015), accounting for 31% of all global deaths.[1]

More than 75% of these cardiovascular deaths occur in low- and middle-income countries, and although CVD is an acknowledged health concern in Africa, this priority area should receive much more attention than it currently does.[2]

Up to 50% of all cardiovascular deaths are a result of a sudden cardiac death (SCD), defined as 'a natural death due to cardiac causes, heralded by abrupt loss of consciousness within 1 hour after the onset of symptoms'. The consequences of these deaths, particularly in the young, have a greater impact and health burden in terms oflife years lost than all individual cancers and other leading causes ofdeath. The fact that nearly 90% of SCDs are caused by an inherited disorder justifies the international focus on, and prioritisation of, the underlying genetic causes of these cardiac disorders.[3]

Disorders linked to SCDs vary greatly between different age groups, with ischaemic heart disease being the most common cause of death in the older population. In comparison, the majority of SCDs in the younger population (≤45 years) are due to inherited cardiomyopathies and arrhythmogenic disorders. Unfortunately, there is a lack of clinical symptoms or warning signs, with research showing that in 75% of SCD cases, death is the first 'symptom'.[4]

Inherited cardiomyopathy- and arrhythmogenic-related SCDs result from lethal arrhythmias. These are caused by alterations (genetic variations) in genes that all play a role in cardiomyocyte excitability and contractility. Cardiomyopathy-related genetic variations affect the structure and function of the heart muscle, whereas cardiac arrhythmogenic genetic disorders are generally associated with isolated electrical dysfunction. The four most common inherited arrhythmogenic disorders include long QT syndrome, Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia and short QT syndrome. Although each of these has a characteristic electrocardiogram (ECG) profile when experiencing an arrhythmic episode, their spontaneous and sporadic nature results in a difficult clinical diagnosis.[5,6]

The same gene can be altered in different ways (different genetic variations), which can lead to vastly different clinical manifestations of a disorder. These differences are important when considering available and effective treatment for patients, as each treatment is designed to target a certain defect and/or function. Fortunately, there are various types of treatment available, which can range from anti-arrhythmic medications to implantable cardioverter defibrillators and pacemakers.[1,7]

Therefore, even for general practitioners (GPs), there is clinical importance in determining the underlying genetics of an inherited cardiac disorder, to allow for effective and individualised treatment of a patient and/or affected family members. Since diagnosis can be challenging, be it due to an absence of ECG abnormalities, overlap of clinical phenotypes or lack of symptoms, genetic testing in all individuals at risk for an inherited cardiomyopathy or arrhythmogenic disorder is crucial. This is reiterated by the marked reduction in mortality associated with the administration of proper treatment.[7]

The general medical practitioner, in particular, is in a central position in SCD prevention, and plays an essential role in the multidisciplinary team tending to affected family members. GPshave a greater personal connection to the community, and oftencare for different generations of the same family, which allowsfor earlier recognition of subtle warning signs suggestive of aninherited cardiomyopathy or arrhythmogenic disorder. Clinical practitioners should especially be cognisant of any family history of syncope, epilepsy, sudden death, deafness, heart failure or pacemaker implantation at a young age (<50 years). Primarily, the GP will be the first to recognise a possible inherited cardiac disease in an individual or family, and through appropriate genetic testing may provide the only opportunity for an early diagnosis and proper clinical management.

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Viewpoint

Long QT syndrome and sudden unexpected infant death

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ABSTRACT

Long QT syndrome (LQTS) is an inheritable primary electric disease of the heart characterised by abnormally long QT intervals and a propensity to develop atrial and ventricular tachyarrhythmias. It is caused by an inherited channelopathy responsible for sudden cardiac death in individuals with structurally normal hearts. Long QT syndrome can present early in life, and some studies suggest that it may be associated with up to 20% of sudden unexplained infant death (SUID), particularly when associated with external stressors such as asphyxia, which is commonly seen in many infant death scenes. With an understanding of the genetic defects, it has now been possible to retrospectively analyse samples from infants who have presented to forensic pathology services with a history of unexplained sudden death, which may, in turn, enable the implementation of preventative treatment for siblings previously not known to have pathogenic genetic variations. In this viewpoint article, we will discuss SUID, LQTS and postmortem genetic analysis.

SUDDEN UNEXPECTED INFANT DEATH

In most countries, sudden and unexpected death cases will be referred for routine medicolegal autopsy. Unfortunately, 70% to 80% of sudden unexpected deaths in infants (SUDIs) will remain unexplained, even after thorough investigation, which include a detailed postmortem examination including macroscopic examination with evisceration of all organs and all ancillary investigations such as histology, microbiology, virology and toxicology.[1-3] The Centre for Disease Control and Prevention estimated in 2016 that 3500 infants die suddenly and unexpectedly each year in the USA.[4] A review study conducted in Wales reported the approximate prevalence of SUDI was 14% of all infant deaths recorded over a 2-year period (2010–2012).[5] These unexplained deaths were previously defined as sudden infant death syndrome (SIDS).[6-9]

In 2013, Byard indicated a possible diagnostic shift in SIDS cases. During the 1990s, the continued monitoring of diagnostic practices and trends in infant deaths revealed the extent to which pathologists contributed to this diagnostic shift.[10,11] An increased awareness of the infant's position in relation with many of these sudden deaths enabled the pathologists to identify more cases of accidental asphyxia in relation to unsafe sleeping environments. Furthermore, Byard also documented an opposing component of the diagnostic shift, which involved the subjective reassignment of causes of death.[12] A specific trend was detected where many pathologists refrained from attributing the cause of death to SIDS and rather used terms such as undetermined cause of death or asphyxia-related death.[12] Reasons for this shift include the absence of pathognomonic diagnostic features for SIDS and the insufficient findings that may be present in cases of accidental or intentional smothering.[12-15] Pathologists have rather taken to determining these deaths as sudden unexplained infant deaths (SUIDs), which are defined as 'the death of an infant less than one year of age in which investigation, autopsy, medical history review and appropriate laboratory testing fail to identify a specific cause of death. SUID includes cases that meet the definition of sudden infant death syndrome.[16]

AETIOLOGY OF SUID

Studies show SUID occurred more frequently in infants between the age of 2 and 4 months and rarely after the age of 8 months.[1,5,17-20] Death apparently occurs during periods of sleep, suddenly and without warning.[1,17] A uniformly accepted triple-risk model was first introduced in 1994 by Filliano and Kinney, and highlighted the interaction of multiple risk factors that increase the probability of SUID.[21] These risk factors are divided into three groups: a vulnerable infant, a critical developmental stage and exogenous stressors.[21] Current theories still suggest that SUID is a complex event and infants may die when risk factors in each of these groups occur at the same time: a vulnerable infant (which can include an underlying genetic mutation/predisposition) in a critical developmental stage (peaks at 3 months) with an exogenous stressor such as asphyxia challenges from unsafe sleeping practices, soft bedding, the exposure to second-hand smoke as well as bacterial and viral infections.[5,17-22]

In the 1990s, there was a decrease in the number of SUID cases, which could probably be attributed to the introduction of the 'back-to-sleep' campaign. However, since then, the SUID rate has remained stable and is the number one cause of death in postneonatal infants in most developed countries.[5,18,19] The large number of published studies strongly suggests that SUID may be multifactorial and may include metabolic and genetic disorders, as well as deficits in serotonin receptors in the brainstem,[23,24] which motivates for the continuous research into possibly preventable causes.[1,5,17-19] Fortunately, with the rapid development in technology and continued studies on genetic risk factors, postmortem molecular analysis proved to be an invaluable tool in determining a possible cause of death in many SUID cases.[25,26]

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A postmortem genetic study conducted by Wang *et al* showed that in their cohort of infants, African-Americans had the highest risk of dying suddenly, followed by Hispanics and Caucasians, with the Asian population at smallest risk.[25] Arnestad *et al* suggested an intriguing hypothesis with regard to possible modulating factors involving specific genetic variants and the associated ethnicity of the individual.[18] Comparing the ethnic/racial differences as described above with the occurrence of SUIDs indicates that the rate of SUIDs among lower income/socioeconomic deprived racial and ethnic groups showed an increase compared with groups within a higher income bracket.[11] American Indians, African-Americans, Maoris from New Zealand as well as Aboriginals in Australia all have a higher incidence of SUID.[1,17] No definitive explanation for this increased occurrence could be found; however, a complex interaction between genetic and environmental risk factors may be the underlying basis—in keeping with the triple-risk model.

SUIDS AND CHANNELOPATHIES

Numerous studies have been done on the association of serotonin receptor deficits in SUIDs.[2,5,8,27] In addition to serotonin receptor deficits, other studies, which have also received increased attention over the past few years, have shown that one of the possible preventable causes of SUIDs is that of inherited, life-threatening cardiac arrhythmic disorders, commonly referred to as cardiac channelopathies.[26,28-30] These channelopathies, which include long QT syndrome (LQTS), Brugada syndrome (BrS) and catecholaminergic polymorphic ventricular tachycardia (CPVT), are a result of pathogenic variants in genes that code for cardiac ion channels.[25,26,28,30] These genes play a role in the cardiac electrical conduction physiology, thus affecting the normal heart rhythm.[8,31-35]

The first evidence pertaining to cardiac conduction disorder in SUIDs is that of Keeton *et al*,[34] who in 1977 reported on the diagnosis of severe conduction disorders in six cases of acute life-threatening events (ALTE) in infants. These infants received proper treatment before any fatalities occurred.[34] Data obtained from six separate studies indicate that the overall prevalence of pathogenic variants in cardiac ion-channel-related genes in SUID victims may be 20%. These variants seem to have a fatal outcome when coinciding with certain stressors/triggers such as fever and asphyxia,[18,35-39] which is especially relevant when considering that asphyxia is commonly encountered in SUID especially in a so-called unsafe sleeping environment. The American National Society of Genetic Counselors,[40] Ackerman,[35] Michaud *et al*,[33] Arnestad *et al*[18] and Davis *et al*[29] all reported that an average of 15% of SUID cases occurred due to inherited cardiac arrhythmic disorders. It was suggested that the putative cause of death in one of every five SUIDs may be the result of pathogenic variants in a cardiac ion-channel-related gene.[15,25]

The 'peak' age of SUIDs is commonly accepted as 3 months.[1,5,17-20] However, in infants identified with a channelopathy, the age range at time of death varies greatly between each study cohort, with no peak age of death noted among all the studies. Some recorded a range between 4 days and 12 months while others recorded median ages at death varying from 2 months up to 6 months.[18,20,25] The exact mechanism to which this relatively broad span of age range can be attributed to is still unknown. It should be kept in mind that the broader definition of SUID includes all infants up to the age of 1 year.

Some variants in genes linked to the different channelopathies seem to be more prevalent in certain population groups while rare in others.[18,25] A number of studies indicate a higher prevalence of certain genetic variants among the Maori population,[1,17,20] whereas other specific variants, especially the *SCN5A*-H558R amino acid replacement, are associated with a higher prevalence in the Caucasian population group.[41] In contrast, certain common variants found in the Hispanic and Asian populations are identified as disease-causing variants in the Caucasian population.[18] The *SCN5A*-A572D variant, which has previously been described as disease-causing, is a common variant found in the Norwegian population.[18]

LONG QT SYNDROME

The channelopathy that has the strongest link to SUIDs is LQTS.[2,26] LQTS is an inherited arrhythmogenic disorder associated with the ionic control of the cardiac action potential. Clinical outcomes include syncope, seizures and sudden death, especially in young and apparently healthy individuals. Of note, all LQTS features, including a postmortem examination that remains unexplained, are similar to SUID.[2,26]

LQTS is a genetically heterogeneous condition, with the majority of cases inherited in an autosomal dominant manner. The less common recessive forms of LQTS are associated with severe cardiac phenotypes and congenital deafness.[31,42,43] The characteristics of LQTS are represented by a delayed repolarisation of the ventricular cells. This is attributed to the reduction in repolarising (outward) currents, or an increase in depolarising (inward) currents, and is associated with ECG manifestations of prolonged QT intervals and T wave abnormalities.[43-45] The prevalence of inherited LQTS is estimated to be 1 in 2500 live births.[18,26,28] However, reports have indicated that this number might be an underestimation since the likelihood for a misdiagnosis exists in approximately two-thirds of patients with LQTS due to the heterogeneity of the disease.[15,25,26,28] In addition, an estimated 10%–35% of patients present with a normal QT interval when measured on a resting 12-lead ECG. This further contributes to the underestimated prevalence of inherited LQTS in the general population.[28,42-44,46] The onset of symptoms usually occurs at a mean age of 12 years, with an earlier onset of symptoms typically associated with more severe outcomes.[42-44,47]

To date, a significant number of genetic variations have been associated with LQTS.[5,18,48] According to the Human Gene Mutation Database, more than 600 long QT variations have been identified in several ion-channel-related genes.[49] Three major genes are responsible for 75%–90% of these variants: the potassium voltage-gated channel subfamily Q member 1 (*KCNQ1*), the potassium voltage-gated channel subfamily H member 2 (*KCNH2*) and the sodium voltage-gated channel type V alpha (*SCN5A*) gene.[45,44,50] Loss-of-function variants in *KCNQ1*, encoding for the ion channel that mediates the slow delayed rectifying potassium current (I_{Ks}), cause long QT type 1 (LQT1) syndrome. Most arrhythmias experienced in LQT1 patients are triggered by exercise-related stress.[31,33,45,51] Loss-of-function variants in *KCNH2*, encoding for the ion channel generating the rapid delayed rectifying potassium current (I_{Kr}) during repolarisation, cause long QT type 2 (LQT2) syndrome. In LQT2 patients, the majority of events are triggered by emotional stress.[45,44,47] Gain-of-function variants in *SCN5A*, encoding for the sodium channel that generates the depolarising I_{Na} sodium current, cause long QT type 3 (LQT3) syndrome.[42,45,52-54] The cardiac events in LQT3 patients, which are considered the most lethal among LQTS, occur during a period of sleep/rest and have been reported in SUID cases.[20,26,44,55] The higher lethality rate can be best explained by the 20% increased risk of sudden death presenting as the

first clinical manifestation in LQT3 patients versus the 4% risk among LQT1 and LQT2 patients.[20,25,44]

LONG QT SYNDROME AND SUID

Of all the channelopathies, LQTS is the most prevalent disorder associated with SUIDs[5,20,26,55,56] as well as sudden death in the young.[28,55,57,58] Postmortem genetic testing in SUID cases demonstrated that 13.9% of cases with identified variants in the LQTS genes have pathogenic clinical significance.[15,28]

A large population-based study conducted on the clinical association between a prolonged QT interval in ECGs and an increased risk of SUID analysed 33 034 ECGs of healthy Italian babies, which were taken on the third or fourth day of life.[51] In each case, the QT interval was measured and the infants were followed for 1 year. In total, 34 infants died, of which 24 deaths were attributed to SUID (incidence of 0.7 per 1000 live births). A prolonged QT interval was recorded in 12 of the SUID cases (50%), whereas none of the survivors, or infants who died of other causes, demonstrated a prolonged QT interval.[51] As a result, Schwartz *et al*[51] calculated the OR for SUID in infants with a prolonged QT interval as 41, an OR significantly higher than that of prone posture and maternal smoking.[15]

A more recent follow-up study on the association of LQTS with an increased risk for SUID involved a comprehensive 19-year prospective review of ECGs, which were recorded between 15 and 28 days of life in more than 44 000 infants.[59] Molecular screening was performed in 28 infants who presented with a marked QT interval prolongation, which showed that 14 of these infants (50%) were carriers of potentially pathogenic LQTS-related variants. All neonates who presented with a prolonged QT interval received successful treatment with a β-blocker (propranolol).[59]

An association between LQTS variations and SUID victims has been recognised by two well-known case studies:[56,60] one on a SUID case and the other on an infant with documented ventricular fibrillation who survived an ALTE. These two studies ultimately paved the way for other cohort studies on SUIDs.[56,60] One study showed a 5.2% prevalence of LQTS causing variations in a study cohort of 68 SUID cases.[55] Another study, composed of 201 SUID cases and 187 controls, found that 9.5% (95% CI 5.8 to 14.4) of SUID cases carried functional LQTS pathogenic variations, whereas none of the controls did.[18] A third study, conducted by Wang *et al*,[25] identified variants of probable pathogenic significance in 19 of 141 SUID cases (13.5%).

Long QT type 3 syndrome seems particularly important in SUID cases as studies demonstrated a link between SUID and a predominance of *SCN5A* gene variants.[18,19,53,54,61,62] In three different studies, molecular screening identified pathogenic variants linked to LQTS in a number of SUID cases, where variations in the *SCN5A* gene comprised respectively 50%, 68.4% and 50% of all identified variants.[28,52,63] This could be ascribed to the known genotype–phenotype correlations that suggest patients with LQT3 (*SCN5A*) variants may experience a higher lethality rate, mostly occurring during sleep, compared with patients who have variants in other genes involved in LQTS.[18,20,24]

The *SCN5A* gene is a member of the voltage-gated sodium channel family, with at least nine sodium channel α-subunits in this family identified from various human tissues.[28,52,61] The genomic location of *SCN5A* is on the short arm of chromosome 3 at position 21 (3p21). It consists of 28 exons with an approximate span of 80 000 base pairs (80 kb).[51,52,61,61] The *SCN5A* gene encodes for a protein (sodium (Nav1.5) ion channel pore-forming α-subunit) of 2016 amino acids with a calculated molecular weight of 227 kDa. The voltage-gated Na^+ channel α-subunit contains six transmembrane-spanning segments (S1–S6) found within each of four homologous domains (DI–DIV).[28,52,52,63] It is restrictively expressed in the myocardium and plays a critical role in heart excitability and conduction.[28,51,46] The integral membrane protein produces the fast inward Na^+ current that is responsible for the depolarising phase of the cardiac action potential.[15,28,46] Variations of this gene cause a persistent Na^+ current with a subsequent prolongation of the ventricular action potential, essentially resulting in an inherited predisposition to ventricular arrhythmias and sudden death, seen in several cardiac diseases, including LQT3.[29,54,65–67]

POSTMORTEM GENETIC TESTING AND SUID

Postmortem genetic testing is increasingly being recommended as a routine procedure in the investigation of any sudden unexpected death.[25,26,63,69] Sudden death is often the sentinel event of 10%–40% of LQTS, as most genetic variant carriers are unaware that the disease is present.[26,68–71] The importance of postmortem genetic testing lies not only in determining the cause of death at autopsy but also serves as a diagnostic tool in identifying relatives (of the deceased) at risk for the same inherited genetic disorder.[26,29,69] Over 95% of cardiac genetic disorders (in the general population) are inherited as an autosomal-dominant trait.[69] Furthermore, the risk for subsequent siblings dying from SUID is reported to be between 3.7-fold and 10-fold (although this is regarded as controversial by some).[2]

Various treatment modalities for channelopathies are available, with the three most common/effective being that of β-adrenergic blockers, antiarrhythmic agents and the use of implanted device therapy.[15,28,63] Although β-adrenergic blockers are still considered the first line of therapy in LQTS, a lower efficacy in treatment for *SCN5A* variant-associated LQTS has been reported.[15,28] Evidence obtained from both clinical and in vitro settings suggests a successful counteraction of mexiletine against the aberrant persistent Na^+ current, which ultimately shortens the QT interval in *SCN5A* pathogenic variation carriers.[28,63] In addition, flecainide also proves to shorten QT intervals in many *SCN5A* pathogenic variation carriers; however, concerns regarding the safety of this specific therapy have been raised.[15,28,63] Quinidine and sotalol, both class III-type antiarrhythmic agents, proved to be beneficial to patients diagnosed with BrS.[15,28]Patients with LQTS and BrS seem to benefit significantly from implantable defibrillators, whereas patients suffering from conduction disorders were managed successfully with pacemaker implantation as treatment option.[15,28,52,63]

The profound value of existing treatment for these arrhythmic diseases may be best portrayed by Wilders' comparison of two similar case studies and their associated clinical outcomes.[15] Both cases involved neonates with documented arrhythmias and a prolonged QT interval, though only one of the cases received treatment on presentation of clinical symptoms.[15,72] The first case was reported by Southall *et al*[72] on a neonate who presented with arrhythmias in utero and bradycardia for the first 9 days of life; however, on day 10, a normal heart rate was recorded and the baby was discharged from hospital. Unfortunately, the baby suffered a sudden and unexpected death 3 days later, which, after an autopsy investigation, remained unexplained. On retrospective analysis of the available ECG recordings, a substantial QT interval prolongation was observed.[15,72] In contrast, a second neonate who also presented with arrhythmias in utero and a 24-hour ECG illustrating a prolonged QT interval with frequent premature ventricular beats received a β-blocker

(propranolol), which proved to be successful in treatment.[15,72] Since the disease is potentially treatable, the ability of molecular testing to identify these channelopathies as a cause of death in SUID cases will allow for testing and initiation of preventive therapies not exclusively to just family members at risk but even in future pregnancies.[26,65,69] Unfortunately, as a consequence of the almost silent nature of the disorder (sudden death being the first 'symptom'),[26,69-71] genetic testing would be difficult to implement as a preventative measure before any SUID occurrence or without strong suspicion due to known family history. The role of postmortem genetic testing in this age group will be to establish the prevalence of these variations in the general population.

THE ROLE OF MOLECULAR TESTING

Considering all the data, the question arises as to whether a routine postmortem genetic analysis should be implemented in all sudden infant deaths that remain unexplained after a thorough autopsy investigation.

First, as described by Skinner,[52] the identification of pathogenic variations in SUID victims does not necessarily prove causality even if their clinical significance has been proven to be disease causing in other families or by in vitro testing. This leads to the old dictum where the forensic pathologists need to decide if the person died with the disease or as a result thereof. However, evidence exists (referenced throughout this paper) that SUID may be due, in a minority, to cardiac channelopathies such as long QT syndrome.

Second, the question arises as to what extent forensic pathologists are legally and ethically bound to conduct these tests. It can be argued that the forensic pathologists need to determine the cause, and in some cases the manner, of death. The next-of-kin in these cases might benefit tremendously from testing, which in some instances could include ECG screening followed by genetic testing.[45,46,71] This would necessitate close working relationships between forensic pathologists and a team of other experts including molecular biologists, cardiologists and genetic counsellors. The importance of findings by forensic pathologists over the years has drastically led to the reduction of certain mortalities—for example, the implementation of restraint devices in road traffic accidents—and cannot thus be negated.

Third, in many instances, finances are not available to routinely conduct these tests. On average, screening only for variations in the *SCN5A* gene, which is reported to be found in 5.2% of SUID victims,[15,28,52,65] would cost approximately US$570 per case in South Africa (the cost of similar genetic testing may differ between countries). However, these costs will be dramatically reduced in the event of implementation of routine genetic testing in all unexplained SUID cases, as targeted genetic testing of known hotspot regions will be used instead of whole exome sequencing. Research should also focus on screening the general population to determine which variations occur naturally in any given population. A recent molecular study conducted on South African SUIDs (unpublished data) revealed eight specific exons of the *SCN5A* gene as definite hotspot regions particular to this population. In effect, the costs of postmortem genetic testing, refined to those eight hotspot regions, in a single SUID case, would amount to approximately US$143. Considering the reduced costs, which should continue to decline due to advances in technology, one might argue that ethical issues far outweigh financial concerns with regard to targeted postmortem genetic testing in applicable SUID cases.

The question will always remain as to which genes should be tested for in each case. According to the Heart Rhythm Society/European Heart Rhythm Association guidelines, targeted postmortem mutational analysis in all sudden unexpected deaths between 0 and 40 years of age is recommended.[50,75] In countries such as Australia and New Zealand, all sudden and unexpected deaths are mandated to undergo targeted postmortem genetic testing.[50,69] In 2015, the Swiss Society of Legal Medicine recommended that all sudden unexpected deaths under the age of 40 should be subjected to postmortem genetic testing.[75] In a recent study conducted by Sanchez *et al*,[75] next-generation sequencing (NGS) postmortem genetic analyses showed that in 13.4% of sudden unexplained death cases (between 0 and 10 years of age), a disease-causing variation linked to an inherited cardiac arrhythmic disorder (LQTS, BrS and CPVT) was identified and diagnosed as the cause of death.[75] In the remaining 31.9% cases, in which variants considered possibly pathogenic could not be fully defined as the cause of death, a necessity for family members to consider further genetic evaluation was established.[75] As a result of their findings, they recommend that NGS genetic analyses should be performed on all unexplained sudden deaths below the age of 40.[75]

In our opinion, interdisciplinary centres should conduct large studies in order to attempt identifying the true incidence of these cases. Prospective and retrospective studies could be undertaken. At most large medicolegal death investigation centres (which are often linked to tertiary academic institutions), forensic pathologists have established archives of formalin-fixed, paraffin-embedded (FFPE) tissue samples, which can serve as a (sometimes only) source of material that contains critical genetic information valuable to molecular testing.[74,75] Several studies have reported the successful, though not necessarily ideal, use of FFPE tissue samples in retrospective postmortem mutational analysis of previously admitted SUID cases.[53,57,74,75] This raises an important issue pertaining to a possible difference in cost between the usage of FFPE tissue samples versus more traditional samples such as DNA extracted from blood. From experience working with FFPE tissue samples as a source of DNA for postmortem genetic testing, costs increase dramatically compared with using blood samples as the source of DNA. However, the rise in cost almost completely depended on factors associated with the incorrect conditions/circumstances surrounding the retention, fixation and storage of FFPE tissue samples. When prescribed guidelines were followed for the retention and fixation of FFPE tissue samples (fixed in formalin for a maximum of 24 hours, cleared in xylene and embedded in a paraffin block), DNA extraction and subsequent molecular applications were equal in quality, be it at lower concentrations, when compared with DNA extracted from blood. Thus, the difference in cost between using these two sources of genetic material for genetic testing may, in fact, be insignificant and therefore highlights the crucial importance of appropriate sampling/storage of all retained autopsy samples.

Combining resources and including all infants (regardless of the manner/cause of death) in testing for specific genetic variations could provide data on the most commonly encountered variations for each subset. Although this would most definitely be a very costly undertaking, identifying the specific genetic variations and their associated hotspot regions could prove cost-effective in the long term as more focused testing (which will be more affordable) could be undertaken.

Knowledge gained from the results of these tests could be imperative for adequate genetic counselling of parents of subsequent cases and provide closure to families who were previously informed that no cause of death was identified. This will assist in

providing closure and planning options (such as genetic testing) for all siblings, adding significant value in the possible prevention of future similar cases to all individuals involved.[45,73]

Thus, ethical and reasonable justifications compel us to seek a molecular diagnosis of LQTS in an infant whose sudden death remains unexplained despite a thorough autopsy and ancillary investigations, and should therefore be considered in all medicolegal settings.[52]

Correction notice This paper has been amended since it was published Online First. Owing to a scripting error, some of the publisher names in the references were replaced with 'BMJ Publishing Group'. This only affected the full text version, not the PDF. We have since corrected these errors and the correct publishers have been inserted into the references.

Contributors All three authors were involved in the conception, writing and editing of this paper.

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Appendix N

Case Report

The added value of molecular-based diagnostics in the African forensic medical setting

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Abstract

Sudden unexpected infant death (SUDI) is reported to be an extraordinarily high burden in sub-Saharan Africa, with the incidence rate in South Africa among the highest in the world. Advances in molecular-based diagnostics allow researchers to identify numerous underlying inherited cardiac arrhythmogenic disorders in many SUDI cases, with a predominance of variants identified in the SCN5A gene. Such cardiac arrhythmogenic-related sudden deaths generally present with no structural alterations of the heart that are macroscopically identifiable at autopsy, therefore highlighting the importance of post mortem genetic testing. We report on a significant genetic finding that was made on a SUDI case in which the cause was ascribed to an acute bacterial pneumonia but it was still subjected to post mortem genetic testing of the SCN5A gene. The literature shows that many SUDI cases diagnosed with inherited cardiac arrhythmogenic disorders have demonstrated a viral prodrome within days of their death. It is therefore not uncommon for these cardiac disorders in infants to be mistaken for flu, viral upper respiratory tract infection or pneumonia, and without the incorporation of post mortem genetic testing, any other contributory causes of these deaths are often disregarded. This study highlights the need for research reporting on the genetics of inherited cardiac disorders in Africa.

Keywords: channelopathies, dilated cardiomyopathy (DCM), inherited cardiac disorders, post mortem genetic testing, *SCN5A*, sudden unexpected death in an infant (SUDI).

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Sudden deaths in infants are still considered one of the leading causes of infant mortality worldwide and have also been reported to be an extraordinarily high burden in sub-Saharan Africa (SSA).[1-3] According to Duncan *et al*.,[4] for most countries, the rate of sudden unexplained infant deaths (SUIDs) [or the previously termed sudden infant death syndrome (SIDS) cases] is reported at approximately 0.2–0.5 per 1 000 live births. The most recent published incidence rate for South African SUID cases was 1.06 per 1 000 live births for the white population and 3.41 for infants from the mixed-ancestry population group, respectively.[2]

The investigation into SUDIs and child mortality remains a high-priority research area in South Africa.[2,3,5] It has universally been accepted that a SUID case can very rarely be explained by a convenient and simplistic 'single-cause' mechanism, but instead is attributed to a complex event with an increase in incidence when risk factors such as vulnerability, a critical period in development and exogenous stressors all intersect at the same time (triple-risk model proposed by Filiano and Kinney).[4,6,7]

One of these risk factors, and a possible preventable cause of SUIDs, which has received increased attention over the past few years is inheritable cardiac arrhythmogenic disorders.[1,3,8] Although these inherited cardiac disorders in SUID cases have primarily been associated with electrical conditions (channelopathies), recent studies have identified variants in genes encoding structural proteins, thereby suggesting a cardiomyopathy as a possible cause of death as well.[1,9-10]

Previous studies demonstrated a link between SUIDs and a predominance of *SCN5A* gene variants. This could be explained by the known genotype–phenotype correlations that suggest patients with *SCN5A* variants may experience a higher mortality rate, mostly occurring during sleep, compared to patients suffering from variants in other genes involved in inherited cardiac diseases.[1,8]

Advances in molecular-based diagnostics allow researchers to identify numerous underlying inherited cardiac arrhythmogenic disorders that have been misdiagnosed in many SUID cases.[1,11] In many developing countries, including Africa, there is still a significant lack as far as forensic molecular diagnostics is concerned, mainly due to financial and resource constraints.[12] As a result, the cases in this study were subjected to retrospective post mortem molecular analysis of only the most prevalent

gene (*SCN5A*) associated with SUID, in order to identify any possible pathogenic variations associated with an inherited cardiac disease, which may have predisposed this infant to a sudden death.

Case report

We report on a case of a two-month-old male infant of African ancestry whose mother found him unresponsive in his crib during a scheduled nap. Upon emergency medical services (EMS) arrival, the infant was declared dead at the scene without any medical care being administered. No written clinical history/records were available, however his mother reported him having a recent cold for which she administered cough medication. The mother also reported an increase in crying and that the infant struggled to feed.

Due to the sudden and unexpected nature surrounding the death, the body was admitted to the Pretoria Medico-Legal Laboratory for further medico-legal investigation, in accordance with the Inquests Act 58 of 1959. A complete macroscopic autopsy examination was conducted, which externally revealed the deceased to be of average physique and nutritional state. No injuries were noted on the body.

Upon internal examination, the intracranial examination showed no gross pathological changes. Examination of the heart revealed no abnormalities involving the epicardium. The myocardium and heart valves appeared normal. The lungs appeared congested and oedematous. On cut surfaces, the lungs showed sharply defined edges, had a friable texture and contained muco-purulent fluid. Examination of the stomach revealed contents of a milk-like residue and it was noted that the gastric mucosa appeared normal. As a result, no macroscopic cause of death could be identified at autopsy.

Toxicology results revealed only trace amounts of theophylline, a bronchodilator, which is in keeping with the history of cough medication administered to the infant. No sedatives could be detected in the blood specimen.

Histological examination of the thymus, brain and heart showed no obvious pathological changes. Sections of the heart showed no evidence of myocyte hypertrophy, nucleomegally or interstitial fibrosis. Sections of the lungs showed a mild mononuclear interstitial infiltrate with thickening, congestion and focal haemorrhage (Fig. 1). Focal intra-alveolar neutrophilic exudate was also noted in the lungs, as seen in Fig. 2. The features noted in the lungs were found to be in keeping with an acute bacterial pneumonia. Henceforth, the primary medical cause of death was acute bacterial pneumonia.

Genetic testing

Due to the diagnosis of an acute bacterial pneumonia as the cause of death, this infant case study was included in our larger study. For this case, an archived, formalin-fixed, paraffin-embedded (FFPE) myocardial tissue sample, obtained from the original autopsy 10 years prior to this study, for histology purposes, was subsequently subjected to retrospective post mortem genetic testing of the *SCN5A* gene. DNA was extracted from the FFPE myocardial tissue sample using the QIAamp DNA FFPE tissue kit (Qiagen). After extraction, the concentration and purity of the DNA sample was determined spectrophotometrically (NanoDrop spectrophotometer, Thermo Scientific).

Thirty-nine primer pairs were used for amplification of 28 exons of the *SCN5A* gene.[13] High-resolution melt real-time polymerase chain reaction (PCR) amplifications were performed using SensiFast HRM mastermix (Bioline) on the RotorGene Q (Qiagen). Following optimisation, DNA concentrations used per reaction averaged 50 to 80 ng. Final primer concentrations were 10 pmol.

Thermal cycling conditions followed SensiFast guidelines, and annealing temperatures were dictated by the primers. High-resolution melting (HRM) analysis was performed, and control and case study samples were compared. All amplicons that showed variation on HRM were subjected to sequencing (Inqaba Biotec). Sequencing results were analysed using CLC Main Workbench 5 software (CLC Bio®) and were aligned with the *SCN5A* gene sequences from GenBank (SCN5A NG_008934.1; NM_001160161.1 and NP_001092874.1) [National Center of Biotechnology and Information (NCBI)]. Polymorphism phenotyping v2 (PolyPhen2) was used to determine the probability of pathogenicity for novel identified variations.

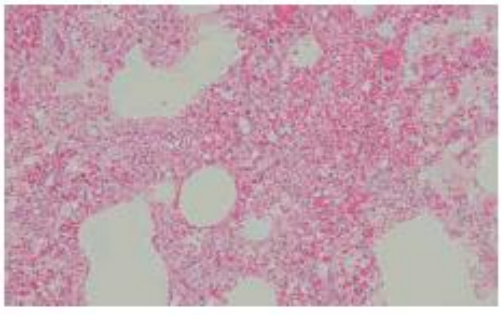

Fig. 1. Haematoxylin and eosin stain of the lungs shows a mild mononuclear interstitial infiltrate with thickening, congestion and fresh focal haemorrhage.

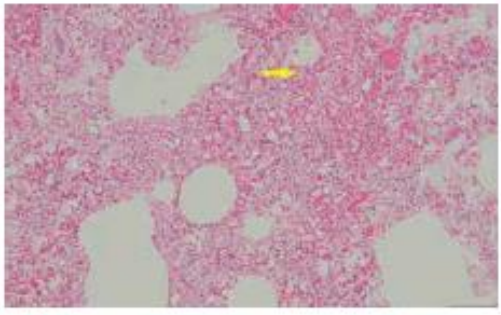

Fig. 2. Haematoxylin and eosin-stained slide of the lungs showing a mixed intra-alveolar infiltrate chiefly composed of macrophages, neutrophils, fresh haemorrhage and oedema.

Results

Genetic analysis revealed two different variations in exon 28 of the *SCN5A* gene. The first was a novel heterozygous variation (c.5566G>A) in the coding DNA sequence. This missense variation leads to a G>A nucleotide change in codon 1856, with an amino acid change of alanine (Ala) to threonine (Thr) (p.A1856T) (Fig. 3). Due to the functional difference between these two amino acids, the possibility of this variation affecting the protein structure is high. The PolyPhen-2 online algorithm predicted this variant to be probably damaging with a score of 1.000.

The second heterozygous single-nucleotide variation, c.5668G>A, is registered on the Atlas of Genetic Cardiac Variation database, with an uncertain clinical significance, likely associated with dilated cardiomyopathy (DCM). The G>A nucleotide change in codon 1890 leads to an amino acid change of glutamic acid (Glu) to lycine (Lys) (p.E1890K) (Fig. 4). This variant is considered by 87.5% of algorithms to be likely damaging, predicting an adverse effect on the protein structure.

The E1890K variant is a non-conservative amino acid substitution, which would likely impact on the secondary protein structure as these residues differ in polarity, charge, size and/or other properties. This substitution occurs at a position that is conserved across species, and *in silico* analysis, predicts this variant to be probably damaging to the protein structure/function. More recently, p.E1890K has been registered on the NCBI database under rs766875593, with an uncertain clinical significance associated with various channelopathies rather than a cardiomyopathy.

Recent studies reported on the identification of this variant in SUID cases associated with the long-QT syndrome (LQTS) as well as Brugada syndrome (BrS). The Genome Aggregation Database (gnomAD) and the Exome Aggregation Consortium (ExAC) reported on an allele frequency of 0.00001 and 0.00002, respectively. However, it is important to note that these allele frequencies are not representative of the African population since no studies have been done to provide statistics on this occurrence.

Wang *et al.*[14] reported on the identification of this variant in a two-month-old infant, whose cause of death could not be determined after a thorough autopsy, scene investigation as well as all ancillary investigations had been conducted. A review of the case history indicated a prone sleeping position at the time of death, with a history of a recent cold, similar to our case study.

Discussion

Two heterozygous missense variations in the *SCN5A* gene were identified in this SUID. Bearing in mind that a cause of death (bacterial pneumonia) had been established for this case prior to genetic testing, the results were deemed significant, although unexpected, in explaining the full circumstances surrounding the death.

The *SCN5A* gene encodes a protein, sodium (Nav1.5) ion channel pore-forming α-subunit, that is expressed only in the myocardium and performs a critical role in heart excitability and conduction.[15-17] The integral membrane protein produces the fast-inward Na+ current that is responsible for the depolarising phase of the cardiac action potential. Variations in this gene cause an increased persistent Na+ current, with a subsequent prolongation of the ventricular action potential, essentially resulting in an inherited predisposition to ventricular arrhythmias and sudden death, seen in several cardiac diseases.[4,18] Previous studies demonstrated a link between SUID and a predominance of *SCN5A* variants, more commonly associated with channelopathies.

Channelopathies are generally described as inherited cardiac arrhythmogenic disorders associated with isolated electric dysfunction caused by variants in genes encoding for cardiac ion channels and regulatory protein receptors, which are involved in the ionic control of the cardiac action potential.[1,14] A link between many human diseases and the dysfunction of ion channels (channelopathies) has been established, either as a result of genetic variants or acquired malfunctions of ion channels.[13,17]

The three most common and epidemiologically relevant genetic heart channelopathies include LQTS, BrS and catecholaminergic polymorphic ventricular tachycardia (CPVT).[1,13,16] Although the involvement of numerous susceptibility genes has been identified, most of the variants (especially in SUID cases) have been located in the *SCN5A* gene, predominantly linked to LQTS and BrS.[15-18] Post mortem genetic studies have implicated channelopathy-associated variants in 10 to 15% of SUID/SUDI cases.[1,8,14]

The most common clinical manifestations associated with LQTS and BrS are syncope, seizures and sudden death as a result of ventricular arrhythmias, usually occurring during a period of rest/sleep. Of particular note is that these channelopathy-related sudden deaths generally present with no macroscopically identifiable structural alterations of the heart at autopsy.[4,8,15,16] Our case study was found to carry two variations, of which one has been documented with an uncertain clinical significance, although associated with LQTS, BrS and DCM.

Numerous studies have reported on the diversity of the phenotypic and genotypic expression of the *SCN5A* gene with variations linked to other arrhythmogenic disorders, including DCM, progressive familial heart block type 1 and sick sinus syndrome.[4,16,19] Reports of SUID cases where genetic variations associated with cardiomyopathies are increasingly identified in structurally normal hearts should prevent the tendency

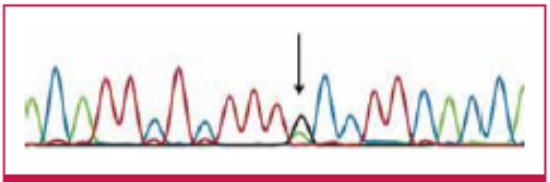

Fig. 3. Heterozygous single-nucleotide variation p.A1856T (c.5566G>A) identified in exon 28 of the *SCN5A* gene.

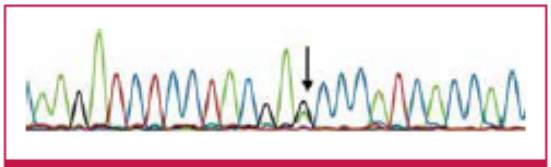

Fig. 4. Heterozygous single-nucleotide variation p.E1890K (c.5668G>A) identified in exon 28 of the *SCN5A* gene.

of eliminating associations between *SCN5A* variations and DCM.[1,8,20-22]

Cardiomyopathies can be described as a group of heart diseases that affect the structure and function of the myocardium, which can all lead to heart failure, arrhythmia and even sudden death.[9,13,23] The most common types of cardiomyopathies include hypertrophic cardiomyopathy, DCM, restrictive cardiomyopathy and arrhythmogenic cardiomyopathy.[13,21,23] Although it is generally associated with cardiac alterations macroscopically identifiable at autopsy, it is not uncommon for a cardiomyopathy to be inadvertently missed in SUID cases, which usually present with a macroscopically normal heart.[1,8,21,23]

Studies have been reported that genetic variations in cardiomyopathy-related genes, which may cause arrhythmia and sudden death, have been identified in SUID cases presenting without any cardiac changes. Research suggest that this might be explained by the progressive nature of cardiomyopathy, whereby in the first stages of the disease the myocardial changes may be so incipient that it may not be visible at autopsy.[1,8,20,24]

The genetic basis of DCM in infants commonly demonstrates phenotypic overlap. Reported cases of DCM due to *SCN5A* variations identified in long-QT syndrome type 3 (LQT3), shows that not only can it result from structural changes in the myocytes, but also from altered calcium ion handling.[10,13,16,20-21] These inherited genetic susceptibilities in infant cases have been proven to play an important role in how the cardiac muscle responds to environmental and infectious factors.[11,22,24]

Researchers believe that variations in the *SCN5A* gene, with its associated higher risk of lethal arrhythmias, are linked to an increase in an infant's critical vulnerability to certain infections. Consequently, acute viral infections are regarded as one of the provocative factors associated with sudden death in infant channelopathy and/or DCM cases.[11,20,23,24] In fact, many of these SUID cases (diagnosed with inherited cardiac arrhythmogenic disorders) demonstrated a viral prodrome within days of their death. Such infants often present with respiratory signs, extreme sleepiness, difficulty in feeding and increased fussiness prior to death.[11,23,24]

It is not uncommon for inherited channelopathies and/or cardiomyopathies in infants to be mistaken for flu, viral upper respiratory tract infection or pneumonia, and without the incorporation of post mortem genetic testing, any other contributory causes of these deaths are often disregarded.[1,8,11,20] Consequently, it is even of greater importance for countries with a high burden of infectious diseases to be especially aware of these findings, as there might be a reasonable tendency to overcall minor findings of viral infection in these SUID cases.[1,2,11,20]

Genetic testing is considered an ideal risk-assessment tool, not only for channelopathies, but for cardiomyopathies as well, due to its ability to identify patients at risk prior to overt disease development.[1,10,23-24] The use of post mortem genetic testing in SUID cases can benefit family members, especially those from poor communities, by providing the first indication of a familial cardiac arrhythmogenic disorder. Ultimately this will allow for the opportunity of preventative intervention, which can be used to avoid the progressive onset of the disease.[8,10,15,22,23]

For decades now, the undeniable benefit of post mortem genetic testing in SUID cases, especially those that remain unexplained, has been widely recognised worldwide.[1,4,8] The continued advancement in molecular diagnostics and its associated decrease in costs has allowed for expanded molecular testing using cardiac gene panels and next-generation sequencing.[1,4,8,24] Although this is not a novel concept to most first-world countries, it still eludes the radar of many medical professionals practicing in an economically and resource-strained country. These countries, including South Africa, have not yet been conducting post mortem genetic testing in unexplained SUID cases, at least not routinely.[2,3,7,12] The greatest benefit of such testing is not to define the cause of death, but rather the highly disease-specific diagnostic, therapeutic and prognostic benefit derived from subsequent genetic screening of family members of the deceased.[1,8,15,16]

In addition, disease-causing variants in the *SCN5A* gene have been reported as a possible predisposing factor of SUID, providing an apparent aetiology of arrhythmias due to secondary challenges/risk factors such as complicating lower respiratory infections, which are generally tolerated in infants not carrying such genetic variations. Considering South Africa's burden of infectious diseases coupled with a high infant survival rate in most of these cases, a more scrutinised and in-depth investigation into those SUID cases that typically present with no more than minimal findings such as the presence of a mild infection, should be considered.[2,12]

Conclusion

There is a lack of research reporting on the genetics of channelopathies and cardiomyopathies in Africa. The fact that cardiomyopathies are deemed an endemic form of non-communicable diseases, of high importance in the largely low-income communities in SSA, proves the need for local research on this topic. The results from this case study demonstrate the possible impact molecular diagnostics can have on identifying potential inherited cardiac disorders. Additionally, it highlights the occurrence of misdiagnosis of SUID cases in our population, or the possibility of an incomplete understanding pertaining to the circumstances surrounding these deaths. Further molecular testing may provide better knowledge as to why certain infants do not survive these viral and/or bacterial infections.

This case study aimed to create awareness on this subject among medical professionals, especially those practicing in resource-strained countries. Hopefully, this will motivate for more collaborative research and investigation to gain a better understanding of the unique genetic diversity and its associated inherited diseases in SSA.

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Appendix O

SAMJ CME

Postmortem genetic testing in young individuals: What clinical medical practitioners need to know

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The death of a young person is most often a tragic occurrence, more so when this death was unexpected. Forensic pathologists are mandated to investigate such deaths and internationally, there has been a strong move towards genetic testing as an additional investigative tool. The aim of our article is to bring the advantage of implementing the so-called molecular autopsy in a local setting, to the attention of medical practitioners. When a multidisciplinary approach is taken in cases of sudden unexpected death, the benefits to family members, and society as a whole, is irrefutable.

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A general misconception regarding the field of forensic medicine seems to be that the purpose thereof is only to conduct medicolegal autopsies on cases that are criminal in nature, and to deal with the subsequent judicial matters resulting from such cases. However, forensic medical practitioners are in fact in a most fortunate and unique position, as they observe the exact pathology of various diseases (whether or not these are attributed as the cause of death) in thousands of autopsies performed each year. In most cases these medicolegal autopsies reveal an underlying, natural disease as the cause of death. All information pertaining to the cause and mechanism of these deaths are relayed to several entities, including the Department of Home Affairs and Statistics South Africa. This, perhaps, can be considered to be one of the most valuable contributions from the field of forensic medicine to society. Such data, relayed to the various stakeholders in the public health sector (including clinical medical practitioners), contribute to determining population health status as well as identifying and dealing with priority areas. Currently, one of the most important public health concerns in Africa and considered to be among the priority areas that should receive greater attention is that of the continuous increase in cardiovascular diseases (CVD). A particular subset of CVD in South Africa (SA) that is of great concern is sudden unexpected deaths (SUDs) in the young population.

It is a common occurrence to read poignant media headlines in SA of young, seemingly healthy, individuals who suddenly and unexpectedly die, often with family members in the dark as to the cause of the death. Fortunately, research on these SUDs has shown that postmortem genetic testing can detect if an inherited cardiac arrhythmogenic disease is the cause of death in many (up to 40%) of these cases.[1] Such cardiac diseases often present with no clinical symptoms or warning signs prior to a sudden death (SD), showing the added benefit of conveying autopsy findings to clinicians. The societal benefits from clinicians receiving such evidence-based findings associated with the cause of a patient's SD are currently underutilised in SA. Such practical recommendations are an added benefit for clinicians when evaluating patients presenting with a sudden cardiac arrest. It could even be said that of greater importance is the benefit to patients with a family history of SUD. This matter urgently requires the attention it deserves.

The role of genetic testing in SUDs

Rapid and continuing development in molecular techniques allows the field of forensic molecular biology, also known as forensic molecular pathology, to use a molecular approach in not only studying but also diagnosing the underlying genetic basis of human disease and death processes.[2-4] International reports show that no other scientific discipline has embraced the application of molecular biology techniques for diagnostic purposes more than the field of forensic science and pathology, particularly in the medicolegal investigation of SDs in the young.[2,5]

Approximately 70 - 85% of SDs in young populations are cardiac related (termed sudden cardiac death (SCD)), and up to 90% of these SCD cases are potentially caused by inherited cardiac diseases, making this an important global topic in molecular research.[4-8] Molecular screening in cases of SCD has provided an explanation to the aetiology underlying the inherited cardiac disease, ultimately leading to an increased understanding of critical conditions and the clinical management thereof.[2,9,10] Most international medical associations deem SCD a global public health issue, and are currently advocating for prioritising and aligning their research plans and healthcare delivery objectives to utilise the diagnostic benefits that molecular analysis can provide.[8,11,12]

The growing global burden of noncommunicable diseases, of which CVD is the predominant cause, is one of the United Nations' health priorities.[13] A published editorial in SA Heart addresses the topic of global health and, more specifically, the CVD burden in Africa. Not only is CVD one of the leading causes of death worldwide, but it has also been reported that ~85% of these deaths occur in low- and middle-income countries.[14,15,16] Apart from CVD being the second biggest killer in Africa, the mean age of these deaths has also been recorded as the youngest in the world.[15,16]

One of the greater health priorities in sub-Saharan Africa should be a focus on preventing and treating cardiac diseases. There are key

gaps in knowledge, and especially on research priorities on genetic causes of cardiac diseases specific to the African population.[13,16,17]

The SD conundrum

SD is a leading cause of mortality in the young, and considered a public health problem worldwide owing to its prevalence and significant impact on society.[18-20] The global incidence of SD in the young ranges between 1.3 and 8.5 per 100 000 person years.[4]

In SA, a SUD of a person is classified as an unnatural death, and therefore mandated in terms of the Inquests Act No. 58 of 1959 to be investigated in the medicolegal environment, where the forensic medical practitioner will conduct a thorough autopsy of the case in order to determine a cause and mechanism of death. However, in many instances, a clinician has previously known a patient who presented with features of a heart disease, and consequently classifies these cases as natural, attributing the sudden death to ischaemic heart disease (acute myocardial infarction). In decedents who have documented clinically investigated coronary artery disease, this would be the reasonable cause of death. However, in a subset of individuals, ischaemic heart disease might not have been the most accurate cause of death to explain the SCD.[12,21]

It has long been assumed that ischaemic heart disease is responsible for most SDs. However, data from the young population obtained in the last decade have refuted this assumption.[22,23] Fortunately, with the rapid development of technology and our increase in genetic knowledge, postmortem genetic testing (the so-called molecular autopsy) has been an invaluable tool in identifying inherited cardiomyopathies and arrhythmogenic disorders as the cause of death in many SDs, including infant cases.[23-26] The American Heart Association, European Heart Rhythm Association and the Royal College of Pathologists of Australasia have published recommended guidelines that they consider the minimum standard required in the routine autopsy practice for adequate investigation of a SCD.[7,8,11,12]

SCD

Approximately 5 000 000 lives per year are lost to SCD globally, with an annual incidence rate that ranges between 50 and 100 per 100 000 in the general population.[27,28] It has been reported that SCD accounts for 15 - 20% of all international deaths. Although the true incidence remains unknown, the Heart and Stroke Foundation estimated that approximately 2 000 young and healthy South Africans die suddenly each year as a result of SCD.[28] An increase in the incidence of SCD has been observed worldwide, regardless of socioeconomic status and ethnicity and this creates a public health burden. In fact, the impact of SCD in the young has created a premature death burden exceeding any other cause of death, except those attributed to all types of cancers combined.[8,13]

The causes of SCD are greatly dependent on the age of the deceased. Although the incidence of cardiac-related death increases with age, the proportion of SDs is much higher in the young population.[4,23,30] Ischaemic heart disease is the most common cause of SCD in individuals >40 years old, but inherited cardiomyopathies and arrhythmogenic disorders rank higher than ischaemic heart and valvular disease in individuals <40 years of age, with up to 75% of SCD in the young a result of the former.[8,14,25,28,31]

Using the molecular autopsy to identify causes of SUD

Although the molecular autopsy is not a novel concept to most first-world countries, it still eludes the radar of many clinicians practicing in an economically and resource-strained country. The term 'molecular autopsy' can be described as the use of postmortem genetic testing to identify genetic variants associated with, or causative of, a lethal disease, in order to help determine or better understand the cause of death (usually that of a SD).[28,32] Although its causes may vary, it has been determined that ~85% of all SDs are of cardiac origin.[4-8]

Inherited cardiomyopathies

The most prevalent cardiomyopathies implicated in SCD can, in most cases, be macroscopically identified at autopsy.[33] Every so often, these cardiomyopathies will be described as idiopathic, postpartum or a consequence of chronic alcohol abuse, only to be recognised, after examining the relatives, to be familial.[34,35] A further challenge often experienced by forensic pathologists is the fact that these cardiomyopathies may at times present with very subtle or even absent cardiac alterations at autopsy, especially in infant cases.[33,34] This, in combination with sometimes minor, potentially misleading findings, substantiates the need for postmortem genetic testing.[32,34,37]

Channelopathies

Alarmingly, not all SCDs in children, adolescents and young adults have an obvious cause of death that can be determined at autopsy. Research has estimated that between 3% and 53% of SCD cases have no identifiable abnormal morphological findings at autopsy and remain unexplained, whereas the number of unexplained sudden deaths in infants (SUDI) may rise to 80%.[24,38,39] Only through postmortem genetic testing has it been shown that inherited cardiac arrhythmogenic disorders, commonly referred to as channelopathies, are the cause of a large number of these unexplained cases.[24,36,38]

The benefit of molecular autopsy

It could be argued that the greatest benefit of such testing is not to define the cause of death, but rather the highly disease-specific diagnostic, therapeutic and prognostic benefit derived from subsequent genetic screening of family members of the deceased.[40-42] Considering the high heritability of cardiac disorders and the fact that they are often treatable, genetic analysis of SUD/SUDI victims provides significant clinical benefit with regard to the diagnosis and treatment of family members at risk for the same disease. Over 95% of these genetic cardiac disorders are inherited in an autosomal dominant manner, leading to a 50% chance for first-degree relatives to inherit the same genetic variant.[41] Several authors have reported on studies that evaluated family members of SUD cases, and found that up to 53% of family members tested positive for an inherited cardiac disease.[39,42,43] In the majority of those affected family members, considerable lifesaving interventions such as β-blockers and implantable cardioverter-defibrillators proved to be highly beneficial.[40,41] Family members of SUD/SUDI cases are usually unaware of carrying a disease-causing variant associated with an arrhythmogenic disorder. With few, if any, clinical symptoms or warning signs (family history of syncope, SD, epilepsy, deafness or early pacemaker implantation) before a SUD, the critical importance of postmortem genetic testing cannot be overstated.[22,36,41] The confirmed marked reduction in mortality associated with the administration of proper treatment in such cases leaves no ethically arguable justification for allowing family members at potential risk to remain undiagnosed and untreated.[41,44]

Postmortem genetic testing is recommended (in published guidelines) in all SUDs in the young (0 - 40 years of age) and in all cases suggestive of cardiomyopathy.[7,8,11,12] The minimum requirements involve only targeted genetic testing of the major genes. However, the use of commercial panels consisting of a combination of up to 100 cardiomyopathy and channelopathy genes is becoming

more common. These minimum guidelines aim to prevent criticism of case analysis in the medicolegal setting and protect surviving family members with possible genetic disorders.[7,8,23]

With advancements in technology and the associated decrease in cost, the forensic medical profession is increasingly becoming aware of the dangerous implications that an unidentified aetiology of a possible inherited disorder can have on family members at risk.[45,46] The ethical duty and legal liability pertaining to the 'failure to diagnose' and 'duty to warn' in forensic pathology is currently debated on an international level. According to the Code of Medical Ethics of the American Medical Association, the implications of genetic information for the biological relatives of a patient must be included in the pre and post genetic counselling process.[47] Consequently, it is argued that the legal precedent laid down by two primary American court cases (Pate v Threlkel and Safer v Estate of Park) should be, and most probably will, be applied to the forensic pathology profession. The court held that the physician has a duty to warn biological relatives of an inheritable genetic condition, if the standard of care available will be to their benefit, and if the physician is aware of the existence of these biological relatives.[47] Forensic pathologists in the USA already recognise their duty to warn, and are currently in the process of drafting a standard national set of guidelines for the notification of family members of all cases where a possible genetically heritable aetiology is found.

The way forward

Internationally, the application of postmortem genetic testing as routine investigation in all unexplained SUD/SUDI cases has been adopted.[7,8] The results produced by these molecular autopsies have successfully contributed to the public health sector in improving the population's health status by diagnosing and treating at-risk family members. Inevitably, it raises the question as to the current stance on the implementation of the molecular autopsy in African forensic medical institutions.

In SA, there is no medicolegal mortuary that offers targeted genetic testing in SUDs in the young, even though the departments of forensic medicine at the University of Pretoria and University of Cape Town both conduct valuable research on this topic.[48,49] Our pilot study, conducted on one gene linked to unexplained SUDI, yielded interesting results, with 22.5% of cases having possibly pathogenic *SCN5A* variants considered to be associated with the cause of death.[49]

SA needs to realise that SCDs, especially in the young, should be deemed a clinical health priority, and urgently treated as such. It can be inferred that a high proportion of these SD victims were actively occupying the workforce, and thus contributing to the national economy, emphasising the impact on society. Tackling this health concern can only be successful through a multidisciplinary approach, where all relevant stakeholders, including forensic medical practitioners, clinicians, governmental agencies and funding bodies, to name a few, accept their responsibilities and play their part. With adequate funding and resources, and stricter referral, according to legislation, of SUDs for medicolegal death investigation, a significant increase in molecular research can be conducted into these deaths. A direct result of this will be to enable researchers to detect with greater certainty the most prevalent genes associated with inherited cardiac diseases specifically targeted towards the SA population. The ultimate aim, through adequate research, is to reach that point of targeted genetic testing that can be used as affordable point-of-care testing, which will be of immense value to clinicians, forensic medical practitioners and society as a whole.

African medical professionals have often been at the forefront when it comes to innovative and ground-breaking medical procedures. Therefore there is no excuse not to excel at the implementation of the molecular autopsy and reap the clinical benefits it has to offer.

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