
Sudden unexpected death in infants: a forensic genetic investigation in a South African cohort

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This thesis is submitted in fulfilment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Division of Human Genetics | Department of Pathology | Faculty of Health Sciences

UNIVERSITY OF CAPE TOWN

February 2019



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1. Heathfield, L.J., Martin, L.J., and Ramesar, R. A Systematic Review of Molecular Autopsy Studies in Sudden Infant Death Cases. *J Pediatr Genet* 2018;7:143–149. doi:10.1055/s-0038-1668079.
2. Heathfield LJ, Maistry S, Martin LJ, Ramesar R, de Vries J. Ethical considerations in forensic genetics research on tissue samples collected post-mortem in Cape Town, South Africa. *BMC Med Ethics*. 2017 Nov 29;18(66):1-8. doi: 10.1186/s12910-017-0225-6
3. Heathfield, L.J., Martin, L.J., and Ramesar, R. Considerations of molecular autopsies in forensic cases of sudden unexpected infant death in South Africa. *S. Afr. J. Child Health*. In press.

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Date: 09 February 2019

Acknowledgements

I would like to express my sincere gratitude towards my supervisors, Professor Raj Ramesar and Professor Lorna Martin for their guidance and mentorship throughout the project. Doing a PhD whilst working full time was challenging at times, and I wish to also thank them for their understanding, patience and encouragement along the way. I would also like to acknowledge Dr Sairita Maistry, who began as a co-supervisor (later immigrated) whose enthusiasm and determination helped to drive this project in its early stages. I am also indebted to Professor Hugh Watkins (University of Oxford) for hosting my research visit in 2017/2018, whose expertise and experience was invaluable to this research project and shaping its future direction.

This research was funded by the RCUK Newton PhD Partnership award, the National Health Laboratory Service Development Trust grant and the Research Office, University of Cape Town. I am sincerely grateful for these sources of funding.

This project would also not have been possible without the co-operation and assistance from Salt River Mortuary, Forensic Pathology Service, Western Cape Government, Department of Health. I am forever indebted to the forensic pathologists, forensic pathology officers and of course, the participants and their families.

I would also like to acknowledge the Division of Forensic Medicine and Toxicology, UCT; the Division of Human Genetics, UCT; the Wellcome Trust Centre for Human Genetics, University of Oxford; and the Molecular Genetics Laboratories, National Health Services, Oxford University Hospitals for the facilities which enabled the laboratory work of this project.

Further, I greatly appreciate the support from the following individuals: Tracy Bennett, Chanté du Toit, Bronwen Davies, Yolanda Davies, Jantina de Vries, Caitlin Herbert, Mia Kruger, Susan Louw, Lerato Majara, Surita Meldau, Calvin Mole, Michelle Perrins, Kate Reid, Mmannini Rangwaga, Petra Schwab, Kate Thomson, Dennis Wan and Andrea Young (in alphabetical order of surname). You have all assisted me in a meaningful way, including technical-related queries, advice and/or support; for this I am truly thankful.

Lastly, I would like to thank my colleagues, friends and family for all their support; in particular, Joan Heathfield, John Heathfield, John Cowlin, Carol Royle and Matthew Royle. Your constant encouragement and motivation has been inspiring, particularly during challenging moments. Thank you for believing in me.

Dedicated to my Uncle, John
for instilling my passion for science.

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Abbreviations

Notes: The International System of Units (SI) units and associated spacing conventions are used throughout. All genes names are written in italics, whereas the protein encoded by the gene is not italicised. This allows easy differentiation as to whether the gene or the protein is being referred to, and is a common convention within the genetics field. Since this thesis contains publications, acronyms which appear in the thesis and manuscripts may be defined more than once.

Symbol, acronym or abbreviation	Definition
%	percentage
~	approximately
<	less than
=	equals to
>	greater than
≤	less than or equal to
≥	greater than or equal to
°C	degrees
μl	micro litres
μM	micro molar
3'	3 prime end
5'	5 prime end
A	adenine
ABI	Applied Biosystems
ACADM or MCAD	medium-chain acyl-CoA dehydrogenase
ACMG	American College of Medical Genetics and Genomics

AKAP9	A-kinase anchoring protein 9
Ala (A)	Alanine
am	ante merīdiem
AMP	Association for Molecular Pathology
AMPD1	adenosine monophosphatede aminase 1
ANK2	ankyrin 2
Arg (R)	Arginine
ARVC	arrhythmogenic right ventricular cardiomyopathy
Asn (N)	Asparagine
Asp (D)	Aspartate
BAM	binary alignment/map
bp	base pairs
BR	broad range
BRCA2	breast cancer type 2
BrS	Brugada syndrome
BTHLM1	Bethlem myopathy 1
BWA	Burrows-Wheeler Alignment
C	cytosine
CACNA1C	calcium voltage-gated channel subunit alpha1 C
cAMP	cyclic adenosine monophosphate
CDH2	cadherin 2
CDK5RAP2	CDK5 regulatory subunit associated protein 2
CMD	congenital muscular dystrophy
COL6A3	collagen type VI alpha 3 chain
CPVT	catecholaminergic polymorphic ventricular tachycardia

Cys (C)	Cysteine
DCM	dilated cardiomyopathy
DFMT	Division of Forensic Medicine and Toxicology
DI	degradation index
DMP1	dentin matrix acidic phosphoprotein 1
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSP	desmoplakin
<i>E. coli</i>	<i>Escherichia coli</i>
<i>e.g.</i>	for example
EDTA	ethylenediaminetetraacetic acid
ESP	Exome Sequencing Project
etc.	etcetera
exo1	exonuclease 1
FFPE	formalin fixed paraffin embedded
FGx	forensic genomics
FPO	forensic pathology officer
FPS	Forensic Pathology Service
FTA	Flinders Technology Associates
FUT1	fucosyltransferase 1
G	guanine
g	grams
GALT	galactose-1-phosphate uridylyltransferase
GCDH	glutaryl-CoA dehydrogenase
Glu (E)	Glutamate

Gln (Q)	Glutamine
Gly (G)	Glycine
gnomAD	The genome aggregation database
GO	gene ontology
HCM	hypertrophic cardiomyopathy
HCN4	hyperpolarization activated cyclic nucleotide gated potassium channel 4
His (H)	Histidine
HIV	Human immunodeficiency virus
HPLC	high-performance liquid chromatography
HREC	Human Research Ethics Committee
HS	high sensitivity
<i>i.e.</i>	that is
IgG	immunoglobulin G
IL-1	interleukin 1
IL-10	interleukin 10
IL-6	interleukin 6
Ile (I)	Isoleucine
IMR	infant mortality rate
indel	insertion/deletion
INF- γ	interferon-gamma
JAK2	Janus kinase 2
KCNE1	potassium voltage-gated channel subfamily E regulatory subunit 1
KCNQ1	potassium voltage-gated channel subfamily Q member 1
KMC	kangaroo mother care

Leu (L)	Leucine
LMICs	low- and middle- income countries
LMNA	lamin A/C
Lodox	low dose X-Ray
LQTS	long QT syndrome
LRTI	lower respiratory tract infection
Lys (K)	Lysine
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
MAF	minor allele frequency
MASP1	mannan binding lectin serine peptidase 1
MBG	molecular biology grade
MBL	mannose-binding lectin
MCPH	microcephaly primary hereditary
MDG	Millennium Development Goals
Met (M)	Methionine
MgCl ₂	magnesium chloride
min	minutes
MMDD	muscle AMP deaminase deficiency
mtDNA	mitochondrial DNA
ng	nanogram
NGS	next generation sequencing
NHLS	National Health Laboratory Service
NHS	National Health Service
nM	nanomolar
NPHP3	nephrocystin 3

OXCT1	3-oxoacid CoA-transferase 1
PAMPs	pathogen-associated molecular patterns
PCDH15	protocadherin related 15
PCR	polymerase chain reaction
pg	picogram
Phe (F)	Phenylalanine
PID	primary immunodeficiency disease
PKP2	plakophilin 2
pm	post merīdiem
pM	picomolar
POMGNT1	protein O-linked mannose N-acetylglucosaminyltransferase 1
Pro (P)	Proline
QFI	quantitative functional index
qPCR	real time PCR
RE	restriction enzyme
Ref	reference
REM	rapid eye movement
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROM1	rod outer segment protein 1
rSAP	recombinant shrimp alkaline phosphatase
RSV	respiratory syncytial virus
RYR2	ryanodine receptor 2
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SA	South Africa

SAM	sequence alignment/map
SAPS	South African Police Service
SCN10A	sodium voltage-gated channel alpha subunit 10
SCN3B	sodium voltage-gated channel beta subunit 3
SCN5A	sodium voltage-gated channel alpha subunit 5
SCOT	succinyl-CoA acetoacetate transferase deficiency
SDS	sodium dodecyl sulfate
Ser (S)	Serine
SI	International System of Units
SIDS	sudden infant death syndrome
SNP	single nucleotide polymorphism
SRM	Salt River Mortuary
STRs	short tandem repeats
SUD	sudden unexpected death
SUDI	sudden unexpected death of infants
SUDY	sudden unexpected death in the young
SWGDM	Scientific Working Group on DNA Analysis Methods
T	thymine
T _a	annealing temperature
TE	Tris-EDTA
Thr (T)	Threonine
TLR4	toll-like receptor 4
T _m	melting temperature
TMEM43	transmembrane protein 43
TNF- α	tumor necrosis factor alpha

Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
UCT	University of Cape Town
UK	United Kingdom
USA	United States of America
V	Volts
Val (V)	Valine
VCF	virtual card file
VUS	variant of unknown significance
w/v	weight per volume
w/w	weight per weight
WES	whole exome sequencing
WHO	World Health Organisation
Wnt	Wingless/Integrated

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Abstract

Sudden unexpected death in infants (SUDI) is a devastating event, and unfortunately occurs frequently in South Africa. The emerging molecular autopsy has added value to SUDI investigations by revealing genetic variants which contributed to their demise. Motivated by the value of this concept to family members as well as the limited research of SUDI locally, the aim of this study was to explore molecular autopsies in the medico-legal investigation of SUDI cases in South Africa. A 5-year retrospective study of 1 199 SUDI admissions to Salt River Mortuary, Cape Town showed that 110 (9 %) cases were still under investigation, while most had infectious causes of death. An ethical framework was established and used to prospectively recruit 201 SUDI cases from Salt River Mortuary. A pilot quality assessment of DNA from blood, buccal cells and formalin fixed paraffin embedded tissue motivated the prospective collection of blood samples. Three variants previously associated with the risk of infections (*IL-6* rs1800795 G>C; *TNF- α* rs1800629 G>A; *TLR4* rs4986790 A>G) were genotyped in the sampled cohort. The allele frequency data generated suggested a possible association between each of these variants and an infection-related cause of death in SUDI. Targeted genotyping of candidate variants revealed several pathogenic mutations, including a twin who was homozygous T/T for a founder mutation, *GALT* rs111033690 C>G/T, causative of galactosaemia (previously undiagnosed). Follow up with the family revealed that the other twin had subsequently demised. Additionally, 43 genes previously associated with cardiac arrhythmias, were sequenced in a subset of cases (n = 19) and parental samples. Putative pathogenic variants were identified in four infants, and four additional novel variants were found. Lastly, using a hypothesis-free approach, clinical exome sequencing was performed on two cases, which suggested one infant was immune-compromised and the second may have had bronchopulmonary dysplasia. The findings in this study highlight possible new candidate

variants to assess in SUDI cases, and has directly contributed to the development of a molecular autopsy which is locally relevant. It is evident that until newborn screening becomes routine and accessible in South Africa, molecular autopsies should include testing for inherited metabolic disorders, as it holds potential to save lives.

Preface

BACKGROUND

Rationale, aim and study design

Motivation

Sudden unexpected death of infants (SUDI) is the rapid and unanticipated death of an infant, less than one year of age. In most countries, these cases are mandated to undergo a medico-legal investigation for cause of death determination [1]. If the cause of death remains unexplained following an exhaustive investigation, including a full autopsy, death scene investigation and clinical history review, the death is classified as ‘sudden infant death syndrome’ (SIDS) [2]. SIDS is the leading cause of death of infants in developed countries [3–7].

The mystery of SIDS has fascinated medical professionals for decades and consequently, has been extensively researched in some countries and regions. In the 1990s, there were two major developments in the field: (i) the discovery that prone sleeping was significantly associated with SIDS [8–12]; and (ii) the revision of the triple-risk hypothesis, which suggested that SIDS was the occurrence of a trigger event in an already predisposed infant who was at a vulnerable and critical stage of their development [13,14]. These findings prompted a surge of research on sleeping and household environments to identify risk factors or trigger events for SUDI and/or SIDS [15–18].

Various awareness campaigns were launched to educate caregivers about safe sleeping practices and other risk factors, which have seen a substantial reduction in the number of SIDS in developed countries [19]. However, assessment of these rates has revealed that the rate of decline has plateaued [20], suggesting that the residual burden of SIDS may be attributed to factors not yet identified. This may include environmental circumstances or possibly genetic factors; the latter has only recently been explored in the context of forensic post-mortem investigations.

A molecular autopsy refers to analysis of the deceased individual's deoxyribonucleic acid (DNA) with the aim of identifying genetic variants which may have contributed to death. While still a relatively new concept in the forensic pathology setting, molecular autopsies have resolved cases which were previously unexplained [21–23]. In some of these investigations, deaths which were diagnosed as SIDS were later resolved using a molecular autopsy, as the genetic variants identified sufficiently explained the death [24–26]. Other studies have found associations with genetic variants and SUDI and/or SIDS, which provides insights into possible compromised pathways in these infants [27–29].

Due to the wide range of possible genetic mutations to test for, it would be extremely time consuming to implement a molecular assay to test for genetic variants one by one, or even a few at a time. Fortunately, with the increased use of 'next generation sequencing', entire panels of genes or even whole exomes (all coding regions of the DNA) can theoretically be analysed in a single assay [22,30]. The costs of these assays are also becoming more affordable with time.

The accurate classification of cause of death is important for reliable reporting and statistical purposes, but moreover, has value for families and public health [25,31]. From the parents' perspective, a specific cause of death provides a reason as to why their infant passed away, which is important for closure and acceptance of the death [31]. Identification of genetic variants in the deceased also has potential health value for blood relatives, who may carry the same genetic variant and be at risk for sudden death themselves. The genetic variant identified in the deceased infant could be screened for in parents, siblings and other family members. Together with a genetic counsellor, the risk of carrying the variant(s) as well as the probability of future infants also carrying the variant(s) could be communicated to the family [32]. Linking to clinical screening (*e.g.* echocardiograms in the case of channelopathies) and health interventions (*e.g.* implanting a defibrillator) could be introduced, with the potential to save lives.

Molecular autopsies have not yet been established in developing countries, including in South Africa. Further, SUDI and SIDS are largely understudied on the continent of Africa. A multicentre retrospective study in South Africa indicated that SIDS comprised up to 24 % of SUDI cases, which is comparable to that reported internationally [33]. To our knowledge, there has been no study in Africa, comprising indigenous African cohorts, which has prospectively investigated the use of a molecular autopsy in the forensic post-mortem setting. Furthermore, before a molecular autopsy is validated and implemented in these regions, it is imperative that the assay is relevant and informative to the local community. Therefore, retrospective and prospective research needs to be carried out to determine the burden of SUDI, understand local risk factors as well as explore what genetic variants should be included in a routine molecular autopsy.

It was hypothesised that a molecular autopsy would be of value to at least some SUDI cases in South Africa; and given population demographics, the molecular autopsy utilised locally would need to consist of genetic variants that are different to those routinely included in developed countries.

Aim and objectives

The aim of this study was to explore the use of molecular autopsies in the medico-legal investigation of SUDI cases in Cape Town, South Africa.

To this end, six objectives were defined:

Objective 1: Retrospectively investigate SUDI cases that were admitted to Salt River Mortuary between 2013 and 2017 to establish the (i) burden of SUDI, (ii) scope of the post-mortem examination leading to the final cause of death outcomes, and (iii) risk factors in the local community.

Objective 2: Perform a systematic literature review to determine (i) the scope of molecular autopsies conducted elsewhere, (ii) what disease groups were tested, and (iii) what resolution was achieved by the performance of a molecular autopsy.

Objective 3: Establish a research collaboration with Salt River Mortuary and an ethical framework to prospectively recruit SUDI cases for forensic genetics research, and then recruit a statistically significant cohort of SUDI cases.

Objective 4: Assessment of the quality and quantity of DNA obtained from two different biological samples (blood and non-invasive buccal cells) from a subset of SUDI cases where informed consent by next of kin was given, and compare these results to those obtained from formalin fixed paraffin embedded tissue (FFPE) from retrospective cases.

Objective 5: Design and optimise molecular assays, and then genotype candidate variants in the DNA obtained from the cohort of SUDI cases recruited from Salt River Mortuary, to (i) assess if the variants should be included in the molecular autopsy, and (ii) determine if any death could be further explained by the genetic analyses.

Objective 6: For SUDI cases which remained ambiguous after post-mortem investigation and genetic screening, follow up with parents and obtain parental DNA samples. This was to perform next generation sequencing on DNA samples from these infants together with their parents to investigate possible genetic causes of death. This also required bioinformatic analysis and assessment of pathogenicity of variants of unknown significance (VUS). Functional studies on VUS were outside the scope of this study.

Study design and setting

These objectives were studied using a quantitative research paradigm and a cross-sectional approach. The study comprised both retrospective and prospective components, as aligned with the objectives. The target population was SUDI cases between one day and one year of age, admitted to Salt River Mortuary, Cape Town. A power calculation indicated that 162 SUDI cases was sufficient (Appendix 1).

Salt River Mortuary forms part of the Forensic Pathology Service (FPS) within the Department of Health, Western Cape Government. The mortuary is classified as an M6 academic facility (the highest rank) which, by definition, means that it investigates more than 2 000 deaths annually. The caseload at Salt River Mortuary, however, is actually 3 500 to 4 000 cases per annum, with an increase in cases each year. In the Western Cape, there are sixteen forensic mortuaries, of which Salt River Mortuary and Tygerberg Mortuary are the only two M6 facilities. Salt River Mortuary is linked with the University of Cape Town, and Tygerberg Mortuary with the University of Stellenbosch; teaching and academic activities take place at these mortuaries as a function of their respective academic institutions.

Organisation of thesis

This thesis has been structured into ten chapters. Chapter 1 introduces the key concepts and critically evaluates international literature pertinent to SUDI and SIDS. It provides clarity on definitions used in the thesis and explores the causes and risk factors for SUDI.

The South African literature is put into the context of the body of international literature in Chapter 2, and a 5-year retrospective analysis of infant death cases admitted to Salt River Mortuary is presented. The demographics, clinical features and risk factors for local SUDI cases were investigated, and compared to national and international studies. This chapter strongly argues for an awareness campaign to be carried out in South Africa to reduce the burden of infant death. A version of this chapter has been submitted to Forensic Science International and is currently under peer-review.

Chapter 3 is a systematic literature review which explored the scope of molecular autopsies conducted elsewhere and what proportions of cases were resolved abroad. This chapter has

been published in the Journal of Pediatric Genetics [34], and thus the manuscript forms part of this thesis.

The foundation of the prospective part of the project is presented in Chapter 4, whereby logistical and ethical considerations surrounding forensic genetic research in a mortuary environment are presented, discussed and debated. Part of this chapter has been published in BMC: Medical Ethics [32], and the manuscript forms a section of this chapter.

In a pilot study, blood and buccal cells were collected from SUDI cases ($n = 52$) where parents gave informed consent. Chapter 5 presents the results pertaining to this phase of the study, where the quantity and quality of DNA obtained from these biological samples were optimised and assessed. DNA results were also compared with those obtained from FFPE tissue from the infants, where this material was available.

The primary cohort of SUDI cases ($n \geq 162$) was subsequently recruited using the guidelines presented in Chapter 4 [32]. A total of 201 SUDI cases were recruited, out of which 169 were appropriate for genetic and statistical analyses. Several candidate genetic variants were then genotyped in the cohort. Chapter 6 describes the investigation of three variants associated with suboptimal immune response, and Chapter 7 describes the investigation of four variants causative of inborn metabolic diseases.

A subset of cases which did not have a specific cause of death ($n = 19$) were investigated further using next generation sequencing, whereby a panel of 43 genes associated with inherited cardiac disorders was analysed. Parental DNA was included in the analysis where available.

This molecular analysis identified a candidate variant in four infants, one of which had parental samples available. This analysis is presented in Chapter 8.

The remaining two ‘trios’ (*i.e.* infant and parents) were prioritised for additional next generation sequencing, whereby the entire ‘clinical exome’ was sequenced and analysed. The clinical exome represents all 4 811 genes known to have variants of functional and clinical importance. These investigations are presented as case reports in Chapter 9.

Finally, Chapter 10 provides a synthesis of the findings, critically evaluates them in the context of international findings and recommends a way forward. Overall, this study strongly argues for the inclusion of a molecular autopsy in selected SUDI cases. Molecular analysis should be prioritised when the deceased is part of a set of identical twins and the second twin is still alive. The findings of this study also advocate for local awareness campaigns about risk factors for SUDI as well as suggests that newborn screening for metabolic disorders should be implemented, in order to reduce the number of infant deaths.

Ethics approval

This study received institutional ethics approval by the Faculty of Health Science Human Research Ethics Committee (HREC: 445/2015) (Appendix 2). It was executed in accordance with the Declaration of Helsinki (1964) as amended most recently at the 4th WMA General Assembly in Fortaleza, Brazil, October 2013.

Chapter 1

INTRODUCTION

Definitions, causes and risk factors of sudden unexpected death in infants

1.1 Sudden unexpected death

When a death is thought to be unnatural, a medico-legal investigation is carried out to determine both the cause and manner of death. According to the World Health Organisation (WHO), cause of death is defined as the disease, trauma or incident which leads to the death or lethal injury, while the manner of death is the category describing the method of death; *i.e.* natural, homicide, suicide, accidental or undetermined [35]. In South Africa, this medico-legal process is governed by the Inquests Act No. 58 of 1959 [1] and the National Health Act No. 61 of 2003 [36].

As the name suggests, a sudden unexpected death (SUD) is a fatality that occurs rapidly and unexpectedly, specifically within 24 hours of the onset of symptoms. In South Africa, SUDs are referred to the FPS for a medico-legal investigation and it is the mandate of the forensic pathologist to determine the cause of death [36]. Although the circumstances around these deaths are initially unknown, the cause of death is established in many of these cases via the post-mortem examination. However, if the cause of death cannot be established after all possible and available investigations, then the manner of death remains ‘undetermined’, as a

matter of exclusion. A retrospective study in the Eastern metropole of Cape Town showed that SUD cases comprised 6.1 % of their caseload, of which 8.8 % remained undetermined [37]. This was more than double the proportion of undetermined SUD cases reported in England (4.1 %) during a similar time period (2002) [38].

1.2 Sudden unexpected death of infants

SUDI is a subset of SUD, but specifically refers to cases where the individual is an infant less than one year old. While SUDI describes the sudden and unexpected *nature* of the death, SIDS is specifically reserved for deaths that remain undetermined after a full investigation [2]. The formal definition of SIDS was first proposed in 1969 [39] and has since been modified to incorporate additional, relevant information [2,40], including the specific age of the infant as less than one year old and the stipulation that the death scene and clinical history must be reviewed [40]. In 2004, the definition was refined as well as stratified into sub-categories to enable appropriate classification of SIDS cases that fell short of meeting the full definition [2]. This 2004 San Diego definition is used in this study:

“SIDS is defined as the sudden unexpected death of an infant < 1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history” [2].

Although the most recent (2004) definition was established more than ten years ago [2], much of the current literature still does not conform to it [41] and often refers to SIDS when actually meaning SUDI under this definition. For example, researchers refer to the “cause(s) of SIDS” [3,42–44], but by its very definition, SIDS is a matter of exclusion; specifically when *no* cause

of death is established. As Williams (2008) summarised, SIDS “is a description of an outcome, a term used to acknowledge the failure of a diagnosis” [45]. It is therefore contradictory to classify deaths with a known cause as SIDS [46]. Establishing causes of SUDI will automatically reduce the number of SIDS, and when all SUDIs can eventually be accounted for, it will eliminate the need for a definition of SIDS altogether [47].

The progression of definitions however, needs to be considered when reviewing literature, and when evaluating any trends associated with SIDS. For example, SIDS may appear to increase or decrease, but it may be the mere result of defining SIDS differently. The current definition at the time of the publication should therefore be kept in mind; and ideally, authors should define SUDI and SIDS upfront in their publications, especially if the article is around a time when definitions are revised. Byard and Marshall (2007) audited 50 publications from 2005 that related to SIDS and found that 58 % of publications did not specify their definition or used a non-standard one. Only 10 % of publications used the most current definition from 2004; 2 % used the original 1969 definition and the remaining 30 % used the intermediate 1989 definition [48]. More recently, there has been a call to abandon the term ‘SIDS’ altogether, and rather refer to undetermined SUDI cases as simply ‘undetermined’ rather than SIDS [49].

As previously identified by many experts in the field, the lack of conformity in terminology and definitions not only leads to confusion among forensic pathologists and scientists, but also makes research difficult to compare, integrate and critically analyse [2,20,41,48,50–52]. This inconsistency is not only the result of revised definitions over time, but also due to the misuse of standard definitions. This might be partly attributed to the complexity of the results from ancillary tests, as well as the scope of full autopsy, death scene investigation and clinical history review, as defined in each country. The thoroughness of these investigations are, in turn,

dependent on the experience of the forensic pathologists, the resources available as well as standard operating procedures which specify what analyses constitute a full post-mortem investigation in that country or setting [53]. For example, South Africa does not have a standard operating procedure for the investigation of SUDI cases and some medico-legal facilities in the country do not have access to full body X-ray machines or microbiology testing due to limited resources [33]. Further, while a metabolic screen is one of the required tests prior to diagnosing SIDS [2], it is unclear what constitutes a metabolic screen and if these tests are available in low and middle income countries. These sorts of variables contribute to inconsistencies in terminology use as well as the difficulties in interpretation of the literature.

1.3 Understanding the causes and risk factors of SUDI

1.3.1 Causes of SUDI

To elucidate causes of death underpinning SUDI, retrospective analyses of post-mortem records have been carried out; revealing inborn errors of metabolism [54], cardiac abnormalities [55], infections [42,56,57], alterations in neurological regulation pathways [58] and defective mitochondrial function [29]. A retrospective review of SUDI in Canada, between 1987 and 1996, showed that out of 623 SUDI cases, 7.1 % of the deaths were infection-related, 2.7 % were due to cardiovascular abnormalities, 2.6 % resulted from child abuse or negligence and 2.1 % were linked to metabolic or genetic disorders [18]. Infection-related deaths, metabolic disorders and cardiovascular abnormalities will be discussed in detail in Chapters 6, 7 and 8, respectively. However, the majority of SUDI cases (80 %) were diagnosed as SIDS [18]. This finding in Canada is supported by numerous studies [18,20,59], making SIDS the leading cause of infant death in most developed countries.

1.3.2 Triple-risk models

Due to the high rates of SUDI cases without a known cause (including SIDS and undetermined cases which fall short of the standard definition), research has focused on identifying risk factors for sudden infant death. Risk models for SIDS were loosely proposed by Wedgewood in 1972 [60], as well as Rognum and Saugstad in 1993 [61], and were more formally refined by Filiano and Kinney in 1994 [13]. They attempted to collate the risks for infant death into a single model to describe the situation when SIDS is most likely to occur. The 1994 triple-risk model for SIDS proposed the simultaneous occurrence of the following three aspects: (i) a vulnerable infant, (ii) a critical stage of development in homeostatic control, and (iii) a trigger event or stressor. [13]

Filiano, Kinney and colleagues suggested that neuropathies and brain abnormalities, such as prenatal brainstem injury or lesions in the brain, contribute to SIDS by making an infant vulnerable [13,62–68]. Factors that could contribute to the ‘vulnerable infant’ include maternal behaviour such as maternal smoking or drug use, and epidemiological factors such as prematurity and low birth weight. According to Filiano and Kinney (1994), these would yield a suboptimal intrauterine environment, and consequently promote abnormal brain development in some infants [13]. The abnormal brain was proposed to be insufficient in supporting an infant’s homeostatic control, but the specific vulnerability would remain silent unless a corresponding stressor was applied to the infant [13]. For example, an external stressor could be an extra blanket on a sleeping infant, and if the infant could not regulate its own temperature due to an impaired brainstem (vulnerability), then the infant would demise suddenly and unexpectedly.

The critical stage of development in the triple-risk model refers to the period where major physiological changes occur in infants, such as adapting to their own homeostasis control (*e.g.* breathing, temperature regulation and autonomic control), which requires regulation by the brainstem [13]. This stage is between one and six months of age, and specifically between two and four months. This age range mirrors the most common ages of SUDI cases.

Guntheroth and Spiers (2002) argued that the proposed critical period of infant development is not between one and six months, but rather during the neonatal stage [68]. During the first month of life, neonates experience instability and many demise from congenital defects during this period. It is consistent then, that if damage to the infant's brainstem occurred during pregnancy, their death is more likely to occur in the extremely sensitive neonatal period, rather than when they are between one and six months old [68].

1.3.3 Pathophysiological factors during sleep

A common finding across SUDI cases is that the majority of events occur during the infant's sleep [2,69–73]. Hence, to better understand the circumstances leading up to death, numerous 'sleep studies' have been conducted with the use of monitors. Such monitors can record physiological functions such as breathing and heart beat and are often placed on infants who had a previous apparent life-threatening event. Some infants who were monitored, unfortunately later died suddenly and unexpectedly during infancy, while the majority continued as normal. Evaluation of such recordings has provided valuable insight into the physiological changes and possible mechanisms that are present in infants who later are victims of SUDI [74–77].

In a series of sleep studies conducted in laboratories, Kahn *et al.* (2004) observed a cohort of infants, some of who later demised suddenly and unexpectedly and were diagnosed as SIDS [74]. Infants who subsequently demised had significantly more and longer obstructed mixed apnoea than those experienced by matched controls. In addition, the control infants moved more during sleep, woke up more frequently and spent more time in rapid eye movement (REM) sleep compared to infants who subsequently demised [74]. The researchers observed that impaired breathing of infants who later experienced SUDI, occurred in parallel to decreased heart rate during REM sleep [78]. This suggested that there may be a deficiency in cardiorespiratory control during sleep. Further, the lack of arousal potential of a sleeping infant could play a role in the cascade pathway ultimately preventing autoresuscitation and thus leading to death. Risk factors such as maternal smoking and the infant's sleeping environment (including their sleeping position) could contribute to decreased arousal status of an infant [74,78].

Sridhar *et al.* (2003) observed that healthy infants less than one year old who gasp during sleep is fairly common and can lead to partial or complete autoresuscitation; perhaps failure of this autonomic gasping may have resulted in death [75]. However, this was not the case, as the SUDI cases exhibited significantly more double and triple gasps than control infants, and thus it was not due to a lack of gasping that infants subsequently died [75]. Rather, such infants may have had a suboptimal autoresuscitation mechanism; for example, infants began gasping too late to revive their normal physiological functions [76].

A review article by Poets (2004), showed that prolonged apnoea was actually infrequent, but hypoxaemia (very low amounts of oxygen in the blood) was more common in physiological recordings of sleeping infants who later experienced SUDI; however, the mechanism leading

to this was unclear [77]. For example, death could have resulted from airway obstruction or rebreathing expired air. Furthermore, although gasping was observed in the majority of recordings from these infants, there was a consistent failure of arousal and autoresuscitation [77]. Intentional suffocation, however, was reported to be difficult to identify during autopsy, but was estimated to account for less than 5 % of all SUDI cases [47,79]. In some instances, accidental asphyxia might be established from death scene investigation, particularly with the use of a re-enactment of the events leading up to the death, with the use of a doll [80–82].

Randall *et al.* (2009) investigated the use of a different classification system for sudden infant death in Cape Town (n = 10), where the cause of death indicated a possible relation to asphyxia [83]. This accounted for cases where the cause of death was not SIDS, nor was it known, but rather it was unclassified and possibly related to asphyxia. The application of this classification system could potentially change the way cases were previously classified. The conventional system classified the cases as SIDS (n = 6) and as known causes (n = 4), whereas the new system would classify the cases as SIDS (n = 2), known causes (n = 4) and unclassified but related to asphyxia (n = 4). As demonstrated in their pilot study, this approach would allow further investigation into apparent asphyxia-related causes of death as well as highlight the possible need for relevant molecular testing associated with brainstem abnormalities [83].

1.3.4 Risk factors

Research into the circumstances during sleep has been extensively studied to elucidate risk factors pertaining to sleeping and household environment [17,72]. As alluded to previously, one of the major findings in this regard was that the prone sleeping position is reproducibly and significantly associated with SIDS [8,17,72,84–86]. In the prone position, the trachea is underneath the oesophagus which increases the chance of breathing in something other than

fresh air, resulting in airway obstruction. Sleeping prone also decreases the mobility of the head, making it more difficult for the infant to lift and move their head to get fresh air. This is especially the case if the infant's face becomes covered with bedding or a toy, and also leads to a greater chance that the infant will breathe in expired air and develop a hypoxic coma. Lastly, suboptimal control of autonomic functions (such as cardiac control and regulation of body temperature) is also associated with the prone sleeping position [17,72].

It follows that risk factors associated with the sleeping environment include elements which could obstruct the infant's breathing, such as soft bedding, soft toys [87,88] as well as excess clothing and/or blankets which could contribute to decreased heat loss and hyperthermia [84,89]. Co-sleeping of an adult next to the infant could also result in overheating of the infant, or obstruction of the infant's breathing if their face is turned towards the adult [90,91]. A retrospective study of SUDI cases in Northern Ireland between 1996 and 2001 showed that co-sleeping was reported in two thirds of all cases ($n = 30/45$). The authors suggested that co-sleeping might not increase the risk for infant death directly, but the families who do co-sleep with their infant may also possess other behaviour that is associated with SUDI, such as smoking [90].

Exposure to tobacco smoke is a major risk factor for SUDI, as prenatal exposure results in an infant with decreased lung capacity, decreased heart rate variability and decreased arousal in response to hypoxia [92–94]. Such suboptimal functionality could be fatal in an event of stress, especially while the infant's autonomic control has not fully developed [13]. The risk for SUDI also increases with prematurity and low birth weight [95,96]. Such infants are often placed in the prone sleeping position in the intensive care unit, where they have been observed to sleep for longer periods in this position [97]. During these longer periods of sleep, the infant was less

aroused and more central apnoea was observed [98,99], all of which were previously associated with the SUDI sleep studies [74].

Increased SUDI rates have also been associated with certain demographic factors, including age, sex and population group. Male infants between the age of two and four months are most at risk for SUDI, with a ratio of males to females at approximately 3:2 [3]. African Americans, Aboriginal Australians and Maoris are at two to six times higher risk compared to other citizens of the United States of America (USA), Australia and New Zealand, respectively [4,100]. This has been attributed to the cultural behaviour of certain ethnic groups, which includes co-sleeping [101,102] and maternal smoking [103]. There is also a higher prevalence of SUDI among lower socio-economic groups, which could possibly be attributed to their limited access to education about risk factors for SUDI [104]. These and other risk factors have been extensively reviewed by several authors [74,91,105–112]. However, there is stark gap in the literature pertaining to SUDI and associated risk factors in the South African setting.

1.4 Conclusion

A considerable amount of research has been done internationally to better understand SUDI and the causes thereof. SIDS is the leading cause of infant death in developed countries and associated risk factors have been identified. While SIDS is considered a natural manner of death, and thus removes blame from caregivers [16], it would be desirable for more specific causes of death to be identified. Due to the minimal use of genetic testing in a post-mortem forensic setting historically, the statistics pertaining to causes of SUDI reported in this chapter may be an underestimation of the true genetic contribution underlying these deaths.

The recognition and understanding of risk factors for SIDS have resulted in campaigns to create awareness pertaining to safe sleeping environments [19], which will be discussed in Chapter 2. The implementation of these campaigns has seen a reduction in SIDS rates, yet the burden of such cases remains [5]. One hypothesis is that genetic variants may contribute towards this residual burden, and evidence in support of this hypothesis is being steadily generated abroad [21–23]. Even in cases where cause of death is known, some involve complex physiological pathways, which are controlled by genes. Genetic variants that underlie suboptimal physiological mechanisms may contribute to the primary causes of SUDI or infant vulnerability [27,113–115]. However, the lack of research in South Africa hinders our ability to fully understand the burden, causes, risk factors and potential genetic contributions pertinent in our local communities.

Chapter 2

RETROSPECTIVE STUDY

A 5-year retrospective analysis of infant death at Salt River Mortuary (2013 – 2017): the need for a local education campaign about risk factors

2.1. Introduction

2.1.1 Background

Infant death is a global concern and much research has been undertaken to better understand the causes and risk factors thereof [18,27,70,116]. The infant mortality rate (IMR) has been recognised as an appropriate indicator to measure population health, as it reflects factors which affect the population at large [117]. The IMR in South Africa is high at an estimated 31 deaths per 1 000 live births in 2017. However, this represents an improvement since it is lower compared to 1990 when 47 deaths per 1 000 live births was recorded [118]. Comparatively, in 2017, the IMR ranged from 1.8 in Monaco (Europe) to 110.6 in Afghanistan (Asia) (publicly accessible: <https://www.cia.gov/library/publications/the-world-factbook/fields/2091.html>; accessed 31/08/2018).

The ‘Millennium Development Goals’ (MDG) were established following the Millennium Summit of the United Nations in 2000, and target 4A aimed to reduce the under-five (years of

age) mortality rate by two thirds, between 1990 and 2015 (<http://www.un.org/millenniumgoals/>; accessed 9 June 2015). The MDG statistics reported that despite a global reduction in child deaths, 80 % of deaths of children below five years of age occur in sub-Saharan Africa and Southern Asia. Further, the proportion of child death in the first month of life was increasing (<http://www.un.org/millenniumgoals/>; accessed 9 June 2015).

In response to the MDG, several projects were established in the Western Cape, and one of these was the local mortality surveillance system, implemented in 2007 [119]. The surveillance system reported that 2 412 children below five years of age died in the Western Cape in 2011, of which 1 109 were less than one year of age. In these children, lower respiratory tract infection (LRTI) was found to be the leading cause of death (34.8 %), only after mortuary data was linked to the information on the death notification form [119]. Before the linking of mortuary data, the leading cause of death was ‘ill-defined’ (58.7 % of cases), highlighting the importance of collating mortuary data to improve vital statistics [119,120]. Other causes of death were diarrhoea (11.6 %), road injuries (5.6 %), accidental threats to breathing (3.8 %), fires (3.7 %), congenital heart disease (3.4 %), drowning (2.8 %), meningitis (2.6 %), prematurity (2.2 %) and protein energy malnutrition (1.9 %) [119].

Another response to reduce childhood mortality was the Child Death Review project, which was initially piloted in Cape Town (Western Cape Province) and Durban (KwaZulu Natal Province) from 1 January 2014, and has now been implemented across the Western Cape and in some areas in Kwa-Zulu Natal. This initiative entails the monthly meeting of a multi-disciplinary team of experts to review all child deaths (under the age of 18 years) with the aim of expediting medico-legal investigations, potential criminal investigations, legal consequences and social responses to families in need. A review of the 2014 data showed that

LRTI accounted for most infant deaths in parts of Cape Town and Durban, and approximately 44.3 % of the infant deaths caused by LRTI in these regions were associated with prematurity [121].

2.1.2 International trends

These results are in contrast to developed countries, where SIDS is deemed the leading cause of infant death and has a reported incidence of approximately 0.33 per 1 000 live births [5,59]. In response to research which showed a significant association between SIDS and sleeping environment, many countries introduced awareness campaigns in the 1990s in an effort to educate mothers about the risk factors of infant death (*e.g.* the ‘Back to Sleep’ campaign) [19]. These countries have seen a significant decline in SIDS, which has been attributed to these campaigns [5]. The rate of SIDS in the USA has decreased from 1.20 per 1 000 live births in 1992 to 0.54 per 1 000 live births in 2005 [122]. However, although absolute numbers decreased, approximately half of SUDI deaths in the USA in 2006 were still attributed to SIDS [122].

Hauck and Tanabe (2008) reviewed the international SIDS trends between 1990 and 2005 [20]. Thirteen countries were included in the study, all of which introduced an awareness campaign within this period. An overall reduction in the number of SIDS was observed in all countries, with more than a 50 % decrease in most countries, and the largest decline occurring within the first few years of launching the awareness campaign. However, many of these observed declines had reached a plateau by 2005, which suggested that there may be other factors which largely affect infant death, and further action is required to continue to decrease infant death [5,20].

These declining and plateauing trends were noted at an international meeting in 2003 where the latest consensus regarding the definition of SIDS was reached [123]. Experts observed that although SIDS rates had decreased substantially around 1990, the total number of SUDI had remained similar, and the relative proportion of explained infant death cases had increased [123]. Therefore, while it appeared that SIDS decreased, infant death was still occurring, but their causes of deaths were being classified differently.

Although there are published guidelines with regards to classifying ‘SIDS’ which fall short of the full definition [2], the term “unascertained” has also been used to classify such cases [52]. This carries its own disadvantages, as there is no formal definition of “unascertained” in this context. Sheehan *et al.* (2005) showed that if SIDS cases in the Republic of Ireland were to be hypothetically reclassified with the introduction of “unascertained” SUDI, then SIDS rates would decrease from 0.71 to 0.08 deaths per 1 000 live births during the period 1994 to 2000 [52]. Fleming and Blair’s (2005) commentary on the Sheehan *et al.* (2005) article provides further evidence of this trend, where SIDS rates have decreased by 0.35 deaths per 1 000 live births in the United Kingdom (UK), while “unascertained” SUDI has increased five times to 0.2 deaths per 1 000 live births during a similar period, 1996 - 2003 [53]. These articles have raised concerns regarding the reliability of reported SIDS rates internationally, and again calls for a standardisation of definitions relating to sub-SUDI classifications.

2.1.3 SUDI in South Africa

A similar situation regarding the classification of SIDS in the South African forensic mortuaries has also been observed. A 5-year retrospective analysis of SUDI cases from Tygerberg Mortuary and Pretoria Mortuary (in the capital city of South Africa) between 2000 and 2004 showed that 161 out of 413 (40.25 %) SUDI cases were diagnosed as SIDS in Tygerberg,

compared to 10 out of 99 (9.17 %) in Pretoria [124]. This was observed in another retrospective study, which spanned the years 2005 to 2009, and analysed SUDI data from five of the seven major M6 forensic mortuaries in the country. The latter study showed the following numbers of SUDI cases and SIDS classifications: (i) Pretoria: 24 SIDS out of 213 SUDI (11.3 %); (ii) Johannesburg (Johannesburg and Sebokeng mortuaries): 0 SIDS out of 228 SUDI (0 %); (iii) Durban (Gale Street, Pinetown and Phoenix mortuaries): 4 SIDS out of 683 SUDI (0.6 %); (iv) Bloemfontein: 7 SIDS out of 128 SUDI (5.5 %); and (v) the eastern metropole of Cape Town (Tygerberg mortuary): 189 SIDS out of 1271 SUDI (14.9 %) [33].

The reasons for the large difference in SIDS diagnoses was attributed, among other factors, to the lack of a uniform, national investigation protocol [33,124]. These retrospective studies also highlighted that death scene investigations were largely neglected in South Africa. In this regard, a local study by Dempers *et al.* (2016) demonstrated the value of death scene investigation in reducing the number of cases which would have otherwise been classified as SIDS, and that this aspect should be incorporated into a standard operating procedure [81].

Besides the published data from the Child Death Review study from 2014 [121], there is very little representation of infant death at Salt River Mortuary in the literature. In other parts of Cape Town, SIDS rates were reported to be some of the highest in the world [81]; however, it is unclear if this also applies to the areas of Cape Town which Salt River Mortuary serves. It is imperative that the causes and risk factors of infant death are captured accurately such that locally relevant interventions may be designed and implemented. As such, the aim of this chapter was to retrospectively review infant deaths investigated at Salt River Mortuary in order to better understand forensic investigation procedures, circumstances at death, demographics and risk factors in the immediate local community of the City of Cape Town.

2.2. Methodology

Cases investigated consisted of all infants who were admitted to Salt River Mortuary for forensic investigation between 1 January 2013 and 31 December 2017. These deaths would include all those thought to be due to unnatural causes, as defined in the National Health Act No 61 of 2003 [36]. The internal Office Autopsy Database (Ethics approval HREC: R036/2014) was filtered for cases whereby the individual was less than or equal to one year old at the time of death. The unique case number of each case was then used to retrieve the medico-legal case folder from the archives in order to collect data pertaining to each case. After the data was collected, the case number was removed and a unique study number was assigned in order to preserve confidentiality.

For each case, data pertaining to demographics, environment and the death scene, circumstances surrounding death, risk factors, post-mortem investigations and cause of death were collected. For most cases, particularly those admitted as ‘sudden and unexpected’, additional documentation was available; this included the infant’s clinic card, which is locally known as the Road to Health card (Appendix 3), and which records vaccines, milestones and clinical consults from birth; as well as a comprehensive questionnaire for SUDI cases, the FPS006(b) (Appendix 4), which was specifically designed in accordance with international guidelines (publicly accessible: <https://www.cdc.gov/sids/pdf/suidi-reporting-form-508.pdf>; accessed 23/02/2018). The FPS006(b) form also documents information about the scene, and if the forensic pathology officer (FPO) was called to the scene, contemporaneous notes of the death scene are taken. These documents were used to assist with the data collection above, as well as to review clinical history and social circumstances of infants.

At Salt River Mortuary, ancillary tests mainly included histopathology, toxicology and microbiology, and to a lesser extent, virology and biochemistry. External autopsies included a full body low dose X-ray (Lodox) scan using the Lodox[®] Xmplar-dr (Lodox Systems, Johannesburg). Autopsies of infant cases were classified as either full, partial or external according to the following criteria:

- (i) full: external and internal examination with ancillary investigations (*i.e.* ancillary investigations which were necessary to determine cause of death. However, in the case of a full SIDS diagnosis, all ancillary investigations had to be performed [2].)
- (ii) partial: external examination with partial internal examination or minimally invasive ancillary tests, or
- (iii) external examinations only.

Data were collected into a Microsoft Excel 2013 spreadsheet and a subset was checked by an independent researcher as a quality measure. Given the nature of retrospective studies, missing data were inevitable, and this was taken into account during statistical calculations. While the number of SUDI cases reported and the demographics thereof were comprehensive (*i.e.* no data were missing), the missing data pertaining to risk factors was a limitation. Graphs were generated using GraphPad Prism v.6 or Microsoft Excel 2013 and statistics were performed using the respective built-in statistical packages. Descriptive statistics were used to summarise the data. The Chi-squared test was used to test for significant differences between categorical variables, where a p-value of less than 0.05 was considered significant.

2.3 Results

2.3.1 Overview: all infants

Over the 5-year period (2013 - 2017), there was a total of 17 730 cases admitted to Salt River Mortuary, of which 1 608 (9.07 %) were infants less than 1 year old. There were between 280

and 344 infant deaths per year (median: 321). Of the 1 608 total infant deaths, 1 199 (74.56 %) were admitted for medico-legal investigation under the category of ‘sudden unexpected death’ [36], thus making SUDI cases 6.76 % of the total caseload. The remaining 25.54 % of infant cases were admitted for investigation under the following circumstances: (i) act of omission or co-mission: abandoned neonates (n = 302; 18.78 %); (ii) application of external force: alleged homicide or accidents (n = 46; 3.87 %); and (iii) procedure-related deaths (n = 61; 3.79 %). The ‘abandoned neonate’ admissions have been reviewed separately as an ancillary study [125].

The causes of death of all infants between 1 day and 1 year old, following post-mortem investigation, were categorised as natural causes (64.18 %), unnatural causes (27.30 %) and under investigation (8.52 %). There were significantly more natural infant deaths compared to those which were unnatural or under investigation ($p < 0.0001$). Overall unnatural death increased, cases under investigation decreased, and overall, infant death decreased (Figure 2.1).

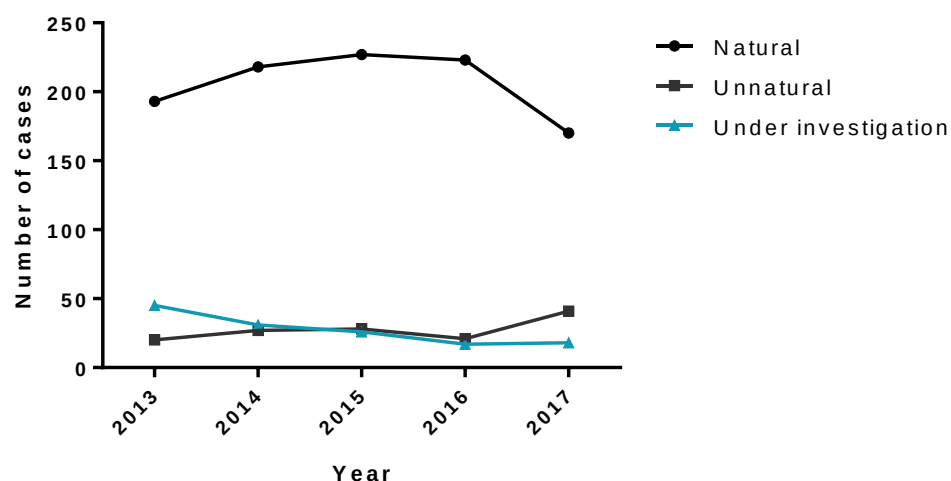


Figure 2.1: Cause of death categories of all infant cases (1 day – 1 year old) admitted to Salt River Mortuary between 1 January 2013 and 31 December 2017.

Infants had significantly more natural causes of death than unnatural or ‘under investigation’. The number of unnatural deaths increased over time, while the number of cases under investigation decreased.

2.3.2 SUDI cases

2.3.2.1 Demographics

Out of the 1 199 cases which were admitted as SUDI, there were slightly more males (51.71 %) than females (48.29 %). The majority of SUDI cases were indigenous Black African infants (57.88 %), followed by infants from the Mixed Ancestry (41.53 %) and European ancestry (0.58%) population groups. No infant of Asian ancestry group was identified. Most infants demised younger than 4 months of age (73.56 %), with a peak of deaths at 1 to 2 months of age (Figure 2.2). Significantly more males demised in this age range compared to females ($p = 0.046$).

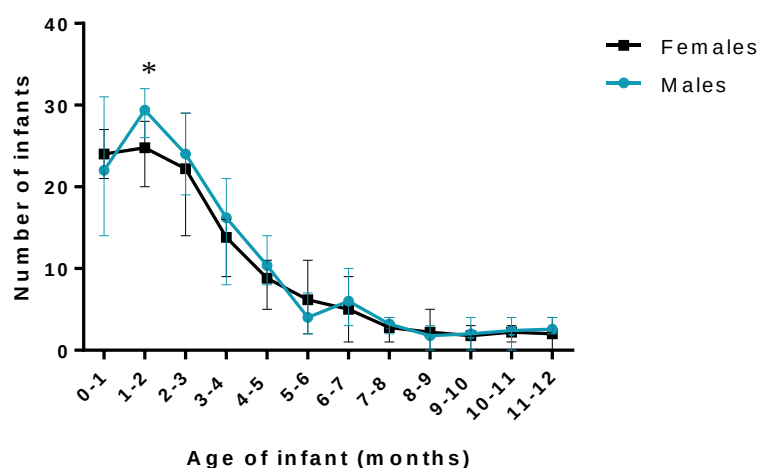


Figure 2.2: Distribution of SUDI cases admitted to Salt River Mortuary between 1 January 2013 and 31 December 2017 according to age at death.

The majority of infants demised younger than 4 months old, with a peak of cases at 1 - 2 months of age. Significantly more males than females at age 1 - 2 months demised.

Approximately half of the SUDI cases occurred on weekends, including the early hours of Monday morning (Saturday – Monday: 50.21 %) (Figure 2.3A). There was a peak in SUDI cases during winter months (June – August), with June being the month with the highest percentage of admissions (13.09 %). August was the only month where there were significantly

more male SUDI cases (than female) admitted to Salt River Mortuary ($p = 0.0479$) (Figure 2.3B).

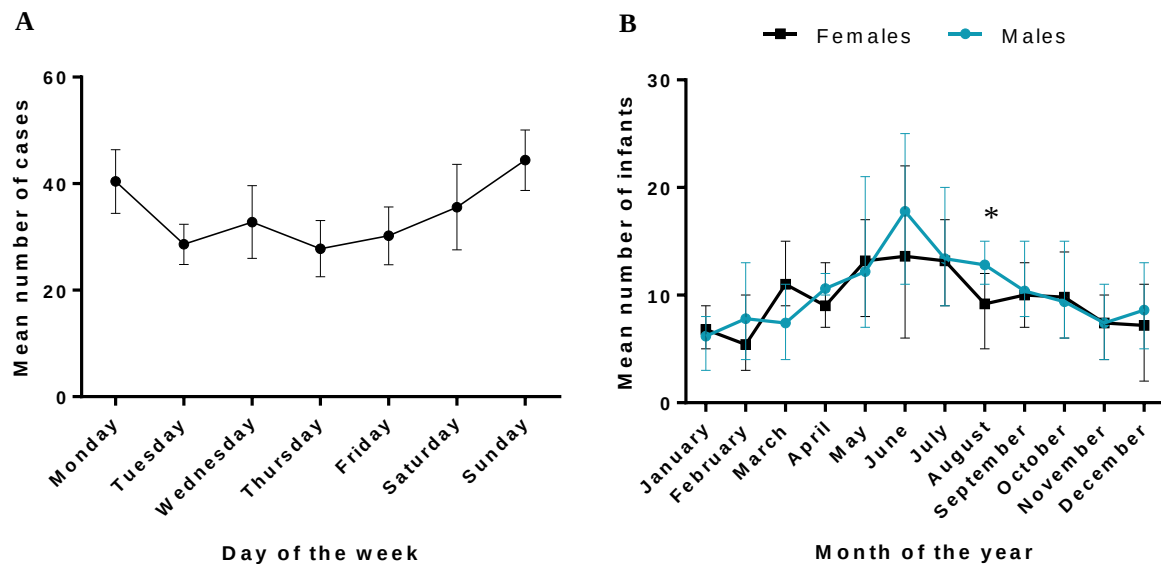


Figure 2.3: Characteristics of the timing of SUDI cases in terms of (A) day of the week and (B) month of the year. Approximately half of the SUDI cases were reported on Saturday to Monday, and winter months saw the highest numbers of SUDI cases. Considering the time period of 1 January 2013 – 31 December 2017, significantly more male cases than female cases were admitted in August.

The area where the highest percentage of SUDI cases was reported was Nyanga (19.02 %), followed by Mitchells Plain (11.01 %) and Gugulethu (10.26 %). Although these are established suburbs, they contain significant tracts of informal settlements, have a high density of residents and are generally classified as ‘low-income’ areas. The heat map below depicts the approximate locations and densities of SUDI reports (Figure 2.4).

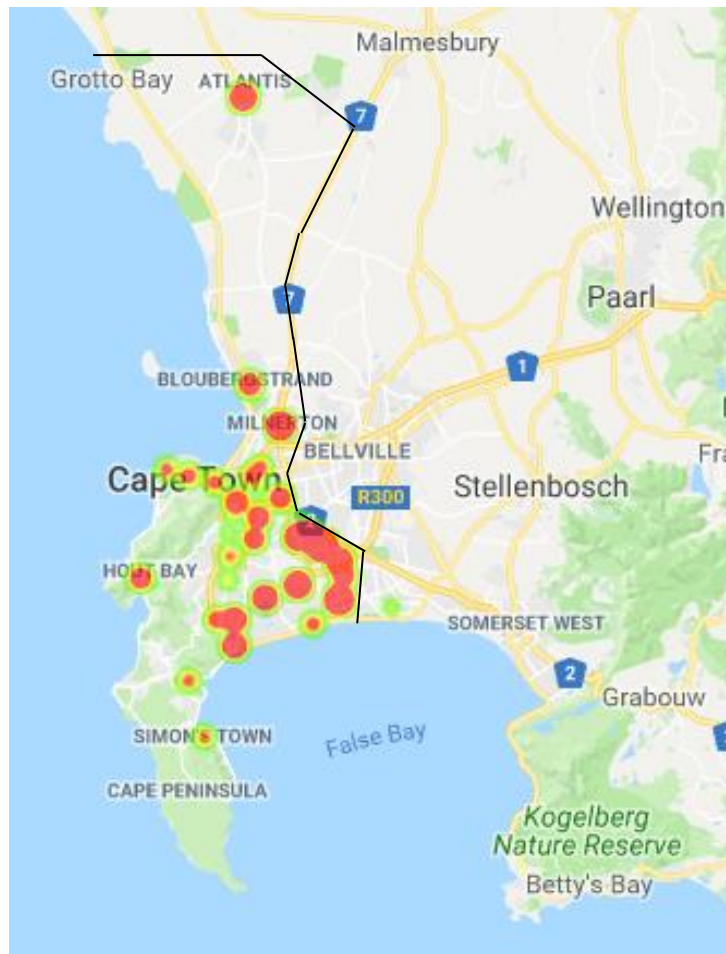


Figure 2.4: Heat intensity map representing the areas where SUDI cases were reported between 1 January 2013 and 31 December 2017, where red and green indicate the most and less dense areas respectively.

The majority of SUDI cases were reported in the Cape Flats region, which is a low-income and socially poor area. The black line indicates the approximate border of the drainage area of Salt River Mortuary.

2.3.2.2 Post-mortem factors

The majority of SUDI cases (78.83 %) were autopsied within four days of death (mode: 3 days), with only 4 % of cases waiting more than seven days (Figure 2.5). A trend in more recent years reflected an increasing delay between death and autopsy, however, this was not statistically significant ($p = 0.7133$).

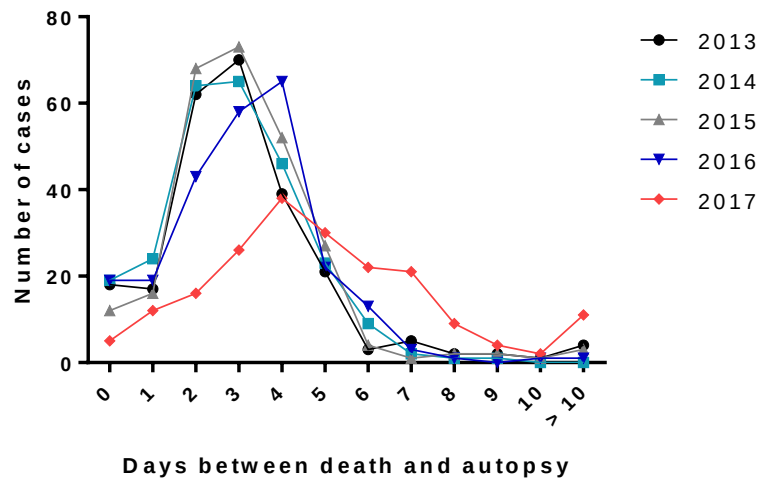


Figure 2.5: Distribution of SUDI cases according to the number of days between death and autopsy.

The majority of cases (78.83 %) were investigated within four days of death.

The scope of autopsy for SUDI cases is portrayed in Figure 2.6, whereby infants received full (19.32 %), partial (15.84 %) and external (64.84 %) examinations. Histology was the most commonly performed ancillary investigation (30.39 %), followed by microbiology (21.77 %) and toxicology (11.72 %) analyses. A full body Lodox[®] scan was performed in 94.91 % of SUDI cases and was not performed only when the Lodox[®] facility was not working.

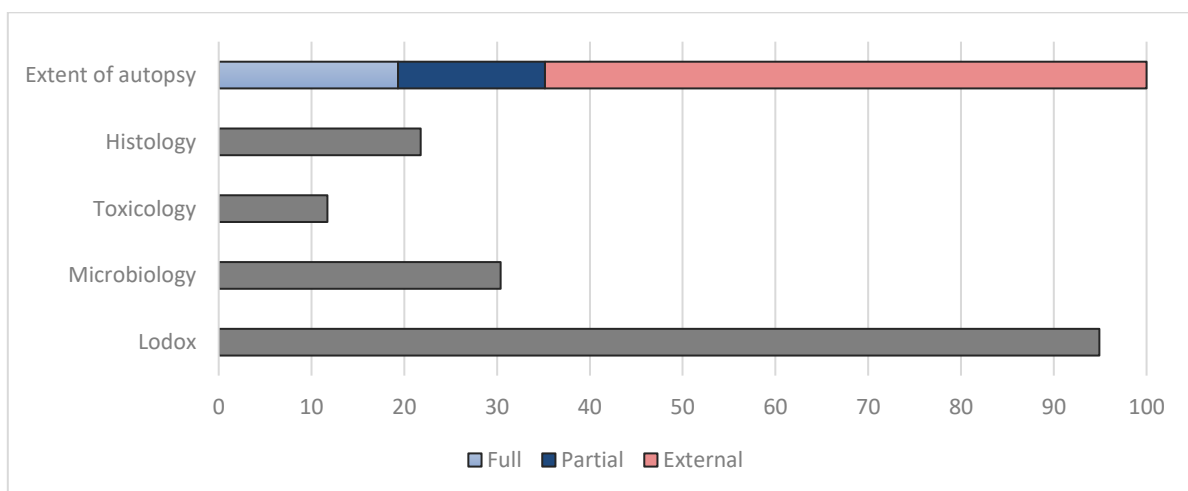


Figure 2.6: Extent of autopsy and ancillary investigations.

Autopsies were either full (19.32 %), partial (15.84 %) or external (64.84 %). Ancillary investigations comprised histology, toxicology and microbiology. A Lodox[®] scan was performed in 94.91 % of cases.

Following post-mortem examination, SUDI admissions were found to have either natural (86 %) or unnatural (3 %) causes of death, while the remaining 11 % of cases were still under investigation at the end of data collection for the present study (31/01/2018) (Figure 2.7). Natural causes of death were mostly respiratory-infection related, followed by gastroenteritis/dehydration and unspecified natural causes of death (Figure 2.7). Unnatural deaths were due to asphyxia (30 % of unnatural SUDI cases), aspiration (60 % of unnatural SUDI cases) and other causes (blunt force trauma, hypothermia, head injury) (together 10 % of unnatural SUDI cases).

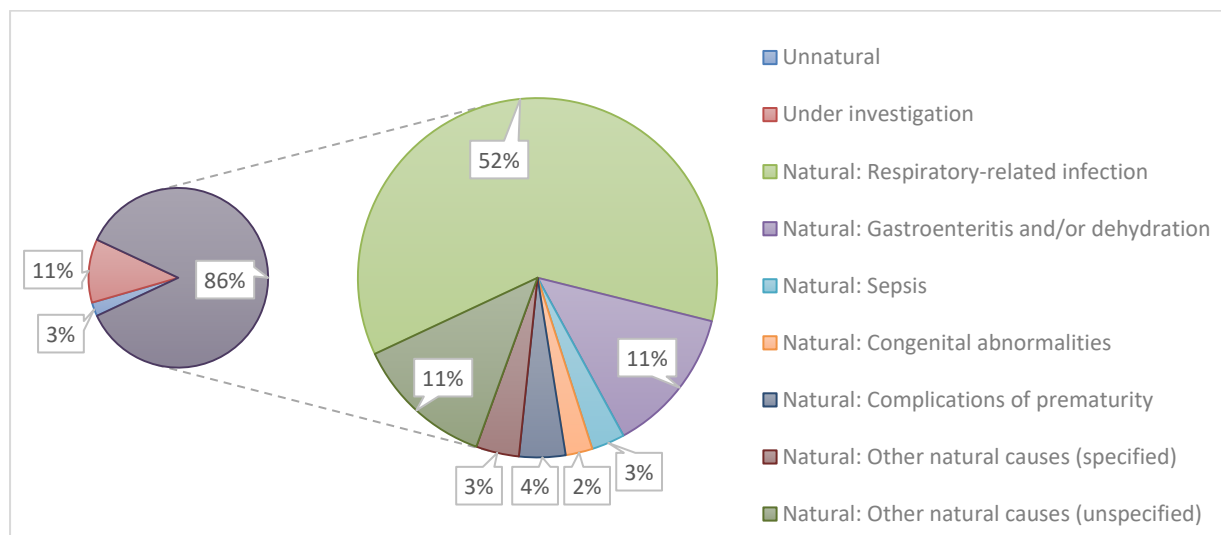


Figure 2.7: Causes of death of all SUDI admissions (n = 1 199) to Salt River Mortuary between 1 January 2013 and 31 December 2017.

The majority of cases had natural causes of death (86 %), of which the leading cause of death was respiratory-related infection. 3 % of cases had unnatural death following post-mortem investigation, while 11 % of cases were still under investigation.

There were a total of 122 (11 %) SUDI cases which remained under investigation, of which 110 had information available pertaining to the scope of post-mortem investigation (Figure 2.8). In addition to the external and internal autopsy, a total of 22 cases (20 %) had all three ancillary investigations requested, whereas the others only had two (n = 34; 31 %), one (n =

53; 48 %) or zero (n = 1; 1 %) ancillary investigations requested. There were 17 cases where no information of this nature was available at the time of this study.

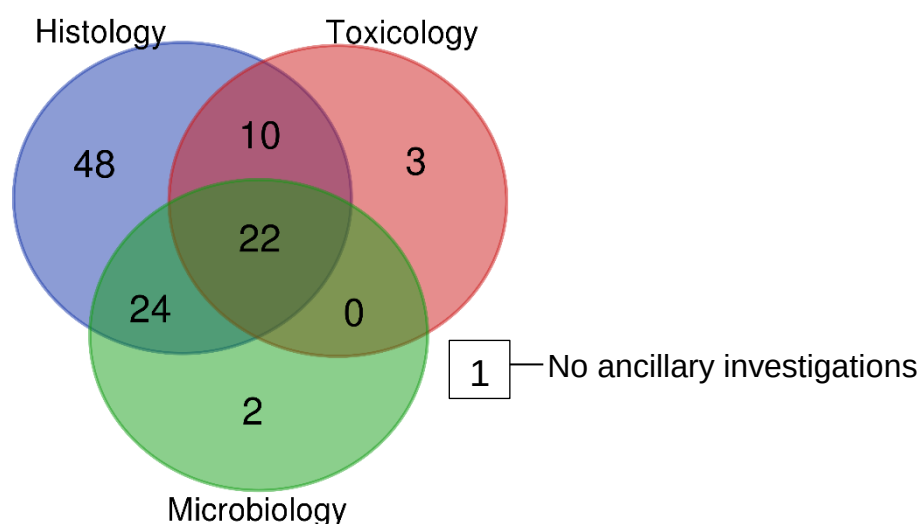


Figure 2.8: Venn diagram showing the scope of ancillary investigations performed in SUDI cases which are still under investigation.

Of the cases still under investigations, a total of 22 cases had all three ancillary investigations performed, whereas in one case, no ancillary investigations were performed.

2.3.2.3 Risk factors

Numerous risk factors for SUDI were identified in the documentation available and these are summarised in Figure 2.9. The majority of infants (94.65 %) co-slept in an adult bed, with at least one, but up to ten, other individuals (mean: 1.77 adults). For infection-related deaths, co-sleeping was significantly associated with the side sleeping position ($p < 0.001$).

In 53.25 % of SUDI cases, symptoms of illness were noted prior to death, yet mothers seldom took their infants to the clinic. Approximately one third (34.90 %) of infants were HIV-exposed while almost one quarter (24.32 %) of infants had vaccines outstanding. A total of 40.55 % of infants were born premature. While most infants were breastfed (51.82 %), significantly more

infants who demised from gastroenteritis were bottle-fed compared to infants who demised from other causes ($p < 0.001$).

More than half of the infants in this study (53.11 %) were exposed to tobacco smoke; mostly from their mothers (68.88 %) or from another family member in the household (32.12 %). Most mothers were unemployed (80.22 %) and single (75.43 %), while 12.83 % of mothers had a previous SUDI. Whilst pregnant, 29.10 % of mothers missed antenatal care, 19.78 % drank alcohol and 9.13 % took drugs.

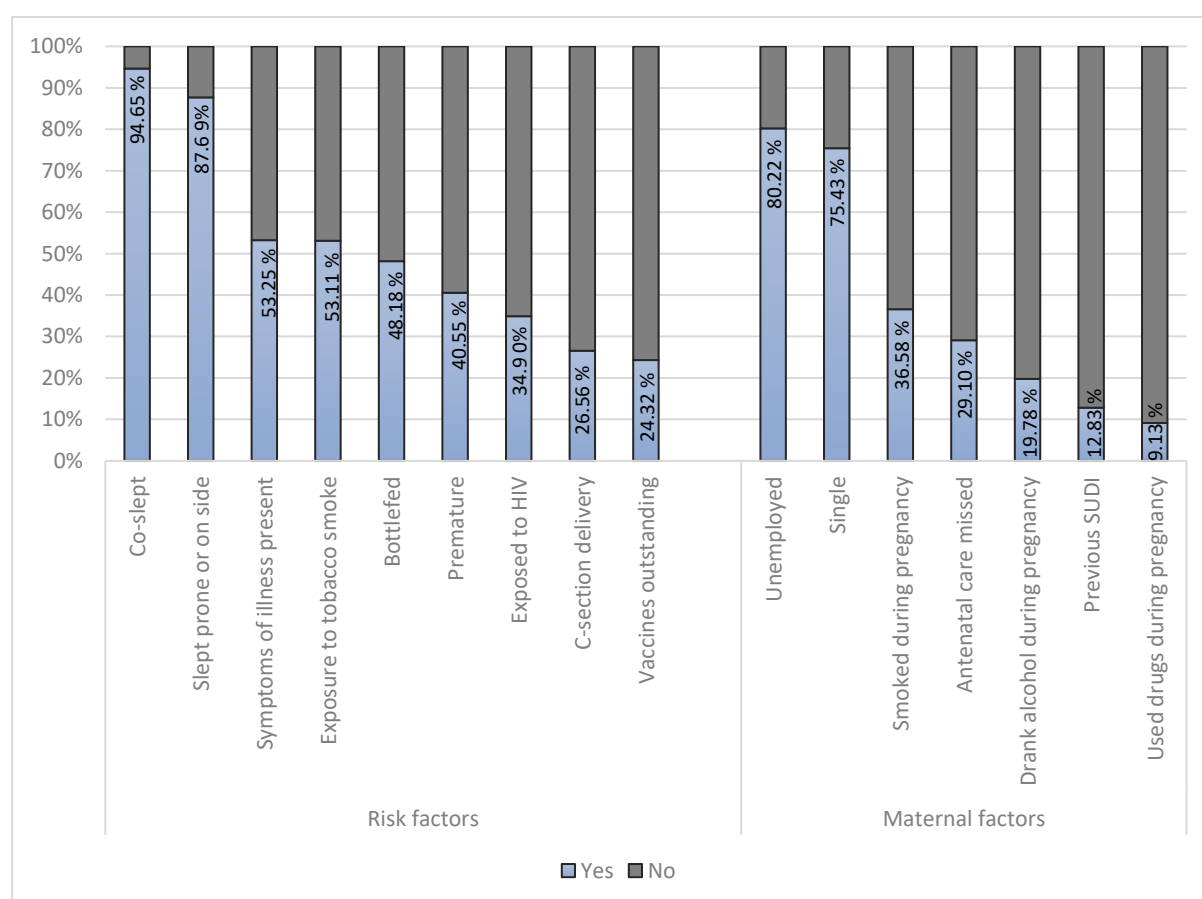


Figure 2.9: Percentage of SUDI cases with identified risk factors.

More than half of the infants co-slept, did not sleep in the supine position, had symptoms of illness prior to death and were exposed to tobacco smoke. More than three quarters of mothers were unemployed and single.

2.4 Discussion

2.4.1 SUDI and SIDS at Salt River Mortuary

The high numbers of SUDI cases admitted to Salt River Mortuary ($n = 1\,199$) over the 5-year period (2013 - 2017) was comparable to those reported at Tygerberg Mortuary, Cape Town in 2005 - 2009 ($n = 1\,271$) [33]. This is expected since Salt River and Tygerberg mortuaries are the two M6 academic mortuaries in the Western Cape, and thus carry the highest caseloads in the province. However, the numbers of SUDI cases admitted to these M6 mortuaries in the Western Cape are higher than those reported in other major cities in South Africa (*i.e.* between 128 and 683 SUDI cases in other South African cities) [33], and in other countries [20].

While the number of SUDI admissions was similar between Salt River Mortuary and Tygerberg Mortuary, the number of cases classified as SIDS was remarkably different. Compared to the 189 (14.9 %) SIDS cases at Tygerberg [33], Salt River Mortuary did not officially report any SIDS cases over a similar 5-year period. While 122 (11 %) SUDI cases remained under investigation, only 22 of these had a clinical history review, death scene investigation and full autopsies with all ancillary investigations; and thus potentially filled the criteria to be classified as SIDS, should the ancillary tests return negative results. This translated to 1.83 % of the total SUDI caseload which was comparable to the low rates of SIDS reported Durban (0.6 %), and may suggest that a similar casework approach is adopted by these mortuaries [33]. This may include avoidance of the term ‘SIDS’ when a full autopsy was not done and therefore, where the full definition of SIDS was not met. It also appeared that the recognised sub-categories of SIDS which fall short of the full definition [2] were also under-utilised at Salt River Mortuary.

In South Africa, the resource-constrained environment unfortunately does not allow for full autopsies in every case; therefore, cases may not meet the investigational requirements to be

classified SIDS, and thus remain ‘under investigation’, or even due to *unspecified* natural causes. At Salt River Mortuary, 11 % of all SUDI cases had an *unspecified* natural cause. One reason that the resources are not available is because it is not the mandate of the FPS to investigate *natural* deaths [1]. As such, if the forensic pathologist is confident that there was no unnatural cause for the infant’s death, there is no obligation to further investigate; hence the death remains due to an unspecified natural cause. However, these cases may have been approached differently in another mortuary, and/or in another country under different legislation, where some may have been classified as SIDS, or a sub-category thereof. Such observations strongly call for the standardisation of procedures to register and investigate SUDI cases in South Africa, as suggested previously [33,81,124]. Since the majority of SUDI deaths admitted to Salt River Mortuary have natural causes of death diagnosed at autopsy, there are, however, important logistical and budgetary considerations for the development of a standard operating procedure. These may include the securing of resources for important ancillary investigations or the creation of additional forensic pathologist posts to enable full autopsies being carried out in more SUDI cases.

Overall, the rates of SIDS observed in this study, as well as at most other mortuaries in South Africa, is in contrast to the rates documented in developed countries where SIDS is the leading cause of infant death. It has been suggested that forensic pathologists internationally may tend to overuse the term ‘SIDS’ to avoid implying that the parents’ behaviour may have contributed to the infant’s death, and to avoid causing more distress to the family [46]. This is because the ‘diagnosis’ of SIDS implies that the parent or carer was not responsible for causing or contributing to the infants’ death [16,45,46]. It has also previously been documented that infants classified as SIDS in other countries may also have had mild infections, but which were not convincing enough to establish a specific cause of death [52]. Such cases would not be

classified as SIDS at Salt River Mortuary, but considered natural, and not investigated further. These are some of the reasons which may explain the large differences in SIDS rates reported between developed countries and those at most South African mortuaries.

It was noteworthy in this study that in more recent years, the number of SUDI cases which have remained under investigation at Salt River Mortuary has decreased. One would expect the opposite; *i.e.* that more cases from earlier years would be resolved (as a longer time period had elapsed since 2013 compared to since 2017). The reason for this may be attributed to the Child Death Review team which was implemented in 2014 [121]; since its establishment, there seems to have been a decreased turn-around time in the number of resolved SUDI cases. This may also be the reason for fewer cases having missing data in recent years, compared to earlier years (data not shown). What is of concern, however, is that the number of unnatural (and potentially avoidable) infant deaths are increasing (Figure 2.1).

2.4.2 Demographics of SUDI cases

The findings reported in this study pertaining to the demographics of SUDI cases are in accordance with those reported in other centres in South Africa, as well as internationally, in that slightly more male SUDI cases were reported, more deaths occurred during winter months and SUDI cases were mostly from regions with a lower socio-economic status [33,104,126]. However, in this study, the peak age of SUDI cases was between 1 and 2 months, which is younger than that reported in developed countries (between 2 and 4 months) [16,126]. The peak of infant death has previously been explained with reference to a decreasing maternal immunoglobulin G (IgG) in the infant after birth [127]. It is our hypothesis that infants in crowded informal settlements in Cape Town are exposed to different microorganisms and the rate of maternal IgG is decreasing faster than those in developed countries. In other words,

vulnerable infants cannot cope with the volume of pathogens and burden of disease associated with overcrowded living conditions [128,129] and are thus succumbing to infections sooner than those in developed countries. This hypothesis would warrant testing by monitoring the levels of maternal IgG in newborns as well as investigating the microbiomes present in crowded and non-crowded living conditions.

The consequences of overcrowded living conditions can be observed in the high rates of infectious causes of death, where 66 % of all SUDI cases had infection-related causes of death (respiratory-related: 52 %; gastroenteritis-related: 11 %; sepsis: 3 %). However, many infants survive these conditions annually, and it thus brings into question if some infants are perhaps less able to cope with these pathogens than others. Such predispositions could be in the form of genetic variants which could make immune response suboptimal in some infants [127]. Such genetic factors have not routinely been investigated in large-scale molecular autopsy studies, but Opdal and colleagues have shown some evidence for associations of SUDI with genetic variants in genes involved in the immune system [28,130–132].

In the present study, only 26.56 % of cases with respiratory causes of death had microbiology testing and 38.66 % had histology; the remainder of cases were diagnosed through a combination of Lodox[®] screening and clinical history. In some of these cases it is plausible that the forensic pathologist decided not to perform the ancillary investigations due to the time interval between death and post-mortem, which would add further challenges to the already difficult interpretation of post-mortem microbiology results. However, the majority of cases were refrigerated within 24 hours of declaration of death and an autopsy was performed within four days of death (Figure 2.5), which has been shown to be a reliable timeframe provided there is refrigeration soon after death [106,133–135].

2.4.3 Risk factors in SUDI cases

The majority of infants in the study slept on their side, and side-sleeping was significantly associated with co-sleeping ($p < 0.0001$). Given the high rates of co-sleeping (94.65 %) observed in this population, it would seem plausible to advocate for a 'Baby Box' whereby infants can be placed to sleep inside the box on the floor next to the parents' or older siblings' bed. This concept was first used in Finland in 1938, and followed by other countries [136–138]. This intervention reduces the risk of infants overheating, or being accidentally smothered whilst sleeping in a bed with adults or older siblings [137,138]; however, there has been some cultural resistance to this idea [139].

The co-sleeping practices in this study's population group are perhaps not as modifiable as one would think; in conversing with mothers of SUDI victims during this study, it became clear that putting the infant in a box on the floor would not be an option because of many homes in Cape Town being infested with rodents. Rodents can easily climb into a box on the floor or even eat through a plastic or cardboard box; which is also a health risk to the infant [140]. In addition to the lack of space and/or cots, rodent infestation appeared to be a burden in informal dwellings [141] as well as a large motivation for infants co-sleeping with parents or siblings in these homes. An alternative solution to this problem of co-sleeping may be in the form of a 'Pepi-Pod' which is a polypropylene container with low sides and a fitted mattress which can be placed on the bed with the parents/siblings, but provides suitable protection for the infant in terms of breathable air and suitable temperature [136].

In the present study, a significant association was observed between 'gastroenteritis' as a cause of death and the absence of breastfeeding. This result is consistent with that reported in other studies, whereby breastfeeding has been found to be protective against gastroenteritis and other

infections [142–144]. Howie *et al.* (1990) showed that infants who were breastfed for at least 13 weeks had significantly less diarrhoeal disease than those who were bottle fed [143]. While Golding *et al.* (1997) showed that breastfeeding may be protective of LRTI [144], this was not observed in this study, where most infants who demised from LRTI were breastfed. Overall, the majority of all SUDI cases were exclusively breastfed (51.82 %). This statistic is probably not due to the mothers' knowledge about the protective factors of breastfeeding, but rather the expense of milk- or feed-formula combined with the high rates of unemployed (80.22 %) and single (75.43 %) mothers, noted in this study. This is further supported by the last full South African Census 2011, where 47 % of the population in the City of Cape Town earned less than the minimum wage and 78.6 % of individuals had not completed secondary school [145].

While infants in this study were mostly breastfed, more than half of the infants (53.11 %) were exposed to tobacco smoke, which is a well-documented risk factor for respiratory-related causes of death [92,93,108,146,147]. Prenatal, perinatal and postnatal maternal smoking has been associated with an increased risk for SUDI [108], and this risk increased when the infant also co-slept [112]. In animal model studies, prenatal nicotine increased the lethal effect of toxins which were injected into chick embryos [148], altered lung development of the foetus in monkeys [149], and showed a reduced capability to tolerate hypoxia in rodents [150].

Of the total SUDI cases, mothers admitted to smoking cigarettes (36.58 %), drinking alcohol (19.78 %) and taking drugs (9.13 %) during pregnancy. However, only 11.72 % of all SUDI cases had samples taken for toxicology analysis. It is likely that the true number of mothers who used substances during pregnancy is higher than reported; hence, the absence of routine toxicology is a limitation in the investigation of SUDI cases. The reason for this may be attributed to the immense backlog in the Forensic Chemistry Laboratory of an estimated 60 000

samples in 2014 [150], and the interpretation of such results years after collection becomes challenging and of little value [151,152]. Consequently, there is a decreasing trend of forensic pathologists requesting quantitative toxicological analysis in our local setting. In the Western Cape, a new forensic pathology institute is currently being commissioned [153], whereby forensic post-mortem toxicology will be undertaken in-house, and thus envisaged to overcome the backlog problem.

A total of 12.83 % of mothers in this study had a previous SUDI, which is higher than that reported in other studies [154,155]. This may suggest recurrent risk factors in households (*e.g.* consistent substance use, co-sleeping *etc.*), but it could also suggest genetic factors. Since genetic variants are inherited, it is plausible that genetic variants which predisposed or contributed towards SUDI in one infant may also affect a sibling. Some genetic variants can cause sudden disease without showing obvious signs or symptoms (*e.g.* inherited channelopathies); thus it is challenging to detect these underlying causes through clinical history or even via autopsy at South African mortuaries, including Salt River Mortuary, which do not currently utilise genetic testing in cases where the cause of death remains undetermined. However, several forensic facilities abroad have recently introduced a molecular autopsy to analyse pathogenic mutations causative of inherited cardiac disease, with promising resolution [22,23]. Given the high number of instances at Salt River Mortuary where there is more than one SUDI in the family, a molecular autopsy, relevant to the South African setting, should be explored.

While some medical factors may not be easily modifiable (*e.g.* possible genetic variants, prematurity, exposure to HIV), some risk factors identified in the present study are modifiable and need to be addressed urgently. These include the mothers' attendance to antenatal care as

well as taking of infants to the local clinic timeously for vaccines, and for treatment when they show symptoms of illness. The latter is particularly the case for premature infants, even with mild symptoms of ill health. Educational campaigns in South Africa need to occur, to inform caregivers about risk factors and the importance of seeking medical care. Such campaigns have shown reductions in infant death in other countries [5]; and given the high rate of SUDI reported in this study, it is a public health issue which requires education, resources and commitment by government.

2.4.4 Conclusion

There is strong evidence in this study which suggests that many infant deaths are avoidable and some risk factors underpinning SUDI are modifiable. Campaigns to educate caregivers about sleeping practices, smoking and medical care (especially for premature infants), needs to occur at a national level. The results of the present study strongly advocate for a campaign to educate mothers and caregivers about risk factors for infant death. There is evidence in other countries that the education of caregivers can reduce infant mortality, and South Africa desperately needs such an intervention. This should focus on safe sleeping practices as well as the recognition and suitable action of mild symptoms, particularly in premature infants. Fundraising efforts should focus on procuring ‘Pepi-Pod’ sleeping aids, given the cultural background as well as social circumstances in at-risk households, which are rodent infested and where space and money is limited. This sleeping solution may add a protective layer against the risks associated with co-sleeping such as overlaying, overheating and asphyxia.

It is also imperative that SUDI cases are investigated consistently within mortuaries and across mortuaries, to allow comparison and investigation of trends on a national level, which will further enhance our understanding of risk factors and necessary interventions. It needs to be

recognised locally that interventions surrounding risk factors can reduce the burden of infant death; as such, forensic pathologists and scientists must recognise their duties to public health and translate these findings into community interventions, particularly pertaining to modifiable risk factors.

Chapter 3

SYSTEMATIC LITERATURE REVIEW

A systematic review of molecular autopsy studies in sudden infant death cases

Original Publication

Heathfield LJ, Martin L, Ramesar R. A Systematic Review of Molecular Autopsy Studies in Sudden Infant Death Cases. J Pediatr Genet. 2018; ahead of print. doi: 10.1055/s-0038-1668079

Article link: <https://www.thieme-connect.com/products/ejournals/abstract/10.1055/s-0038-1668079>

Nature of the publication: Review article

Journal: Journal of Paediatric Genetics ISSN: 2146-4596

Publisher: Georg Thieme Verlag KG Stuttgart, New York

Candidate contribution:

LJH: Conceptualisation; literature search; data curation; formal analysis; investigation; writing of original draft (lead author).

Co-authors contributions:

LJM and RR: Supervision; review and editing of original draft.

A Systematic Review of Molecular Autopsy Studies in Sudden Infant Death Cases

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J Pediatr Genet

Abstract

Keywords

- ▶ sudden infant death syndrome
- ▶ negative autopsy
- ▶ medico-legal investigation

Sudden unexpected death is an upsetting event, which can remain unexplained even after post-mortem investigation. Internationally, molecular autopsies have shown to resolve up to 44% of unexplained cases; however, it is currently unclear how many of these were infants. This systematic literature review showed that significantly fewer infant cases were resolved (median: 4%) compared with cohorts of 1 to 45 years old (median: 32%). Further, no study involving indigenous African participants has yet been published. Overall, molecular autopsies hold immense value to living family members and is motivation to explore new avenues in infant cohorts.

Introduction

Sudden unexpected death of infants (SUDI) is a devastating occurrence worldwide. In most countries, such cases are referred for medico-legal autopsy to establish the cause of death; however, in many instances, the cause of death remains undetermined. By a matter of exclusion, these cases are categorized as sudden infant death syndrome (SIDS) and in developed countries, is the leading cause of death in infants.^{1,2} While SUDI refers to the rapid and unexpected fatal episode of an infant less than 1 year old, SIDS refers specifically to those cases which remain unexplained following a full autopsy, clinical history review, and death scene investigation.³

Molecular autopsies have been reported to be of value in cases of sudden unexpected death in the young (SUDY) (individuals younger than 40 years old), particularly in autopsy-negative cases.^{4,5} A molecular autopsy involves the analysis of the deceased individuals' DNA to determine if they had a genetic mutation which contributed to and/or was likely to have caused their death. Most of the research has been focused on channelopathies, which are difficult to detect at a conventional autopsy (as the heart appears anatomically normal), but analysis of candidate genes implicated in chan-

nelopathy diseases (e.g., long QT syndrome and Brugada syndrome) can yield a molecular diagnosis.⁵ Molecular autopsies have shown a resolution of up to 44% in previously unexplained cases (cohort aged 1–19 years old), which brings closure to family members, while also making it possible for blood relatives to be tested for these mutations.⁶

Various case reports and studies have been published regarding the number of sudden unexpected death cases which have been potentially resolved by a molecular autopsy;^{7,8} however, it is currently unclear how many of these cases were infants. Therefore, the aim of this study was to review the literature to assess the percentage of SUDI cases resolved by molecular autopsies. The objectives were to assess which diseases were most commonly inferred by molecular results and to compare the resolution rates between SUDI cases and older SUDY cohorts.

Methodology

The PubMed database was searched using the following search terms: ((Sudden[All Fields] AND unexpected[All Fields] AND ("death"[MeSH Terms] OR "death"[All Fields]

received

March 30, 2018

accepted after revision

June 25, 2018

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Stuttgart · New York
ISSN 2146-4596.

OR “deaths”[All Fields]) AND (“infant”[MeSH Terms] OR “infant”[All Fields] OR “infants”[All Fields])) OR (“sudden infant death”[MeSH Terms] OR (“sudden”[All Fields] AND “infant”[All Fields] AND “death”[All Fields]) OR “sudden infant death”[All Fields] OR (“sudden”[All Fields] AND “infant”[All Fields] AND “death”[All Fields] AND “syndrome”[All Fields]) OR “sudden infant death syndrome”[All Fields])) AND (molecular[All Fields] AND (“autopsy”[MeSH Terms], OR “autopsy”[All Fields])). No search restrictions were applied on the dates of publication, and all articles up until December 31, 2017 were included.

All articles returned from the search were then scanned for relevance by reading the abstract and were included for further analysis if they met certain criteria, as follows: original research articles and case reports were included, but reviews and opinion articles were excluded. Articles pertaining to the development and/or optimization of molecular methods only were not included. Articles in a language other than English were excluded. Articles that described a cohort inclusive of 0 to 1-year-olds (but not necessarily limited to infants) were included, but the exact number of infants in their studies were also recorded and used in subsequent analysis. Articles whereby the cohort were all greater than 1-year-old were put aside for comparison purposes. It was also noted if a case report was subsequently included in a larger cohort study by the same research group. After all relevant articles were obtained, their reference lists were searched manually for other potential articles, which were subjected to the same criteria as described above.

The following variables were collected from each article: article details, including journal name and date; country of the population studied; total cohort size (including age range) as well as number of infants in the study; scope of molecular investigation (including the number of genes investigated); pathways/disease groups associated with the genes studied; and resolution of cases following molecular analysis. This information was collected for both infants, as well as older cohorts.

Descriptive statistics were applied to the data, and the non-parametric Mann-Whitney test was used to assess for significant differences in the percentages of resolved cases between infants and cohorts of more than 1-year-old, assuming a confidence interval of 95%. A Spearman rank sum test was used to assess for correlation between the number of genes included and the percentages of resolved cases. Graphs were created, and statistical tests were performed in Graph-Pad Prism 6.

Results

A total of 94 articles were returned, of which 55 were subsequently excluded. These were review articles ($n = 15$), retrospective case reviews not involving molecular autopsies ($n = 8$), viewpoint or recommendation articles ($n = 6$), studies involving method optimization or molecular virus detection ($n = 10$), or articles not relevant to the topic ($n = 16$). Only one article was not in English, but was a review article, so was excluded anyway. Numerous articles

were published relating to the Scripps Translational Science Institute study,^{9–11} but only the most relevant article pertaining to this review was included,¹¹ to avoid overrepresentation of the cohort.

A total of 20 original research studies^{11–30} and seven case reports^{31–37} thus met the inclusion criteria for the review, which included at least one infant greater than 1-year-old in their study (►Table 1). One of the seven case reports, however,³⁴ was also represented in one original research article.¹³ The remaining articles ($n = 12$) had cohorts of individuals older than 1 year old only.

Articles that met the inclusion criteria were published between 1992 and 2017, with 23 (85%) published in the past 11 years (►Fig. 1). The articles were published in 21 different journals; most journals published a single article, besides Forensic Science International ($n = 5$), Archives of Disease in Childhood ($n = 2$), and Heart Rhythm ($n = 2$).

Articles represented cohorts from 10 different countries, in addition to the one multi-center study in Europe (►Fig. 2). The United States of America had the most articles published ($11/27 = 40.74\%$). No study was from an indigenous African population. Cohort sizes for infants ranged from $n = 1$ to 292 infants (median: $n = 30$, standard deviation: ± 31.5).

The scope of molecular analysis was between genotyping one variant to whole exome sequencing; however, the majority of studies ($20/27 = 74\%$) researched 11 or fewer candidate genes by a variety of molecular methods. Two studies used whole exome sequencing (infants in cohort: 7–161), while four studies sequenced a panel of 23 genes or more using next generation sequencing technology (cohorts included 6 to 280 infants). In total, however, seven studies utilized next generation sequencing, and these were also the seven most recently published studies (2014–2017). When considering the studies that utilized next generation sequencing, a trend was seen in that—the smaller the cohort, the larger the percentage of potentially resolved cases ($R^2 = -0.7143$); however, this was not statistically significant ($p = 0.0881$).

When case reports were excluded, and the 20 “cohort” studies were analyzed, a mean of 8.6% (median: 4.0%) of sudden infant death cases were potentially resolved after a molecular autopsy. This was compared with the studies involving SUDY cohorts, over 1 year old, whereby a mean of 29.36% (median: 32.13%) of cases were potentially resolved following molecular autopsy (►Fig. 3). A Mann-Whitney test showed that older cohorts of SUDY (1–45 years old) were significantly more resolved by molecular autopsies compared with infants (0–1 years) ($p < 0.0001$).

Out of the seven case reports, one included the analysis of two infants,³⁶ while the remaining six each reported on one infant only. Researchers were successful at identifying pathogenic mutations, which probably contributed toward death, in the six single case reports. These mutations were involved in channelopathies (two case reports), metabolic disorders (three case reports), and autonomic nervous control (one case report). The case report including two infants was unsuccessful at identifying pathogenic mutations in four genes involved in channelopathies.³⁶

Table 1 A total of 20 original articles and 7 case reports met the inclusion criteria for the systematic review

Date	Country	Number of infants (<1 year) in study cohort	Molecular autopsy approach	Number of genes/variants investigated	Sample used from deceased infant	Potential resolution in infant cases (%)	Ref.
Original articles							
2017	United States	8	NGS, WES	Exome & mtDNA genome	Blood	37.5	11
2017	Canada	182	NGS, panel of genes	71 genes	Frozen tissue	6.04	12
2017	Europe (multi-center)	161	NGS, WES	Focused on 192 genes	Tissue	20.0	23
2016	United States	280	NGS, panel of genes	64 genes	Dried blood spot	2.80	24
2015	Germany	38	NGS, panel of genes	86 genes	Frozen blood or tissue	5.00	25
2015	France	6	NGS, panel of genes	23 genes	Frozen tissue	33.3	26
2014	United States	141	NGS, panel of genes	6 genes	Dried blood spot or tissue	13.5	27
2014	New Zealand	102	Sanger sequencing	6 genes	Blood, dried blood spot or tissue	1.96	28
2014	Netherlands	138	Various targeted genotyping methods	25 variants in 11 genes	FFPE tissue	Not available	29
2013	Australia	46	Sanger sequencing	2 genes	Blood or tissue	4.00	30
2013	Italy	10	Real-time PCR	1 gene + gene expression	Tissue	10.0	13
2011	Japan	30	Sanger sequencing	1 gene	Frozen blood	3.33	14
2010	United States	292	HPLC and Sanger sequencing	4 genes	Blood or frozen tissue	1.03	15
2010	New Zealand	1	HPLC and Sanger sequencing	5 genes	Dried blood spot	0.00	16
2009	France	52 of which 32 were SIDS	HPLC and Sanger sequencing	5 genes	Blood	9.38	17
2007	United States	16	Sanger sequencing	2 genes	FFPE tissue	12.5	18
2007	United States	221	HPLC and Sanger sequencing	1 gene	Blood	1.35	19
2007	Germany	6	Sanger sequencing	2 genes	Blood	0.00	20
2001	United States	93	HPLC and Sanger sequencing	1 gene	Frozen tissue	2.15	21
1992	United States	67	Restriction enzyme digestion	1 variant	FFPE tissue	0.00	22
						Mean = 8.62% Median = 4.00%	
Case studies							
2015	United States	1	NGS, WES	Exome	Blood	Yes	31
2014	Japan	1	Sanger sequencing	1 gene	Blood	Yes	32
2014	Spain	1	NGS, panel of genes	104 genes	Blood	Yes	33
2013	Italy	1	PCR and agarose gel electrophoresis	1 variant	Tissue	Yes	34
2009	United States	1	Sanger sequencing	1 variant	Blood	Yes	35
2001	Germany	2	Sanger sequencing	4 genes	Blood	No	36
1997	United States	1	Restriction enzyme digestion	1 gene	Tissue	Yes	37

Abbreviations: FFPE, formalin fixed paraffin embedded; HPLC, high-performance liquid chromatography; mtDNA, mitochondrial DNA; NGS, next generation sequencing; PCR, polymerase chain reaction; Ref., reference; USA, the United States of America; SIDS, sudden infant death syndrome; WES, whole exome sequencing.

Note: Details pertaining to these studies are summarized. Potential resolution is reported to three significant numbers.

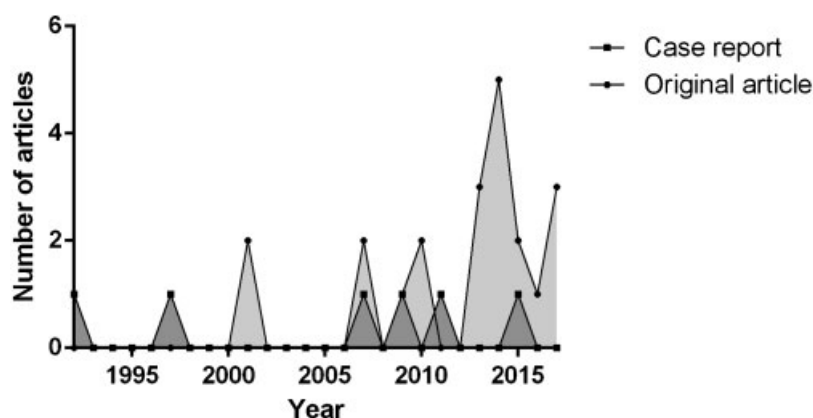


Fig. 1 Number of original articles and case reports published per year. The majority of articles (85%) were published within the last 11 years.

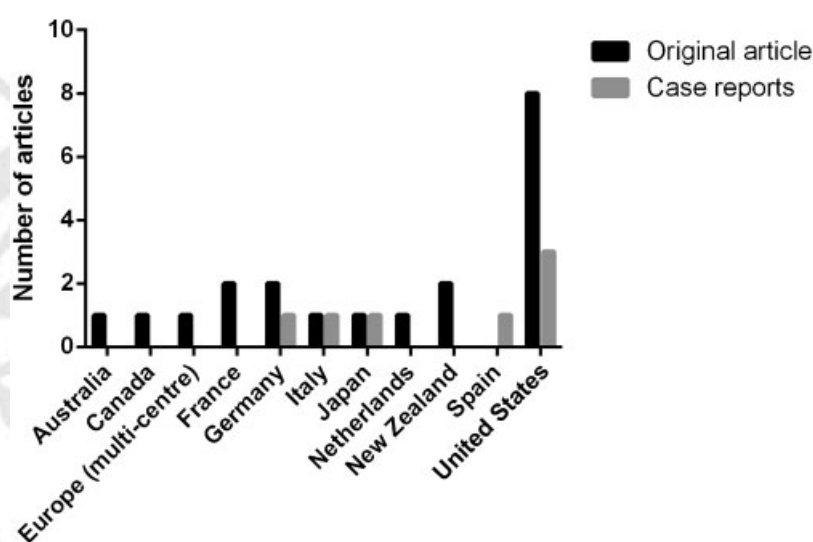


Fig. 2 Number of articles published per country. A total of 41% of articles represented cohorts from the United States.

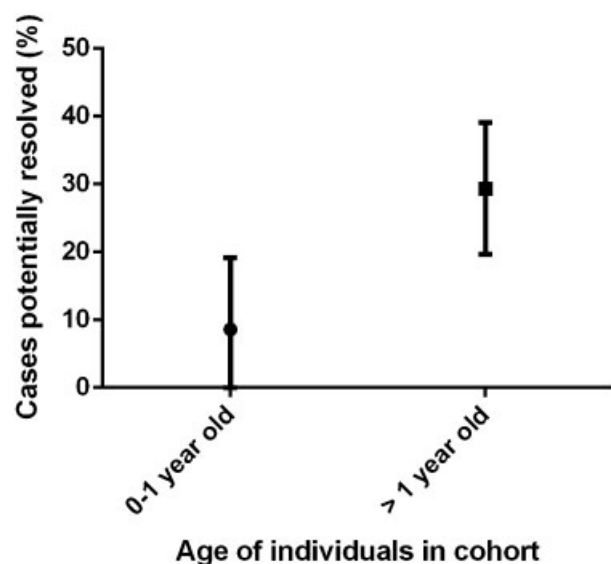


Fig. 3 Percentages of cases potentially resolved following molecular autopsies, which was significantly different between infants (0–1 years old) and cohorts of “sudden unexpected death in the young” (1 to ~45 years old).

Of the 20 original articles, 12 identified putative pathogenic mutations in genes associated with channelopathies, 5 in cardiomyopathies, 4 in metabolic disorders, 2 in autonomic nervous control disorders, and 1 in mitochondrial DNA (mtDNA) disorders. The category of diseases with the highest “yield” of potentially resolved cases was that of channelopathies (6.7%) followed by metabolic disorders (5.08%), with the remaining three categories having a return of less than 1.6% resolution, each. Lastly, within these 20 articles, the number of genes investigated was significantly associated with the percentage of potentially resolved cases ($p = 0.0179$) (→ **Fig. 4**).

Discussion

The role of genetic factors in human disease especially involving metabolic and cardiovascular diseases is well recognized.³⁸ The investigation of genetic factors to explain mortality is less well established; however, this trend is growing for metabolic and cardiovascular diseases. Some of these diseases do not have obvious physical/phenotypic

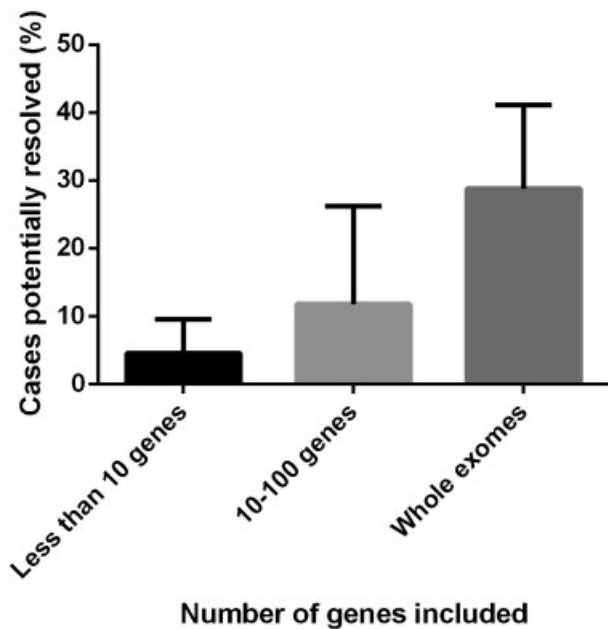


Fig. 4 The percentage of cases potentially resolved by molecular autopsies increased as the number of genes analyzed increased. This is represented in this figure by means of categorizing the data; however, a Spearman rank sum test on the numerical data (► Table 1) showed that this association was statistically significant ($p = 0.0179$).

manifestations, particularly in very young infants and, therefore, may go undetected at autopsy. However, such diseases can potentially be revealed with genetic analysis of the deceased individuals' DNA.

To date, many genes and variants have been identified as candidates for molecular autopsies as reviewed by various authors.^{38–40} Some studies have targeted specific mutations in candidate genes, sometimes on a case-by-case basis, while other studies have screened cohorts for these mutations (► Table 1). In comparison, and more recently, a next generation sequencing approach has been used to identify new mutations which could underlie and perhaps explain sudden unexpected death cases.

One of the challenges in these larger scale studies, as with most next generation data, is the interpretation of rare variants, particularly those that are putatively pathogenic according to *in silico* analysis but lack functional and co-segregation studies to support the predicted pathogenicity of the variant. The value of co-segregation analysis was demonstrated by Campuzano et al³³ whereby the presence of rare variants in asymptomatic family members aided the exclusion of some variants as being causative of the infant's death and ultimately led to the identification of the variant likely related to the infant's sudden death. As Glengarry et al²⁸ noted, however, co-segregation studies are usually challenging to perform especially when the proband is an infant, due to difficulties in tracking families. The implication of this is two-fold; first, if a pathogenic variant is found which explains death, the information cannot be given to the family who is lost to follow up; and second, the lack of co-segregation analysis, together with the absence of functional studies, results in many variants being classified as “variants of

unknown significance” (VUS), and their role within the sudden unexpected death cases remains speculative. Until functional data becomes available, the interpretation of the variant is purely hypothetical and does not warrant clinical testing in living relatives—that is, if family members could eventually be tracked, and they are willing to engage. For the studies that used next-generation sequencing in this review, only one out of seven studies performed co-segregation analysis for at least some of the cases.¹¹ Further, their reported percentage of “potentially resolved cases” was based on putatively pathogenic mutations, which means that the true percentage of actually resolved cases by molecular autopsy is likely to be lower than reported here.

A remarkable finding in this review was that significantly more cases were potentially resolved in older cohorts of SUDY compared with infants. This may have been a consequence of the specific mutations/genes investigated in the respective cohorts and suggests that there may be different mechanisms contributing toward death in the different age groups. For example, genes associated with channelopathies were investigated in the majority of studies in all age groups; however, cardiovascular diseases are probably more advanced and therefore of greater significance in older individuals, whereas other pathways may be more significant in infants, but were just not targeted during the molecular autopsy. Davis et al⁴¹ also proposed that the interplay of genetic variants in long QT syndrome in SIDS cases is part of the vulnerable infant spectrum, and a paradigm shift is needed to fully appreciate the complex interplay of environmental factors. Therefore, while this review demonstrates the potential of molecular autopsies, particularly in the older cohorts, it also suggests that genes associated with other disease categories should possibly be targeted in infants, and environmental factors should also be considered. This notion was supported by Rueda et al¹¹ who showed that it might be worthwhile exploring variants in mtDNA, as four out of eight SIDS cases had an increased ratio of heteroplasmic variants. Out of the cohort studies included in this review, only the Scripps study investigated mtDNA variants and suggested that the systematic collation of data across multiple studies may provide the means to investigate this hypothesis with a larger cohort.

Tang et al⁴² noted that although they were not the only cause of sudden unexpected death, cardiac channelopathies do play a significant role in such cases. Since 2008, the Molecular Genetics Laboratory of the New York City Office of the Chief Medical Examiner has been screening six candidate genes associated with cardiac channelopathies during molecular autopsy of cases of sudden unexplained death. Between 2008 and 2012, 274 sudden unexpected death cases with negative post-mortem investigation were screened for mutations in these candidate genes. A total of 46 genetic variants were detected (of which 24 were novel) in infants (13.5%) and non-infants (19.5%), most of which were in the *sodium voltage-gated channel α subunit 5 (SCN5A)* gene (which encodes for a part of the channel that allows sodium ions into cells). The cohort comprised individuals from diverse ethnic backgrounds, and the authors observed that the group with the highest risk of sudden unexpected death

were African-American infants.²⁷ As observed in this review, however, there is a stark gap in forensic molecular autopsy data pertaining to indigenous Africans.

The finding in this review that significantly more infant cases were potentially resolved with the more genes investigated was not unexpected. Rueda et al¹¹ reviewed the data generated from the Scripps study ($n = 50$; cohort 0–45 years) and analyzed the number of variants obtained following the bioinformatics pipeline on (1) the whole exome sequencing data and (2) a subset of the data, which was a simulated cardiac panel of 233 markers. They showed that there was a median of 354 variants per sample (range: 235–739) from the exome data, compared with a median of 7 per sample (range: 1–18) for the “panel” subset. This vast reduction of variants to consider within the case context is important for time and resource considerations; however, while time may be initially saved, there is also a chance that less cases would be resolved with a panel approach. The advantage of whole exome sequencing in this regard is that the filtering of subsets of genes from the larger dataset is possible, which may be the first step in data analysis; however, this is not true for data from a panel; as such, additional sequencing may need to be performed on molecular autopsy-negative cases. At this exploratory stage, especially in developing countries, it might make sense to strategically consider whole exome sequencing (ensuring good coverage), taking into account that one could resort to different modes of analysis from such data, i.e., looking at just component panels (e.g., channelopathies), while ultimately also having the capability to look for deleterious mutations across the exome. However, one must also consider that a molecular autopsy in SIDS cohorts is not yet a mature test, and relatively, whole exome sequencing is an expensive method to pursue. Recent case-control studies in 2018 (thus not included in systematic search) which used whole exome sequencing on large SIDS cohorts have also shown valuable but very slight differences between cases and controls.^{43,44} Thus, whole exome sequencing should be reserved for large case-control cohorts or cases where families are available for co-segregation analysis.

As noted previously, molecular autopsies may not always have a high diagnostic yield in sudden expected death cases;⁴⁵ DNA from 59 Australian SUD cases underwent genetic testing of two genes encoding ion channels, and 23 cases showed at least one variation in these genes. However, none of them was deemed to be pathogenic. The authors suggested that a more selected approach should be adopted, and these genes should only be screened for those individuals who had a family history of cardiac channelopathy disorders.⁴⁵

This was demonstrated again in a combined “retrospective and prospective” study in New Zealand, where they too showed that a molecular autopsy of genes involved in long QT syndrome does not always yield significant results.²⁸ A total of 102 SIDS cases were included in the study, where 71 cases were “unselected,” and 31 cases were specially selected for a molecular autopsy subsequent to screening by a cardiac genetic service. Long QT syndrome genes were sequenced in these infants, and mutations were observed in

4% of unselected cases compared with 16% in selected cases. Their study showed that there was a significantly lower diagnostic yield among unselected SIDS cases compared with selected cases ($p < 0.05$), and this may be attributed to other common risk factors in the unselected group, such as co-sleeping.²⁸

Overall, the role of molecular autopsies has important consequences for family members of the deceased victim. Apart from providing some sort of answer to the question of “what led to the death of my child?” the results of a molecular diagnosis have clinical value for relatives who may carry the same mutations; some of which are already clinically actionable.^{6,12} This review has collated the results from previous molecular autopsy related studies and showed that a median of 4% of previous SIDS cases can potentially be resolved by a molecular autopsy. While this was significantly lower than the yield from older cohorts of SUDY, these results would have been of particular value to the respective family members. This review has also shown that channelopathies and metabolic disorders yielded the most positive results thus far in this emerging field, but other avenues, such as mtDNA, may be worthwhile to explore. It must be noted though, that a limitation of this study was that not all molecular autopsy studies might have been found using the systematic search methods described here.

While there is a growing momentum of genomics research in large cohorts of adults with various communicable and non-communicable diseases in Africa,⁴⁶ a special effort needs to be made toward developing large cohorts sudden unexpected deaths which remain unexplained. With the anticipated decrease in costs of genomic research and increased capacity in big data analysis and learning from the efforts in the developed world, we will be well-placed to investigate the genetics/genomics that potentially underlies sudden unexplained death. Moreover, we hope this will lead to the effective implementation of a molecular autopsy that will be locally or regionally relevant.

Conflict of Interest

None.

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Chapter 4

ETHICAL CONSIDERATIONS

Ethical and logistical considerations of forensic genetics research on biological samples collected post-mortem

4.1 Introduction

This research study was one of the first projects initiated in our department which involved the use of post-mortem specimens for prospective molecular analysis. Performing such research in an extremely busy post-mortem setting is a complex task. Such research requires the use of body fluids, which means that informed consent needs to be taken from family members. However, this needs to be strongly justified as family members are typically grieving, and there is potential to do more harm than good.

Over and above the primary aims of this research project, this study was a pioneer in our setting to establish the feasibility and acceptability of carrying out research using post-mortem samples in South Africa. While guidelines exist with regard to research of this nature, most have limited applicability to low- and middle income countries, where the access to resources is severely limited. This study thus identified both logistical challenges and ethical considerations in a South African population which is heterogeneous as well as socially and economically diverse. To contextualise this discussion, a summary of the operations at Salt River Mortuary is

provided. After the logistical considerations have been discussed, the ethical aspects will be presented in the form of a published article, which is included as part of this thesis.

4.2 Logistical considerations

4.2.1 Operations at Salt River Mortuary

All deaths in the western part of the Cape Town metropole (Appendix 5), which are thought to be unnatural, are admitted to the Salt River Mortuary. When a body arrives at the mortuary it is accompanied by a scene script, which details the circumstances surrounding the death, and is completed by the FPO at the scene. The case is assigned a unique number, which is captured onto an internal tracking system, along with the admission history.

Each body received at the mortuary is required to be formally identified, which is usually done by the parent(s) in SUDI cases. This process involves the provision of relevant identity documents, of both the deceased and the family member doing the identification, in order to verify the deceased's identity. As previously alluded to, a copy of the infant's Road to Health clinic card (Appendix 3) also needs to be submitted to the mortuary for SUDI cases, and the FPO completes a comprehensive questionnaire with the mother and/or family of the deceased infant using the FPS006(b) form (Appendix 4). The identification session and the completion of the questionnaire takes place in private designated rooms at the mortuary, and is conducted in the language of the family's choice. FPOs at the Salt River Mortuary are able to converse in English, Afrikaans and the main African languages spoken in the Western Cape (notably isiXhosa).

Within one week of admission, an autopsy is performed by a forensic pathologist, and this usually (but not always) occurs after the identification process. Family members typically

arrive approximately three days after the death to identify their deceased family member. Due to this time delay and other logistics, the autopsy is sometimes scheduled to take place before the identification process; in the context of this study, this timing can pose challenges for obtaining informed consent.

The forensic pathologist conducts an autopsy, which can range from an external examination only, to a full autopsy. At Salt River Mortuary, a Lodox[®] scan is routinely performed for all cases. If deemed necessary, the forensic pathologist may take samples for ancillary investigations, and the extent of autopsy varies based on the case and the discretion of the forensic pathologist. The Road to Health Card (in most cases), FPS006(b) and the scene script are available for consideration to formulate cause of death, and additional information may be obtained from the assigned investigating officer at the discretion of the forensic pathologist. As previously noted in the literature, the protocol for the post-mortem investigation of SUDI is not standardised in South Africa [33,124]. Subsequent to autopsy, the body is released for burial or cremation.

4.2.2 Performing research within this environment

To perform research at Salt River Mortuary, permission had to first be obtained from several individuals, including the mortuary manager, the head of the Division of Forensic Medicine and Toxicology at the University of Cape Town and the director of the FPS. During the initial phase of the project, this involved the pitching and discussion of ideas, explaining the applicability and benefit of the research to the FPS, as well as describing all the logistics. Next, communication channels had to be set up between the FPO teams and the researcher. At Salt River Mortuary, there are four teams of FPOs, each with a designated team leader, who work

in shifts. The researcher met with the team leaders over a two week period to introduce the project and explain the necessary procedures, who, in turn, informed their team members.

The FPOs were the main point of contact between the researcher and the mortuary. They would call the researcher when family members of infants who had died suddenly and unexpectedly arrived at the mortuary to identify the deceased. Family members arrived any time between 7 am and 4 pm on weekdays to identify the deceased. They arrive without an appointment, necessitating the researcher going to the mortuary at any time the mortuary called. Since Salt River Mortuary is one of the busiest mortuaries in the country, notices had to be placed to remind FPOs even when they were at their busiest.

In parallel, the researcher briefed the forensic pathologists about the project, as they would be responsible for collecting the biological samples. The researcher prepared packs of collection material, including vials for blood, and swabs for buccal cells. These were packaged into clear plastic bags, labelled and taken to the mortuary in batches, as and when necessary. The researcher asked the chief FPO to hand out the collection packs to the forensic pathologists for cases where informed consent was obtained. After samples were collected, the forensic pathologists handed the samples back to the chief FPO who stored them temporarily. Due to the extreme case load, fridges were often unavailable, so the researcher took cooler boxes to the mortuary daily. After the samples were collected and stored, the samples were transported to the university research laboratory where DNA extraction would be carried out.

4.3 Ethical considerations

Original Publication

Heathfield LJ, Maistry S, Martin LJ, Ramesar R, de Vries J. Ethical considerations in forensic genetics research on tissue samples collected post-mortem in Cape Town, South Africa. BMC Med Ethics. 2017 Nov 29;18(66):1-8. doi: 10.1186/s12910-017-0225-6

Article link: <https://bmcmethics.biomedcentral.com/articles/10.1186/s12910-017-0225-6>

Nature of the publication: Original debate article

Journal/Publisher: BMC Medical Ethics ISSN: 1472-6939

Candidate contribution:

LJH: conducted the informed consent sessions for the SUDI study (investigation); data curation; formal analysis; developed the debate; writing of original draft (lead author).

Co-authors contributions:

SM: conceptualisation; validation; conducted the informed consent sessions for the suicide study and discussed similarities in the process (investigation).

LJM and RR: Supervision; provision of resources; review and editing of original draft.

JdV: conceptualisation; contributed towards the development of the ethical debate surrounding these studies, review and editing of original draft.

DEBATE

Open Access



Ethical considerations in forensic genetics research on tissue samples collected post-mortem in Cape Town, South Africa

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Abstract

Background: The use of tissue collected at a forensic post-mortem for forensic genetics research purposes remains of ethical concern as the process involves obtaining informed consent from grieving family members. Two forensic genetics research studies using tissue collected from a forensic post-mortem were recently initiated at our institution and were the first of their kind to be conducted in Cape Town, South Africa.

Main body: This article discusses some of the ethical challenges that were encountered in these research projects. Among these challenges was the adaptation of research workflows to fit in with an exceptionally busy service delivery that is operating with limited resources. Whilst seeking guidance from the literature regarding research on deceased populations, it was noted that next of kin of decedents are not formally recognised as a vulnerable group in the existing ethical and legal frameworks in South Africa. The authors recommend that research in the forensic mortuary setting is approached using guidance for vulnerable groups, and the benefit to risk standard needs to be strongly justified. Lastly, when planning forensic genetics research, consideration must be given to the potential of uncovering incidental findings, funding to validate these findings and the feedback of results to family members; the latter of which is recommended to occur through a genetic counsellor.

Conclusion: It is hoped that these experiences will contribute towards a formal framework for conducting forensic genetic research in medico-legal mortuaries in South Africa.

Keywords: Vulnerable group, Next of kin, Sudden unexpected death of infants, Suicide, Forensic, Post-mortem, Medico-legal, Biomedical, Research ethics, South Africa

Background

The field of forensic genetics encompasses the analysis of deoxyribonucleic acid (DNA) from human remains for the purposes of identifying an individual or to help understand their cause of death [1, 2]. Although the discipline has a strong service-orientated focus, research and development play an important role in the improvement of this evidence-based practice. Such research requires the use of genetic material obtained from body

fluids and tissue, but the use of post-mortem material in research is a sensitive subject. This is because taking informed consent from grieving family members needs to be strongly justified; there is the potential to do more harm than good and to exploit a vulnerable population. In this manuscript, we will describe some of the most pertinent ethical challenges that we encountered in our ongoing forensic genetics research.

The Division of Forensic Medicine and Toxicology (DFMT) at the University of Cape Town (UCT) leads an active role in service delivery, teaching and research activities. UCT's DFMT is closely affiliated with the Department of Health, Western Cape Government, which oversees the post-mortem investigation of individuals whose deaths are deemed unnatural in the Western part of the Cape Metropole of South Africa. Autopsies have been performed for centuries and serve to establish the nature

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and extent of underlying natural disease, the process and mechanism leading to death, and to enable learning, teaching and research [3]. Post-mortem investigations, from which we derive our genetic research samples, are conducted at the Salt River Mortuary, which conducts more than 3500 post-mortem examinations each year. The DFMT hosts various research projects together with those of the present authors.

Summary of studies

Over the past three years, we initiated two projects that involve the use of post-mortem specimens for prospective genetic analyses. The first involves an investigation of the epidemiology, genetics and neurobiology of suicide. This study seeks to identify multifactorial contributions to suicidal behaviour. Part of this project involved the pilot prospective collection and genetic testing of biological material from suicide victims to establish whether single nucleotide polymorphisms in candidate genes could predispose a person to commit suicide. This study completed pilot sample collection in October 2015 and obtained informed consent to collect brain tissue and/or blood from 37 out of 40 (92.5%) families during a one-year period.

The second study aims to explore the molecular basis of sudden unexpected death in infants (SUDI) at Salt River Mortuary. SUDI cases undergo post-mortem investigation in an attempt to establish cause of death. Most of these cases at Salt River Mortuary end up being classified as natural, with the leading cause of death being infection [4]. However, some deaths remain undetermined and are thus classified as sudden infant death syndrome (SIDS); which is defined when infants less than one year old die suddenly and unexpectedly, and the cause of death cannot be determined after thorough post-mortem investigation, including a full autopsy, death scene investigation and clinical history review [5].

Common risk factors for SUDI are typically environmental, but despite awareness campaigns about these risk factors in developed countries, there remains a residual burden of SUDI which suggests a genetic basis for at least a component of these deaths [6]. This has led to genetic testing in the form of molecular autopsies to try and classify the cause of death, particularly in SIDS cases [7]. These have taken on the form of genotyping known mutations, sequencing candidate genes or massively parallel sequencing of exomes and genomes. Internationally, this has shown potential in not only classifying the direct genetic cause of death, but also establishing genetic factors that could predispose the infant during their critical stage of development [7–11]. This project focuses on exploring whether specific genetic variants identified elsewhere are also found in our population. The first sample collection phase for the SUDI project was between March 2016 and June 2016, where 38 families out of 40

(95%) gave informed consent for blood and/or buccal swabs to be obtained from their deceased infants for molecular analysis. Thus together, the researchers have spoken to a total of 80 families during the pilot collection phases.

These two studies are among the first prospective molecular studies on deceased individuals conducted in South Africa and as such are novel both to the research regulatory infrastructure (including ethics committees) and to the forensic services. One important secondary function of these research projects is their role in establishing the feasibility and acceptability of carrying out genetic research using post-mortem samples in South Africa. While guidelines exist with regard to research of this nature, most have limited applicability to low- and middle- income countries (LMICs), where the access to resources is severely limited [12, 13]. When speaking to the 80 families of deceased individuals about collecting samples for genetics research, and in seeking regulatory approval for these studies, we noted some remarkable similarities in our experiences, which are described and discussed in the current manuscript.

Ethical considerations

As we planned and conducted our research, we encountered a number of ethical challenges, some of which were anticipated and others that were not. To our surprise, when looking to literature for guidance about how to address these challenges, we found a scarcity of resources to help us make sense of these challenges.

Institutional review board: Seeking approval

One of the first and arguably most challenging issues we faced related to seeking ethics approval from our Human Research Ethics Committee (HREC) for these studies. In particular, when the first study was proposed – which was also one of the first ever genetic studies on autopsy samples reviewed by our Institutional Review Board – we faced a barrage of critical questions that took over two months to resolve. These included questions surrounding feedback of results, incidental findings and the timing of the consent, all of which are discussed in this article. At the best of times, genomic research raises fears and anxieties for ethics committees in Africa, relating importantly to the potential for exploitation and concerns about identifiability [14]. We suspect that the novelty of this research, combined with taboos surrounding both suicide and SUDI and the fear that grief could influence individuals' ability to give informed consent, made our HREC particularly anxious about the proposed projects. We resolved this challenge by establishing a relationship with the ethics committee which allowed for less formal conversations and explanations to occur, specifically regarding the legal and judicial

mandates under which medico-legal autopsies are performed in the FPS in South Africa. Through this relationship, we worked with selected members of the HREC to design a workable consent strategy for this study. We also indicated that we would continue to engage with the HREC about our experiences and alert them to any particular challenges that may occur.

Informed consent: Process of procurement

Since both of these projects required samples to be collected at the autopsy, informed consent needed to be obtained prior to the autopsy taking place. According to the South African National Health Act (Act 61 of 2003) and the Inquests Act (Act 58 of 1959), it is mandated that all deaths deemed to be unnatural are referred for a medico-legal autopsy, and during this investigation, samples may be retained at the discretion of the forensic pathologist, for the purpose of determining cause of death.

Suicide, which is the intentional taking of one's own life [15], is considered an unnatural death, and therefore requires a mandatory autopsy. SUDI, on the other hand, is referred to for medico-legal autopsy under the circumstances that the death was sudden and unexpected (National Health Act (Act 61 of 2003)); however, the protocols for the post-mortem investigation of SUDI are not standardised in South Africa [16, 17]. In the case of autopsies, the South African laws allow for the obtaining of tissue samples to determine cause of death, but not for research purposes. Hence for these studies to occur, informed consent must be obtained by the individual concerned during their lifetime (or by a minor's legal guardian), or by their next of kin if the individual is already deceased, to retain and use tissue for research purposes [3]. This raises genuine ethical concerns which need to be acknowledged and addressed.

There were also a number of important differences in the consent processes for the two genetic studies we conducted. The first of these is that while parents of infants typically have legal authority to make decisions for their infants, next-of-kin of adults do not normally have such authority; although *de facto* they do make treatment and/or research enrolment decisions on behalf of their family members in some selected instances (e.g. in the case of unconsciousness). In that case, next of kin are approached on the assumption that they are best-placed to make decisions on behalf of the individual and may even have been made aware of the individual's decisions. In other words, for infants, we are asking parents to make a decision based on their own belief system whilst for next of kin of adults we are asking them to make a decision based on what they think the deceased suicide victim would have wanted.

The second important difference relates to the ease with which samples would be obtained during the autopsy.

In the case of suicide, autopsies are legally mandated (because the cause of death is considered 'unnatural') and samples may be obtained for ancillary testing in any case. Seeking an additional sample for research in that process is fairly straightforward. Not so in the case of SUDI. Without evidence of 'unnatural' causes of death, the pathologist is not legally mandated to establish the exact cause of natural death and thus may not need to perform an internal/full autopsy. In that case, the obtaining of a sample may constitute the only surface violation of the corpse, and in the case of very young infants, may require opening up the body to obtain a blood sample from the heart. Since this sample would not have been taken for cause of death determination, there is no legal mandate to take such a sample without consent.

We based the design of our consent strategy on three sources: discussions with the forensic pathology team and the pathologist conducting the suicide genetic study; discussions with the HREC; and on work reported by Odendaal and colleagues [13]. Following these discussions, we agreed that the most appropriate time to obtain informed consent would be before the identification process in a private designated interview. Family members would normally only come to the mortuary once, and for reasons of feasibility and cost, it was important that we approached them at that point. The identification process entails the formal identification of the deceased by the next of kin for legal purposes, and is typically an emotional process, accompanied by paperwork. The researcher/s introduced themselves to the next of kin of the deceased before the identification process and expressed condolences to the family. Next the researcher explained the project, which was also collated into a written 'information form' which described the purpose, background and procedure of the research study. This form was approved by the Institutional Review Board and contained the contact details of its chair and of the researchers. Family members could participate voluntarily, and if they decided not to, there was no consequence whatsoever – this was clearly explained to the next of kin. The most common question asked by the next of kin was if participating would delay the autopsy process and eventual collection of their relative for the funeral. It was explained to the next of kin that the collection of additional samples would only take a few extra minutes. Parents of infants who had demised suddenly and unexpectedly were given the option to only agree to a sample being taken if the pathologist decided to perform an internal autopsy (and to leave the corpse intact where he or she did not). In total, each informed consent procedure took approximately 20–30 min.

If the family spoke a language that could not be spoken by the researcher, a Forensic Pathology Officer would translate; as such, we were able to communicate

all major languages spoken in the Western Cape. In one instance, a family spoke a dialect which none of the Forensic Pathology Officers could speak, and for fear of a misunderstanding, it was decided to not approach them for participation in the study. Similarly, if the consent process became too distressing for the family member, it was stopped immediately. In circumstances when religious leaders, such as the Imam, Rabbi or Hindu priest, accompanied the family to the mortuary, it was decided to include these religious leaders in the consent process to (i) show respect the family wishes and (ii) to ensure that members of these religious groups could also be included in the study if they wished.

The researcher in the first study is a forensic pathologist with over 10 years of experience and skill with dealing with bereaved families of people on a daily basis. During her specialist training, she developed the prerequisite skills to conduct consent for this research project. The researcher in the second study is a biomedical scientist who was specifically trained to talk to families in stressful and emotional situations. Training took the form of observations, role plays and debriefing sessions conducted by a team of trained medical professionals, until the researcher was ready to take consent on her own.

At the mortuary, managers can provide relief grief counselling and support. If this was not adequate then religious leaders were contacted to provide support. One suggestion that emerged from our experiences was to implement a better referral system for family members to speak to qualified grief counsellors in their area, or at Groote Schuur hospital, for a longer time after the identification process, which could be arranged on a case by case basis.

Vulnerability: Recently bereaved individuals under study

South African laws and ethical guidelines define several kinds of vulnerable groups that require special consideration in research and beyond. In relation to research, the Department of Health Ethical Guidelines (2015) define vulnerable individuals as those whose willingness to participate in research might be influenced either by an expectation of benefit or by peer pressure [18]. In the South African legislative and regulatory framework, identified vulnerable groups include children, mentally ill or disabled individuals, prisoners, subordinate members of hierarchies, poor people, patients in emergency situations and ethnic and racial minorities [19]. Neither the South African ethical nor the legal frameworks that describe or identify vulnerable groups explicitly list deceased individuals and their living relatives as vulnerable.

The Belmont Report (1979) introduced the idea of “diminished autonomy”, whereby vulnerable groups

could be identified as those with a “dependent status” and “inability or limited capacity to provide free consent” [20]. In the case of deceased individuals, whether they are children or adults, their capacity to provide consent is non-existent, and they are completely ‘dependent’ on family members or their next of kin to provide informed consent. But the important question is whether the next of kin should not also be considered a vulnerable group. In a period of profound grief, the next of kin is given the added responsibility of making a decision with regards to consent for samples to be taken from their child/family member/friend to be used for research. Such individuals may or may not have a “dependent status”; for example they might be subordinate members of hierarchies, they might be poor individuals or they might be from ethnic and racial minorities. In these instances, they are vulnerable in one sense, but does the fact that they are grieving make this population a vulnerable group in their own right? While grief does not strictly match the definition of a vulnerable group, in our experience the capacity of grieving individuals to freely give consent may be limited due to various reasons including:

- (i) The experience of shock and grief, both of which could have impaired consenting adults’ ability to understand the information provided;
- (ii) An expectation that they ought to do everything possible for their deceased and therefore give consent without fully considering all aspects of the research;
- (iii) Pressures from their community to do what is deemed ‘best’; this could be influenced by religion, culture and what is perceived to be society’s norm;
- (iv) They themselves want to know what happened (peace of mind) and a molecular diagnosis could relieve them from feeling guilty and/or defer them blaming themselves for the death;
- (v) Fear of what the results might reveal about their parenting, in the case of SUDI cases or interpersonal issues in the case of suicide.

From our perspective, the research participant is the deceased individual as the biological sample was taken from them; however, consent was given by their next of kin, who – we propose – should be considered as vulnerable in their own right due to their state of grief. Therefore, the current definition of vulnerability is perhaps too narrow, as it does not cater for other vulnerable groups who are not participants themselves, but who are approached for consent during a time in which they are vulnerable in the same way that participants would be.

There is considerable discussion in bioethics on how vulnerability should be understood and accounted for in

research ethics [21]. What seems clear, however, is that if vulnerability is understood as potentially impairing an individual's ability to give voluntary and informed consent, then there is an argument for 'special arrangements' to be put in place that promote the vulnerable person's ability to understand the information, and give consent without undue pressure. This could, for instance, involve deferral or progressive staging of consent, where individuals are approached for initial consent but then are approached for re-consenting when the factors causing their vulnerability have resolved. A pertinent example for our discussion is the enrolment of paediatric emergency patients in research [22, 23]. The categorisation as a particular group of participants as 'vulnerable' thus seems to open up the possibility that consent processes more explicitly take into account the circumstances of the person consenting. In our experience, consenting grieving adults for enrolment in research, when they have just lost a family member or child and are awaiting autopsy is a really challenging, if not impossible, task and that we should carefully consider more appropriate ways of seeking consent from surviving relatives, possibly over time.

A key feature of discussions around vulnerability is that the exclusion of vulnerable groups from research merely because it is inconvenient may be considered unethical [18, 19]. Instead, research should also target vulnerable groups, but greater care should be taken to specify the best interests of the population, and only research that offers a direct benefit to the population against acceptable risk should be conducted. It emerges that a clear benefit for both the deceased population as well as the family members needs to be clearly defined in the research study.

Regarding the consenting family members as a vulnerable group also highlights the need to ensure that they are satisfied with the consent and research study over time. Specifically, because of concerns about the consenting individual's state of mind at the time of consent, we think it is imperative to engage with those same family members after some time has elapsed, to ensure they are still satisfied with the consent they gave, have any questions about the samples, and to give an update on research progress.

While working with these vulnerable groups has generated concerns about potential exploitation, we have also experienced a reaction of 'empowerment' by the next of kin. Often, family members who are left behind seem to blame themselves for the death, wonder what they could have done to prevent the death and feel responsible for what happened. They feel very helpless following a death. The opportunity to participate in our projects has, at times, given family members a sense of hope and empowerment in that their family member

may not have died in vain, but could be seen as helping others, through this research. The feeling of empowerment has been reported before in other studies including vulnerable populations [24]. Hence, it is important to appreciate the full spectrum of reactions from family members and consequently to design mortuary-based projects with these reactions/feelings/emotions in mind.

Researcher's emotional skills: Pressures of approaching family members for conducting research

Research on this population is challenging and it places considerable pressure on researchers, as speaking to grieving family members about a research study is emotionally and practically challenging for various reasons. These include feelings of instability and uncertainty in order to be available at unpredictable times to go to the mortuary and meet the family, preparation for a wide spectrum of responses from the family (individuals may exhibit a blunted affect, be angry, crying or hysterical), possessing skills to provide articulate information about the research, as well as interview skills, whilst exhibiting strength, patience, sympathy, adaptability, commitment, knowledge, insight and credibility. Speaking to family members who have just lost an infant, for instance, is exceptionally taxing and debriefing after every session is essential for researchers who find themselves in similar situations.

Because of the challenges relating to seeking consent from recently bereaved family members, we consider the following elements of good practice:

- (i) Preference should be for individuals with prior training in the forensic autopsy setting to seek informed consent for such research;
- (ii) Thorough training in how to communicate about the research study is essential before researchers are left to their own devices. Such training is labour- and time-intensive and needs to be done carefully and sensitively. For this reason, it is unlikely that students or short-term research staff can be trained appropriately and these should only be involved if they already have some of the required skills or experience necessary to communicate with grieving family members appropriately;
- (iii) Counselling support for researchers is essential to help them develop coping mechanisms for dealing with the effects of repeatedly engaging with the trauma of families who have recently lost a family member or infant;

Ideally, there should be a designated person at the mortuary for all research studies but this may not be feasible.

Study feedback: Sharing results with family members under study

When designing these studies, one important ethical question that we considered was whether we should share individual genetic research results with family members; and if so, what results would be fed back. It was decided that during the research phase of the studies, information will not be fed back to the families. This was decided upon for various reasons; to work out project logistics, burdens and hindrances; to prevent families from revisiting the trauma in what may be the distant future; as well as due to the lack of clarity regarding what pathogenic mutations existed in the local populations [25]; in other words the initial pilot research was intended to be exploratory. Once clinically significant mutations have been shown to underlie suicide and SUDI, it is intended that a screening molecular assay will be set up and validated to be offered prospectively as part of the post-mortem investigation. In this instance, it is envisaged that a genetic counsellor would be involved to explain the significance of the genetic results with the family. However, it is also possible that aspects of genetic counselling may be introduced into the repertoire of training of forensic pathologists and scientists involved with molecular autopsies.

It is, however, imperative to make it clear in the informed consent process, that the family members will not receive feedback by participating in this research study. It should also be explained that the study is unlikely to benefit them or their family directly, but it is hoped that it will contribute to knowledge about suicide or SUDI in the future.

However, an unresolved challenge in our study is that of deciding what to do if, during exome sequencing of the deceased individual's DNA, an *unrelated* mutation is detected which would have implications on the living family. An example would be a *BRCA2* mutation which is likely to have been inherited from the parent. The ethical question is whether to inform the family of this mutation or not? We have tentatively established a policy that would allow us to return such findings if and only if three criteria are met: a) the mutation must be medically actionable; b) it must be unlikely that the condition would have been diagnosed without the genetic finding, and c) the mutations must have been validated across multiple studies that are relevant to the South African population [25]. Although we have not yet come across this, it is important to consider what would be done in such a case in the future. The anticipated challenges here are manifold, but include: the funding of validation studies, the availability and funding of a genetic counsellor to feedback information to the family, and also the challenge of contacting the bereaved family, again, and with further 'bad news'. These conditions and

limitations must be communicated to the family during the initial, already challenging consent process, which at this stage is exploring a wide range of issues. It is likely that, as the project progresses, and the research team develops, that the issue of unexpected findings may be dealt with more comprehensively.

On another level, because of the vulnerability of the consenting participants and because family members – and particularly parents of SUDI – may never forget about their child's sample sitting in a laboratory somewhere, it is imperative that researchers are conscious of the need for ongoing communication about study progress and generalised study findings. This could take the form of e.g. an annual meeting for all research participants (which may have the added benefit of bringing together people with similar experiences), individual genetic counselling sessions, or phone calls if and when the project reveals new and important findings. In our study, we intend to use a mix of all three approaches.

Conclusions

In order to accommodate these ethical concerns, we have developed a number of practices that aim to ensure forensic genetic studies are conducted without causing unnecessary upset. These include some practices that are part of good practice for research generally, and others which are specific to conducting research on deceased individuals. With regard to the first, it is imperative that researchers are transparent about the study – no matter how difficult it is to speak with recently bereaved individuals. It is also essential to provide contact numbers for the researchers, the ethics committee and also for any support services that are available. Considering the potential vulnerability of the family members, maintaining confidentiality is key, as is providing information about the study in the next of kin's own language. Recruitment processes need to be flexible enough to allow a multitude of individuals to be involved in deciding on research participation. This includes not only multiple family members, but also, for instance, religious leaders who accompany the family to the mortuary.

Specific to research being conducted on deceased individuals, we learned that it is important to provide study information within the identification process, before family members view the infant or suicide victim. It is also important to ensure that the consent process takes place in a suitable environment that is quiet and private – and many mortuaries would have such suitable spaces. Considering the emotional burden of working in these environments, it is imperative that researchers not only receive appropriate training to deal with the range of emotions they may encounter, but that they also receive counselling support to deal with any issue or stresses arising from this process. In addition, we advocate that

projects should consider budgeting for grief counselling support for family members, particularly considering that such services are overstretched in South Africa and likely to be so in other LMICs. Projects should also budget for ongoing information meetings with participants enrolled in the research projects, to update family members on project progress and to reiterate the information provided during the consent process. We would also advocate that 'forensic genetic counselling' is added to the training curriculum of genetic counsellors and potentially other medical genetics professionals.

We hope that our experiences will contribute to the development of a framework for conducting research in mortuaries in South Africa, especially as we get a better understanding of the contribution of genetic factors to death. This paper will feed into a meeting that we are hoping to organise with relevant stakeholders from across the country. During that meeting, we will aim to discuss the ethical challenges of doing mortuary research, gain broad input on the ways in which these challenges could be addressed, and identify points of consensus that can form the basis of a common approach to mortuary research.

Abbreviations

DFMT: Division of Forensic Medicine and Toxicology; DNA: Deoxyribonucleic acid; FPS: Forensic Pathology Service; LMIC: Low- and middle- income countries; SIDS: Sudden infant death syndrome; SUDI: Sudden unexpected death in infants; UCT: University of Cape Town

Acknowledgements

None

Funding

Not applicable

Availability of data and materials

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LJH and SM conducted the research studies; LJM and RR supervised the research.

JD contributed towards the ethical components of the research. LJH was the lead author of the manuscript. All other authors contributed towards the manuscript. All authors read and approved the final manuscript.

Authors' information

None

Ethics approval and consent to participate

These research projects were approved by the University of Cape Town Faculty of Health Science, Human Research Ethics Committee - Reference numbers: HREC: 713/2013 and 445/2015. Ethics approval for this particular study was covered under HREC: 445/2015.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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Received: 8 June 2017 Accepted: 20 November 2017

Published online: 29 November 2017

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4.4 Conclusion

This study was among the first prospective forensic genetic research investigations on deceased infants in a South African setting. This chapter summarised the process and logistical considerations necessary for this type of research. Further, due to the nature of this research, the investigator deemed it is necessary to critically evaluate and reflect on the approach used, particularly with regards to ethical considerations. Factors such as informed consent, dealing with a non-conventional vulnerable group, risks and benefits as well as the feedback of possibly unrelated genetics results were considered, and suggestions have been made accordingly. These factors should be taken into account by researchers wishing to undertake similar research. It is hoped that these experiences will contribute to the development of a framework for conducting research in mortuaries in South Africa.

Chapter 5

PILOT STUDY TO OPTIMISE WORKFLOWS

Assessment of biological sample types obtained from SUDI cases at autopsy for subsequent DNA analysis

5.1 Introduction

5.1.1 Blood samples

The detection of genetic variants in the DNA of individuals who have demised suddenly and unexpectedly has been shown to be of value in a forensic setting, and for living relatives who may carry the same polymorphisms [156]. In order for a comprehensive genetic analysis to be performed, a suitable biological sample needs to be obtained at autopsy. The recommended sample for a molecular autopsy, is 5 ml – 10 ml of blood, collected into a purple-capped blood vial with K₂EDTA as an anticoagulant [157].

In the investigation of SUDI cases in a local forensic setting, a 5 ml – 10 ml blood sample is not always feasible to obtain, as most SUDI cases only require an external autopsy for cause of death determination (Chapter 2). Obtaining a blood sample from such cases often requires an infants' body to be opened, as post-mortem pooling of blood, the size of infants (particularly premature infants) and dehydration of infants prevent such volumes of blood from easily being obtained via a syringe. In some SUDI cases, opening up of the body is necessary for determination of cause of death, and here, it is easier to obtain a blood sample for research

purposes. However, the opening up of infants' bodies which would have otherwise remained closed is undesirable in the local forensic environment due to the immense caseload. It is also unappealing for families of the deceased infants, particularly where the death is due to natural causes and where an internal examination is not necessary. As such, there is a need to investigate an alternative less-invasive biological sample to obtain at autopsy for subsequent DNA analysis.

5.1.2 Buccal swabs

One such sample is a buccal (inner cheek) swab, which is routinely used in forensics for the purposes of human identification [158]. This process entails forensic DNA profiling, which involves the generation and statistical analysis of a string of alpha-numeric characters which can be used to match or link unknown people to reference samples from known origin [159]. It is a sensitive technique which requires as little as 1 pg of DNA.

In South Africa this DNA profiling workflow is regulated by the Criminal Law (Forensic Procedures) Amendment Act 37 of 2013 and a recent amendment in this legislation has allowed for buccal swabs to be taken from individuals for reference identity purposes, in addition to the previously recognised sample type of blood [160]. In this regard, most forensic agencies in South Africa make use of the Whatman® EasiCollect™ Device (GE Healthcare Life Sciences, Buckinghamshire, UK) to collect DNA from living reference individuals, which utilises a Whatman® Flinders Technology Associates (FTA) card and thereby provides a stable long term storage solution for the DNA sample [161].

While the Whatman® EasiCollect™ device was originally intended for use in living individuals, its use in a post-mortem setting was recently investigated in a local setting, and

showed that full forensic DNA profiles could be obtained from deceased individuals (n = 30) older than 6 months of age [162]. However, when collecting buccal cells from deceased infants, especially those younger than 6 months of age, the Whatman® EasiCollect™ device can pose complications, as the foam applicator of the swab does not easily fit into the mouth of a deceased infant. Under these circumstances, and given that most SUDI cases occur when an infant is less than 6 months old, an alternative swab type should be explored. One option is the use of a cotton swab to collect buccal cells, which is smaller in size, non-invasive and cost effective.

5.1.3 Formalin fixed paraffin embedded tissue

In cases where an internal autopsy is performed on SUDI cases, the sample type most often collected for ancillary investigations is tissue (Chapter 2). To this end, tissue is collected into a liquid agent such as formalin, dehydrated with increasing concentrations of ethanol, subjected to a clearing agent such as xylene to displace the ethanol, and finally embedded in paraffin wax. These formalin fixed paraffin embedded (FFPE) tissue blocks can be sectioned, mounted on a slide and stained with a dye to be viewed under the microscope for histopathology investigations [163]. Since these samples are generally stored for long periods of time, they have the potential of being a valuable retrospective sample type for molecular analysis, especially if other samples such as blood or swabs have been depleted or were not obtained at autopsy [164–166]. Indeed, this sample type is currently being explored in a retrospective molecular autopsy study of SUD cases at the Pretoria Medico-Legal Laboratory [167].

While this method of preparation preserves the cytological integrity for histopathology analysis [168], FFPE tissue has been shown to be a challenging sample type for DNA analysis [169]. This is because formaldehyde, the active component of formalin, crosslinks nucleic acids and

proteins, as well as hydrolyses DNA, resulting in its severe fragmentation [170,171] In addition, the chemical modification of DNA, including the deamination of cytosine, have been shown to be inhibitory to polymerase chain reaction (PCR) [172]. The quality of DNA obtained from FFPE tissue is affected further by the exact method of tissue fixation, the age of the tissue at time of DNA extraction and storage conditions [173]. Attempts to increase success rates have included amendments to DNA extraction and PCR protocols, including deparaffinisation method, tissue quantity and thickness, tissue type, and DNA extraction method [173].

5.1.3.1 Tissue type and quantity

FFPE tissue specimens available in a post-mortem context typically include lung, heart, liver, kidney, spleen and at times, colon and brain [174]. Lung tissue is commonly obtained during local SUDI post-mortems as most cases are suspected and are eventually attributed to respiratory-related causes. Tissue types which are cell-rich are preferred for DNA extraction, as more nuclei would be available within a specific section size. Huijsmans *et al.* (2010) investigated four different tissue types (mammary, prostate, colon and lung) but did not report any differences in DNA yield and quality between tissue type [175]. However, they did show that proteinase K digestion was an important step during DNA extraction, particularly when commercial kits are used [175]. Kokkat *et al.* (2013) also reported no difference in DNA and RNA yields between lung, thyroid and salivary gland FFPE tissue [166]. Gillio-tos *et al.* (2007) successfully extracted DNA from 25 year old laryngeal FFPE tissue, but cautioned that not all samples were suitable for PCR amplification; only 69 %, 17 % and 5 % of samples could be amplified for a 152 bp, 268 bp and 676 bp fragment, respectively [176].

For DNA extraction, the FFPE tissue needs to be sectioned into thin slices, and most studies have reported the use of tissue sections between 3 - 20 microns in thickness, with the most

common being 10 microns [175–177]. The choice of tissue thickness was most likely guided by the manufacturer’s instructions, however, the use of thinner tissue sections may be preferred due to more surface area of the FFPE tissue being able to interact with reagents in the thinner FFPE tissue sections. The QIAamp® DNA FFPE Tissue kit (Qiagen, Hilden, Germany) manual also recommended multiple sections of tissue to increase yield [178]. While a multicentre study of 13 European laboratories seemed to all use 2 - 4 sections of 5 microns of tissue [179], overall, there does not seem to be a global consensus regarding the optimal tissue thickness and number of sections to use in DNA extraction.

5.1.3.2 Deparaffinisation methods

The next step in the DNA extraction process from FFPE tissue is the removal of paraffin wax and rehydration of the tissue. The traditional method of deparaffinisation is the reversal of the ‘dehydration process’; that is, the washing of FFPE tissue sections in xylene and then decreasing concentrations of ethanol washes. While this method has been effective [180], xylene is a hazardous chemical [181] and attempts to reduce the overall risk of this process has been made [182]. As such, several non-hazardous methods have been explored, including a commercial ‘deparaffinisation solution’ supplied with DNA extraction kits [178], mineral oil [182] and even distilled water [183].

While one study supported the use of incubation of FFPE tissue in hot distilled water, the methodology followed in the study and the conclusions drawn are questionable as the sample size was very small and matched control samples were not investigated [183]. Further, quantification was only performed via spectrophotometry, which measures gross nucleic acid content and does not represent the amount of usable, amplifiable human DNA within a sample. Molecular biology grade mineral oil has also been used for deparaffinisation, and some studies

have shown equivalent or better DNA results as compared to the traditional xylene and ethanol wash steps, but with reduced health risk and less turn-around time [182,184,185].

5.1.3.3 DNA extraction method

The DNA extraction method has also been shown to influence the amount of DNA obtained, and a study found that commercial kits yielded more DNA when compared to ‘in-house’ methods [175]. Different commercial kits have also been compared to each other and the preferred kit appears to be the QIAamp[®] DNA FFPE Tissue kit (Qiagen, Hilden, Germany) [180]. However, several other DNA extraction kits have also been successful [165], and some kits are less well studied; *e.g.* ZR FFPE DNA MiniPrep[™] Kit (Zymo Research, USA), the Nucleospin[®] DNA FFPE XS Kit (Macherey Nagel, Germany).

Overall, while FFPE tissue is perhaps not a biological sample of choice for molecular analysis prospectively, it does provide rich bank of retrospective samples for SUDI cases, whereby cause of death is undetermined and no other biological sample type is available [186]. There are numerous factors which affect the DNA yield and quality obtained from FFPE tissue and there is no consensus as to which combination of parameters is best [173].

5.1.4 Rationale and aim

Since this project is one of the first forensic genetics investigations at Salt River Mortuary, there is no baseline data regarding the quantity and quality of DNA obtained from biological samples obtained from SUDI cases at this mortuary, and their suitability for a molecular autopsy. In this regard, it is also important to assess the possibility of DNA contamination in these samples, as Salt River Mortuary is not specifically geared towards being a DNA-

controlled environment. While caution is taken when collecting samples for forensic DNA profiling, there is no feedback from collaborating agencies who perform this analysis as to whether there was contamination or not. Further, maternal DNA contamination may be ‘legitimately’ present in the mouths of infants due breastfeeding.

Therefore, a pilot study was undertaken at Salt River Mortuary to assess both prospective (*i.e.* blood and buccal swabs) and retrospective (*i.e.* FFPE tissue) biological sample types collected from SUDI cases at autopsy and their suitability for subsequent DNA analysis. To this end, the objectives were to optimise the DNA extraction protocol from FFPE tissue samples as well as to assess the quality and quantity obtained from all three sample types. These results would be used to inform prospective sample collection as well as to assess the viability to perform molecular autopsies retrospectively on available FFPE tissue samples.

5.2 Materials and methods

5.2.1 Cohort

Infants less than one year old who died suddenly and unexpectedly and who were referred to Salt River Mortuary for medico-legal investigation between 1 March 2016 and 10 June 2016, were included in the pilot study. During this time, a total of 84 SUDI cases were admitted to Salt River Mortuary, of which a subset were entered into this research, as a function of informed consent (Appendix 6) to participate in this research being obtained from the next of kin (see results).

Next of kin had the option of allowing buccal swabs and/or blood samples to be obtained from their infant at autopsy; but were only asked about DNA analysis on the FFPE tissue samples if FFPE tissue was going to be obtained for ancillary investigations, regardless. This approach

was adopted in the pilot study to reduce the stress inflicted on next of kin during this time of grief, as deciding to donate tissue samples for research is an emotional decision [187,188].

If consent was provided, 1 – 4 ml of blood was collected into a purple-capped EDTA collection vial (BD Vacutainer Systems, Plymouth, UK) and buccal cells were collected by wiping a cotton swab (Copan, Italy) on the infant's inner cheek for 10 seconds. Tissue samples were obtained at the discretion of the forensic pathologist for the purpose of determining of cause of death, and were retrospectively used in this study if parents gave informed consent. Collection of tissue was done by sectioning a small piece of the relevant organ(s) and placing this directly into 10 % formyl saline at autopsy. Samples were labelled and packaged appropriately, and transported to the laboratory for downstream processing. For tissue samples, this included the dehydration of tissues and embedding into paraffin wax according to internal standard operating procedures.

5.2.2 DNA extraction

5.2.2.1 Blood

DNA was isolated from blood according to a standard salting out method [189], using an extended 24 hour proteinase K digestion step, and eluting into 300 µl of Tris-EDTA buffer. DNA extraction was performed on average 8.5 days after infant death.

5.2.2.2 Buccal swabs

DNA was isolated from buccal swabs using the Quick-gDNA™ MiniPrep kit (Zymo Research, USA) according to the manufacturer's protocol [190], and DNA was eluted in 50 µl of the supplied Elution Buffer. A kit with spin-columns was used for buccal swabs to maximise the

yields and purity of DNA obtained from swabs. DNA extraction for buccal swabs was performed on average 4.5 days after infant death.

5.2.2.3 FFPE tissue

Optimisation overview

The DNA extraction protocol from FFPE tissue had to be optimised in order to assess variables such as (i) tissue type, (ii) number of tissue sections, (iii) deparaffinisation method and (iv) DNA extraction kit. An optimisation experiment was designed and carried out in two main phases, as summarised in Figure 5.1.

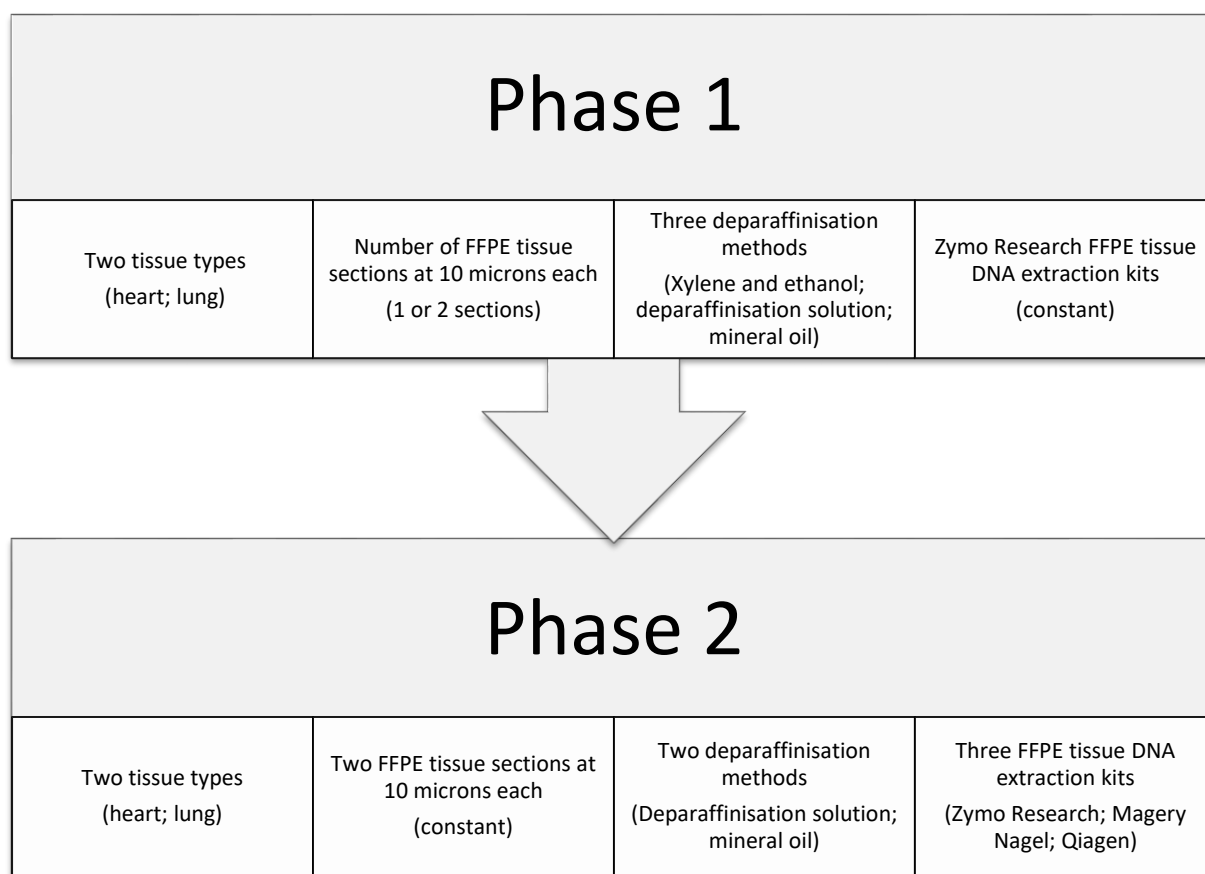


Figure 5.1: Phases of DNA extraction optimisation from FFPE tissue.

In phase 1, two tissue types, two different numbers of sections of FFPE tissue and three deparaffinisation methods were tested, while the kit remained constant. In phase 2, the number of FFPE tissue sections was decided (*i.e.* two sections) and kept constant, while three DNA extraction kits, two tissue types and two deparaffinisation methods were tested.

Optimisation: phase 1

In phase 1, two tissue types, two different section numbers and three deparaffinisation methods were tested in every combination with each other, using the same ZR FFPE DNA MiniPrep™ kit, initially on two of the SUDI cases. Lung and heart tissue were chosen, as they were the tissue types most available from SUDI cases investigated at Salt River Mortuary. These tissue sections were also mounted onto a slide and stained with haematoxylin and eosin (H&E) as per the internal standard operating procedure, and viewed under the Leica DM500 compound microscope (Leica Microsystems, Switzerland) to assess the cell density in each tissue section.

Photographs were taken with the microscope's built-in camera and processed using the Leica Application Suite LAS EZ version 3 software (Leica Microsystems, Switzerland).

One 10 micron tissue section and two 10 micron tissue sections were tested; based on 10 microns being a commonly used tissue thickness in previous studies, and the possibility of multiple sections improving DNA yield. In all instances, heart and lung FFPE tissue from each case was cut into multiple 10 micron sections by the medical technologist at the FPS Histopathology Laboratory, University of Cape Town. This was achieved using the Leica RM2125 RT5 microtome (Leica Microsystems, Switzerland), using clean microtome blades with each new sample. Excess wax surrounding the tissue was removed using a sterile scalpel, after which the FFPE tissue (either one or two sections) was placed into a micro-centrifuge tube.

Lastly, the traditional xylene and ethanol washes, deparaffinisation solution and mineral oil were tested as the deparaffinisation methods. For xylene and ethanol deparaffinisation, the FFPE tissue was incubated in 1 ml of xylene for 1 hour, and then washed with 100 %, 90 % and 75 % ethanol in a stepwise fashion. Alternatively, FFPE tissue samples were incubated in 400 µl of deparaffinisation solution at 55 °C for 1 minute, according to the ZR FFPE DNA MiniPrep™ manual [191]. Deparaffinisation with mineral oil was performed as previously described by Heikal *et al.*, (2014), whereby the FFPE tissue sample was incubated in 300 µl mineral oil at 90 °C for 20 minutes [165]. Following deparaffinisation, the tissue digestion and purification steps were performed using the ZR FFPE DNA MiniPrep™ kit, according to the manufacturer's protocol [191].

Upon analysis of these results, it became clear as to how many sections of tissue should be used in the DNA extraction process, but the results regarding the tissue type and deparaffinisation method were less apparent. These two variables were therefore tested on an additional two cases, using the same methods as described above, but keeping the DNA extraction kit and tissue section number constant. From the results obtained, the xylene and ethanol wash method produced DNA yields equivalent or poorer than the other methods, and given its health risks, was excluded from further analysis.

Optimisation: phase 2

In an attempt to improve DNA yield and quality further, two additional DNA extraction kits were tested: the Nucleospin[®] DNA FFPE XS Kit (Macherey Nagel, Germany) and QIAamp[®] DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The Qiagen kit was chosen as it has been commonly used in other studies; in contrast, there is little literature available on the other two kits were and their comparative performance to the Qiagen kit. For each of the three kits, the two tissue types (heart and lung) as well as two deparaffinisation methods (deparaffinisation solution and mineral oil) were assessed for each of the two original cases analysed in phase 1. In this phase, it was not deemed necessary to further investigate the number of tissue sections, as two sections consistently produced better results than one section (see results). Tissue preparation and DNA extraction was carried out as described above, according to each kits' respective manufacturer's instructions, with no deviations. To assess which variables yielded the best DNA yield and quality, the methods outlined in Section 5.2.3 were followed.

Final DNA extraction procedure

DNA was extracted from the remaining FFPE tissue samples using the procedure which yielded the best quantity and quality of DNA as per optimisation. This included using two sections of

10 microns of lung tissue, incubation in 400 µl of Deparaffinisation Solution (Zymo Research, USA) at 55 °C for 1 minutes and processed with the QIAamp[®] DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. These samples were processed approximately 12 to 18 months after collection at autopsy to simulate the time lapse between obtaining such samples and processing them in the authentic context.

5.2.3 DNA quantification and quality assessment

DNA obtained from optimisation experiments and from all samples (collected from the recruited cases) was analysed using a variety of methods to assess various aspects of the DNA yield, integrity and contamination:

5.2.3.1 Spectrophotometry

To measure the gross nucleic acid concentration, as well as purity of samples, each DNA sample was quantified using the Nanodrop[™] 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Subsequent to blanking the instrument, a volume of 2 µl of DNA was loaded onto the pedestal and measured. Based on Beer-Lambert's law, the absorbance of light at 260 nm was used to calculate the concentration of nucleic acids present in the sample. Absorbance ratios of 260/230 and 260/280 were also recorded to provide information about the DNA purity.

5.2.3.2 Fluorometry

The Qubit[®] 3.0 fluorometer (Thermo Fisher Scientific, Waltham, USA) was used to quantify double stranded DNA. The Qubit[®] dsDNA Broad Range (BR) kit (quantification range: 2 ng – 1 000 ng) was used to quantify DNA from blood samples, whereas the Qubit[®] dsDNA High Sensitivity (HS) kit (quantification range: 0.2 ng - 100 ng) was used for the DNA from the

buccal swabs and FFPE tissue. The quantification kit utilised was aligned with the sample type's DNA concentration range as screened by spectrophotometry, for accuracy purposes. The manufacturer's protocols were followed with no deviations.

5.2.3.3 Quantitative real time PCR

To assess the amount of DNA which was amplifiable in each sample, as well as DNA degradation, absolute quantitative real time PCR (qPCR) was performed. This was achieved using the Quantifiler® Trio qPCR kit (Applied Biosystems, Foster City, USA), as previously described [192]. Prior to amplification, and using the spectrophotometric concentration as a guide, samples were diluted to 5 ng/µl using AccuGENE™ molecular biology grade (MBG) water (Lonza, Basel, Switzerland) in order to ensure that DNA concentrations fell within the range of the standard curve. Amplification was performed on the 7500 thermal cycler (Applied Biosystems, Foster City, USA).

5.2.3.4 DNA profiling

To assess the presence of possible contamination of the samples, either from the mortuary or from the mother during breast feeding, DNA samples underwent forensic DNA profiling using the PowerPlex® ESI 16 System (Promega Corporation, USA), according to the manufacturer's protocol. PCR was performed on the T100 Thermal Cycler (BioRad, Hercules, USA) and capillary electrophoresis was conducted using the ABI 3130xl Genetic Analyser (Applied Biosystems, Foster City, USA) using conditions optimised during internal validation. Results obtained were viewed using GeneMapper® software Version 4.0 (Applied Biosystems, Foster City, USA). Electropherograms were interpreted using an analytical threshold of 32 relative fluorescent units, as determined during internal validation.

As suggested by the manufacturer's protocol, DNA profiling was initially performed according to spectrophotometric concentrations of DNA. After analysing the results, DNA profiling for the buccal swab samples was repeated using the concentration of input DNA as determined by qPCR, which is more conventional when processing forensic samples [193]. DNA profiling on DNA extracted from blood samples was only performed once, using spectrophotometric quantification. Unfortunately, the FFPE tissue samples were too degraded for this analysis.

5.2.3.5 PCR and Sanger sequencing

In order to assess the usability of DNA from these sample types for DNA sequencing or genotyping, the gold standard method of Sanger sequencing was employed. To this end, primers were designed to amplify a 217 bp fragment within the *toll-like receptor 4 (TLR4)* gene and the PCR was optimised (see Chapter 6 and Appendix 7). The choice of this gene fragment is motivated for in Chapter 6, but for the purposes of this analysis, it could have been any gene fragment. The size of approximately 200 bp however, was important, as shorter fragments of DNA (≤ 200 bp) are preferred for analysis of typical forensic samples and for FFPE tissue samples, both of which are often degraded [175].

DNA from each sample was added to a master mix containing 0.4 μ M of each primer and 1X GoTaq[®] Green ReadyMix (Promega, Madison, USA) in a total volume of 25 μ l. For the no template control, MBG water was added instead of DNA. An amount of 50 ng of DNA was preferred, however, if sample concentration was too low, then less DNA was added. Thermal cycling was as follows: initial denaturation at 96 °C for 5 minutes; 30 cycles of 96 °C for 30 seconds, 54.7 °C for 30 seconds and 72 °C for 30 seconds; followed by a final extension at 72 °C for 5 minutes. All PCRs and incubations in this study were performed in a T100 thermal cycler (BioRad, Hercules, USA).

Post-PCR products were electrophoresed on a 1 % agarose gel at 160 Volts (V) for 1 hour, which contained agarose powder (SeaKem[®] LE agarose; Lonza, USA) dissolved in 1X Tris-Borate-EDTA (TBE) buffer (Sigma-Aldrich, St. Louis, USA) and 0.01 % (v/v) SYBR[™] Safe DNA Gel Stain (Invitrogen, Carlsbad, USA) for subsequent visualisation. A 50 bp DNA ladder (New England Biolabs, Ipswich, USA) was also included in the agarose gel process for estimation of amplicon sizes. The gel was visualised under ultraviolet (UV) light on the Chemi Genius 2 Bio-Imaging System (Syngene, Gurugram, India).

Successfully amplified PCR products then underwent a post-PCR clean up to remove unincorporated dNTPs and primers. This included incubation of the post-PCR produce in 0.1 unit of *exonuclease 1* and 1 unit of recombinant shrimp alkaline phosphatase (rSAP) at 37 °C for 1 hour followed by 75 °C for 15 minutes. Cleaned products were then sequenced with the BigDye[®] Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, USA) according to the manufacturer's protocol. Capillary electrophoresis was performed on the ABI 3130xl Genetic Analyser (Applied Biosystems, Foster City, USA).

5.2.4 Data analysis

5.2.4.1 Assessment of DNA degradation

The Quantifiler[®] Trio qPCR chemistry (Applied Biosystems, Foster City, USA) quantifies three target types within human and higher primate DNA using a multiplex TaqMan[®] approach with higher-primate specific primers: (i) a single target on the Y-chromosome; (ii) a multi-copy 214 bp autosomal locus (referred to as the large target); and (iii) a 80 bp multi-copy autosomal locus (referred to as the small target). The presence or absence of amplification of the Y-chromosome target was an opportunity to cross-check the sex of the donors of the samples,

which served as an intermediate quality check. The concentration of DNA as determined by amplification of the small and large fragments of DNA respectively, was compared in a ratio to determine the ‘degradation index’ of the sample (Equation 5.1). If the sample was degraded, it was expected that there would be more amplification of the short fragment compared to the large; thus a degradation index > 1 was indicative of degraded DNA; with the higher the degradation index, the more degraded the sample [192]. A degradation index of ≤ 1 however, was indicative of DNA which was not degraded. This approach was therefore used to determine the usability of the sample for downstream analysis.

$$\text{Degradation index} = \frac{\text{concentration of 80 bp amplicon}}{\text{concentration of 214 bp amplicon}}$$

Equation 5.1: Calculation used to determine the degradation index of the DNA sample.

5.2.4.2 Assessment of DNA usability for next generation sequencing

The quantitative functional index (QFI) was calculated, which indicates the usability of the sample for next generation sequencing (Equation 5.2). If the QFI is above 6.6 %, the sample is considered suitable for next generation sequencing [171].

$$QFI = \frac{\text{Amplifiable DNA concentration as measured by qPCR}}{\text{Total DNA concentration as measured spectrophotometrically}} \times 100$$

Equation 5.2: Calculation used to determine the quantitative functional index, which was used to predict the usability of the sample for next generation sequencing.

5.2.4.3 Statistical tests

Graphical representation of data as well as associated statistical tests were conducted using GraphPad Prism version 6.0. Non-parametric tests were performed since data did not follow a Gaussian distribution, as measured using the Shapiro-Wilk test. Two-way ANOVA tests were performed to assess (i) if two sections of tissue yielded significantly more DNA as compared to one section; and (ii) if the DNA extraction kit significantly affected DNA yield. The Mann-Whitney U tests were used to assess if DNA concentration differed significantly between buccal swabs and FFPE tissue, for each quantification method; while the Wilcoxon rank-sum test was used to assess if DNA concentrations as quantified by qPCR were significantly lower than those obtained spectrophotometrically, within a sample type. To assess if (i) degradation index and (ii) QFI were significantly different between blood, buccal swabs and FFPE tissue, pairwise Mann-Whitney U tests were used. Lastly, a Spearman Rank test was performed to assess the association between obtaining a partial forensic DNA profile and the time between death and DNA extraction for blood and buccal swabs.

5.3 Results

5.3.1 Cohort and samples

During the pilot study period, 84 SUDI cases were admitted to Salt River Mortuary, of which 64 families were approached for informed consent. Out of the 64 families, 3 declined to participate, while 61 agreed. However, in 6 out of 61 cases, consent was obtained but the forensic pathologist did not take the samples, perhaps due to forgetting in the midst of their large caseload (Figure 5.2).

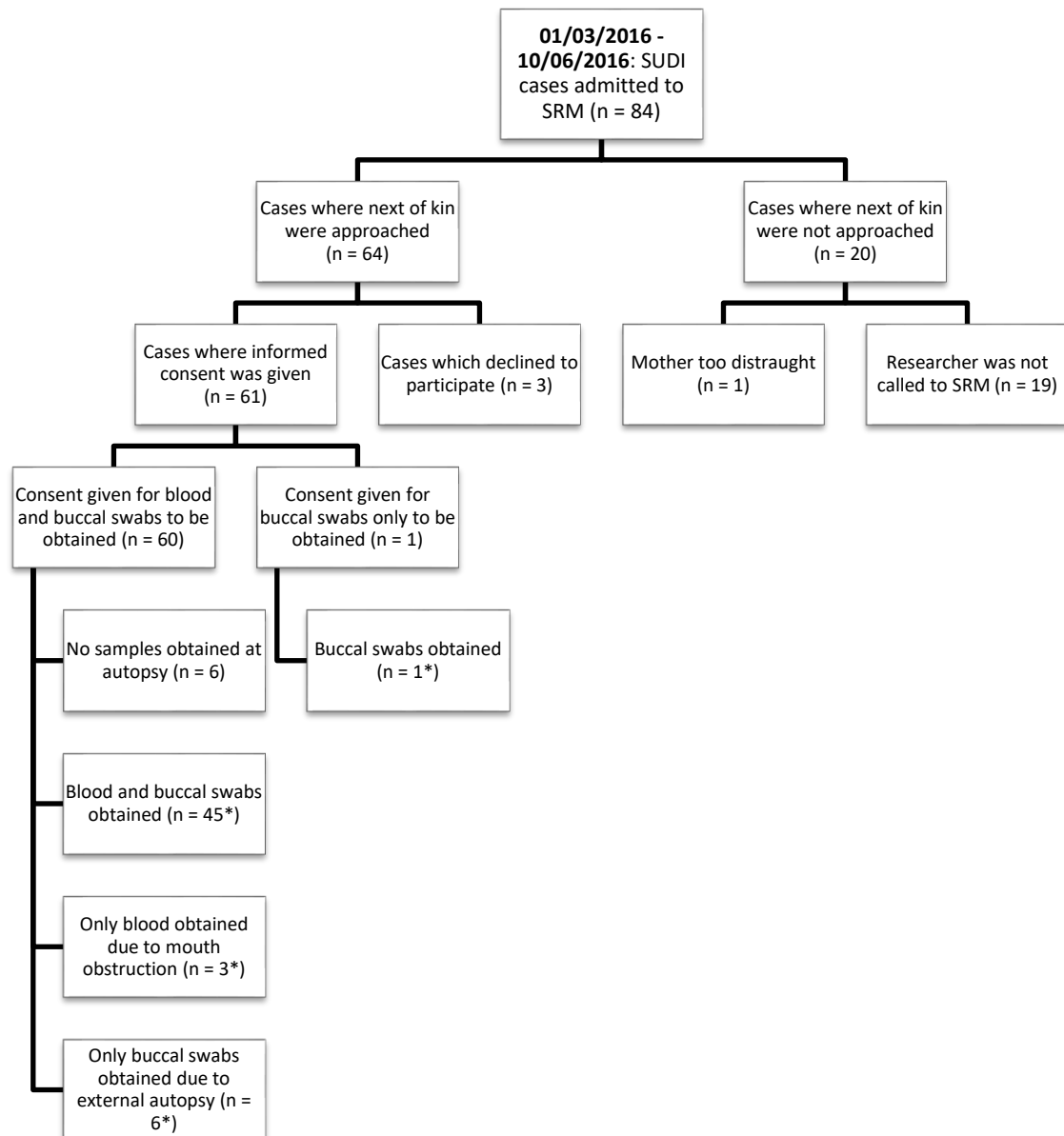


Figure 5.2: Flow diagram depicting the SUDI cases included in this pilot study.

A total of 84 SUDI cases were admitted to Salt River Mortuary between 1 March 2016 and 10 June 2016, of which 55 cases were included in the molecular analysis. * 14 out of the 55 cases included in this study also had FFPE tissue available for molecular investigation.

Thus, of the 55 cases included in the pilot study, blood and buccal swabs were obtained for 45 cases; only blood was obtained in three cases an obstruction (*e.g.* excessive foam) was present in the mouths of the infant; and in the other seven cases, only buccal swabs were obtained. The latter was because it was not possible to obtain blood from the infants ($n = 6$) without an internal

autopsy (which was not necessary to determine cause of death), or the parents only gave consent for buccal cells to be obtained (n = 1). Further, of these 55 cases, 14 also had FFPE tissue obtained for ancillary investigations and permission had been obtained to perform molecular testing on all of these samples (Figure 5.3).

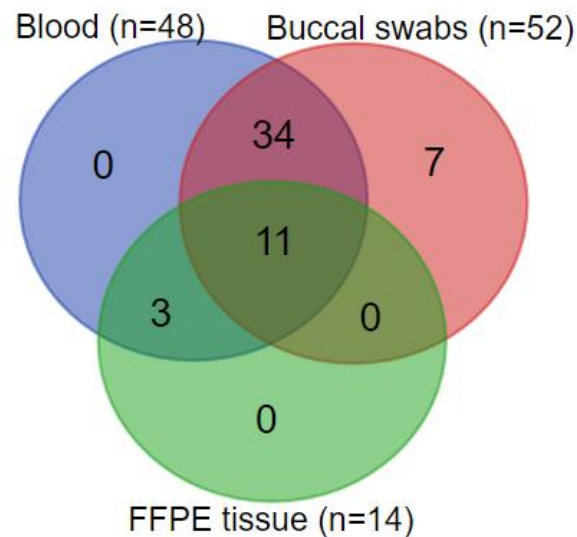


Figure 5.3: Sample types collected from the 55 cases included in the pilot study.

Blood samples were collected from 48/55 infants, buccal swabs were collected from 52/55 infants and FFPE tissue was available from 14/55 infants.

A total of 20 cases were not approached for participation in the study for various reasons: a mother seemed too distraught to be asked (n = 1), the parents came to identify their infant outside of working hours (especially in the case of Muslim infants) (n = 2), while in the remaining cases, the FPOs were too busy at the time to call the researcher to the mortuary to speak to the families about the research study.

5.3.2 Optimisation of DNA extraction procedure from FFPE tissue

The majority of optimisation testing was performed on FFPE tissue from two SUDI cases. Images of representative parts of each FFPE tissue sample is shown in Figure 5.4. In case 1, the lung tissue appeared to have more nuclei, while in case 2, the heart tissue had more nuclei. The lung tissue in case 2 also appeared to be decomposed.

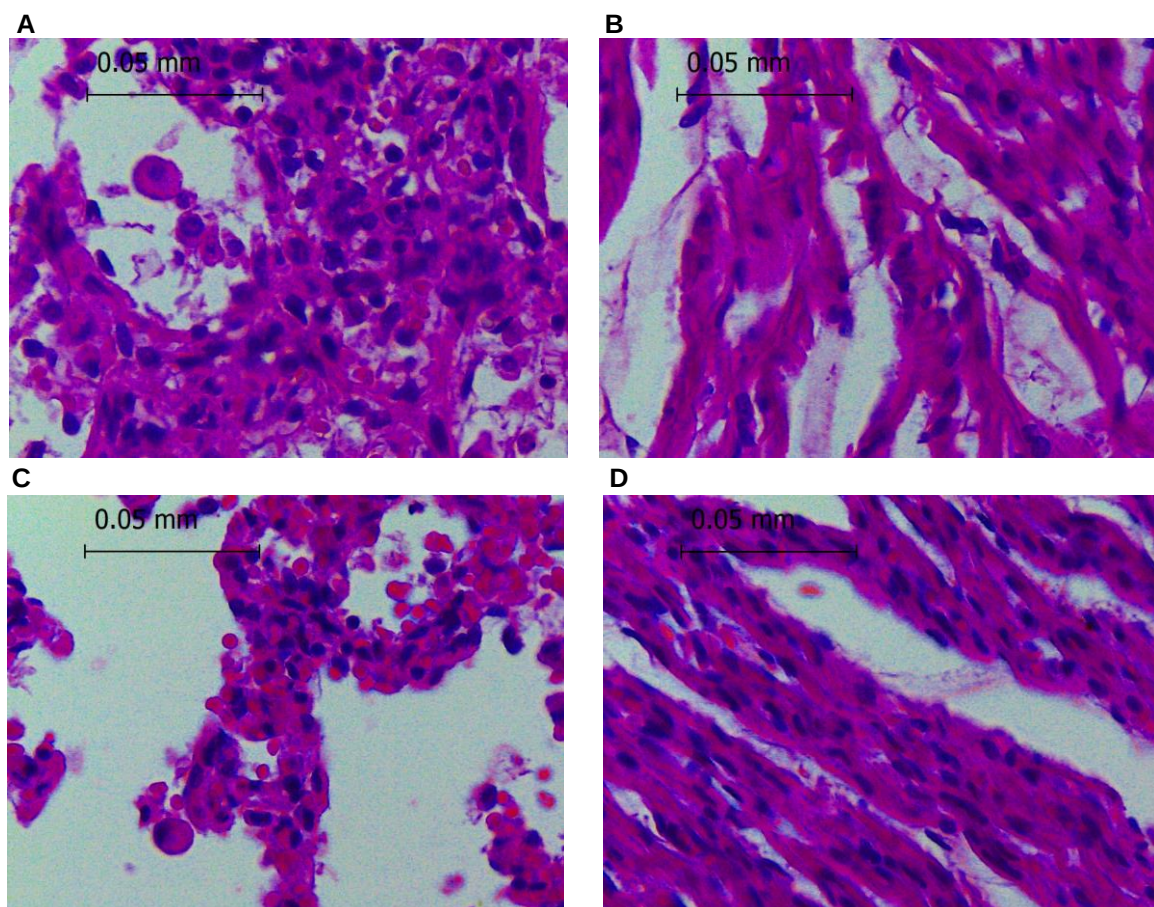


Figure 5.4: Sections of FFPE tissue used for optimisation (40X).

Tissue sections were stained with haematoxylin and eosin. A: case 1 lung tissue; B: case 1 heart tissue; C: case 2 lung tissue; D: case 2 heart tissue

In phase 1 of DNA extraction optimisation from FFPE tissue, three variables were tested on two SUDI cases. The DNA obtained was quantified in various manners and the results compared. In all instances, two sections of FFPE tissue, each at 10 microns, yielded

significantly more DNA compared to matched samples with one section of 10 microns ($p = 0.0006$). Figure 5.5 demonstrates this using the short target (80 bp) qPCR results, but the less specific quantification methods, such as spectrophotometry and Qubit® fluorometry, showed the same trend. As a result, two sections of FFPE tissue, each at 10 microns, was used for subsequent DNA extractions.

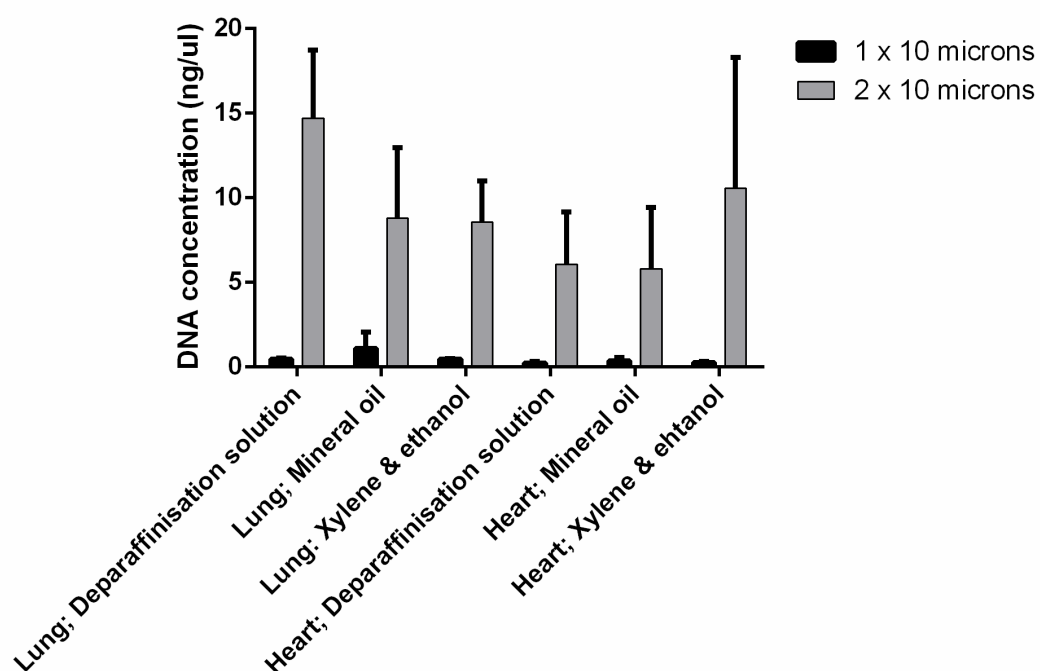


Figure 5.5 Median DNA concentration (with upper range) obtained from one and two sections of FFPE tissue, each at a thickness of 10 microns.

Two sections of tissue, each at 10 microns, produced significantly more DNA than one section of tissue of the same thickness ($p = 0.0006$).

It emerged that lung was a better tissue type in case 1, while heart was a better tissue type in case 2 (data not shown). It was also not apparent which deparaffinisation method was better, as the outcome varied between the two cases. As such, these two variables (tissue type and deparaffinisation method) were investigated further on two additional cases. Figure 5.6 shows

(A) median DNA concentration results obtained from Qubit® fluorometry and (B) DNA degradation index of the four samples. Lung tissue prepared with deparaffinisation solution had the highest DNA concentration and least degradation out of the four combinations (Figure 5.6).

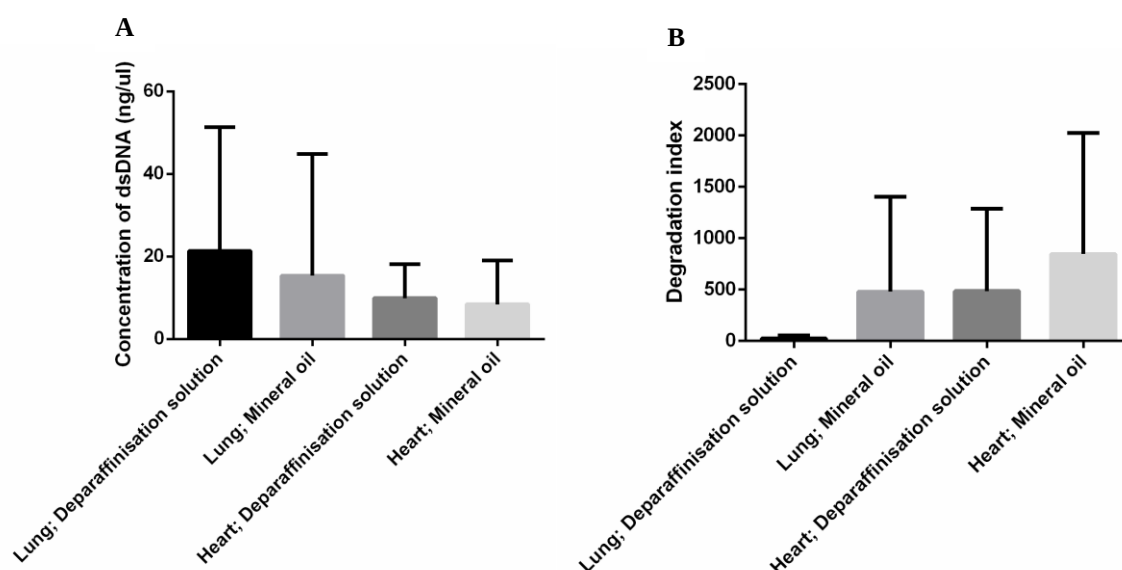


Figure 5.6: (A) The median DNA yield with 95 % confidence intervals as quantified by Qubit® fluorometry, and (B) mean degradation index with standard deviation, of lung and heart tissue obtained from four SUDI cases deparaffinised with different methods.

Lung tissue prepared with deparaffinisation solution produced higher yields of DNA and less degraded DNA compared to the other combinations.

In an attempt to improve DNA yields and quality even further, phase 2 of optimisation was carried out, whereby the objective was to compare the performance of three different DNA extraction kits. To this end, lung and heart FFPE tissue sections from the original two SUDI cases were each prepared using deparaffinisation solution and mineral oil, and extracted using two additional kits.

The concentration of DNA as quantified by qPCR (80 bp fragment) showed that the QIAamp® DNA FFPE Tissue kit (Qiagen, Hilden, Germany) kit produced significantly higher yields of DNA independent of tissue type and deparaffinisation method ($p = 0.0223$) (Figure 5.7). Within DNA extraction kits, all combinations of tissue type and deparaffinisation methods produced results that were not significantly different from another.

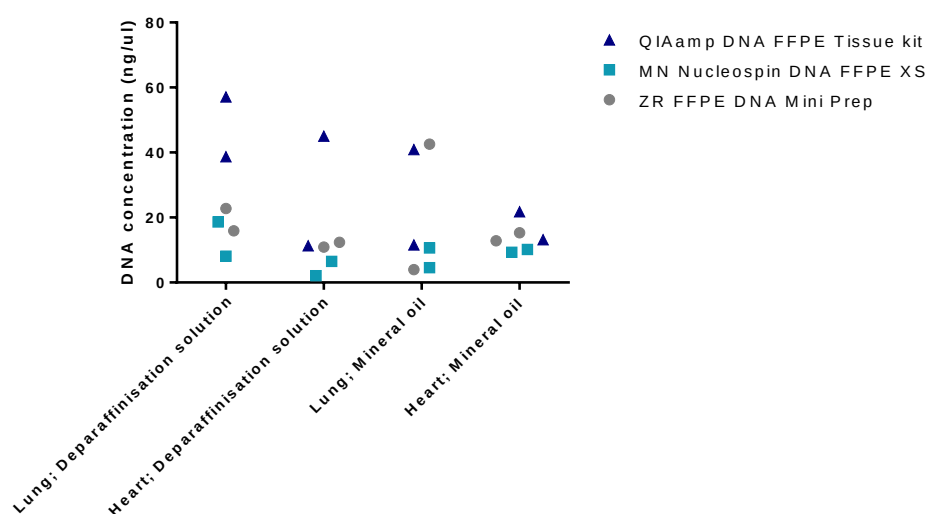


Figure 5.7: DNA concentration of an 80 bp target (measured by qPCR) of samples extracted with different DNA extraction kits.

The QIAamp® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) yielded significantly more DNA than the other two kits ($p = 0.0223$).

To assess the purity of the DNA obtained from the three extract kits, absorbance ratios were analysed. Overall, the absorbance ratio A260/280 was mostly above 1.8 for all samples, which indicated little protein presence, independent of DNA extraction kit. The A260/230 ratio was below the accepted 1.8 threshold for all samples when the Nucleospin® DNA FFPE XS Kit (Macherey Nagel, Germany) was used; and all but one sample when the ZR FFPE DNA MiniPrep™ Kit (Zymo Research, USA) was used. However, when the QIAamp® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) kit was used, the A260/230 ratio was above the 1.8 threshold for all samples, indicating fewer salt and organic compound impurities (Figure 5.8).

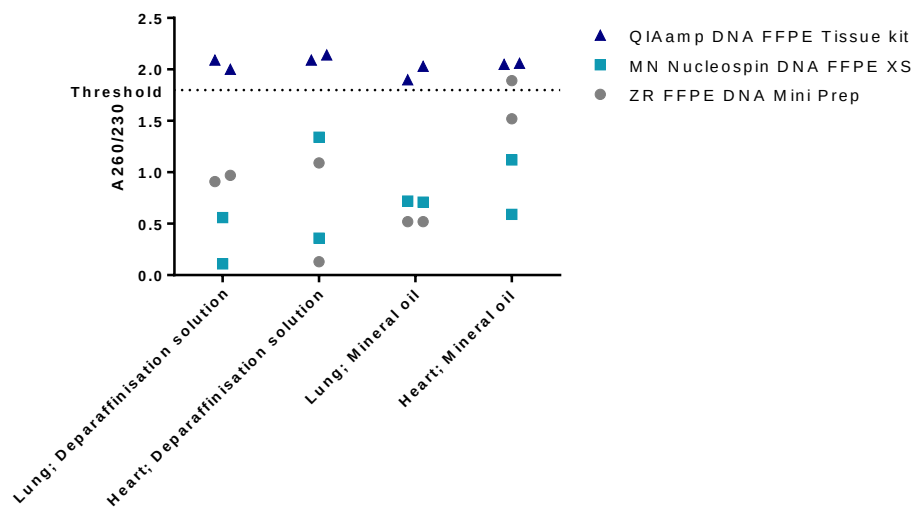


Figure 5.8: The 260/230 absorbance ratio for each sample extracted with different DNA extraction kits. The QIAamp® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) consistently yielded DNA with an A260/230 ratio higher than the 1.8 threshold.

5.3.3 DNA quantity and quality assessment

DNA isolated from biological samples were quantified using three different methods. For the blood samples, the total yield of DNA, measured spectrophotometrically, was 102.9 µg (median), which was 46-fold higher than that obtained from buccal swabs (median = 2.2 µg); and 11-fold higher than that obtained from FFPE tissue (median = 9.1 µg). The yield of DNA obtained from these three sample types were significantly different from each other ($p < 0.0001$ for each pairwise test).

A similar trend was observed when sample types were measured fluorometrically; the median DNA concentrations between blood (192.4 ± 469.2 ng/µl), buccal swabs (14.38 ± 23.86 ng/µl) and FFPE tissue samples (44.45 ± 35.2 ng/µl) were also significantly different from each other ($p < 0.0001$ for each pairwise test).

Diluted DNA from blood samples all amplified as expected at both the 80 bp and 214 bp targets during qPCR. Similarly, DNA from all buccal swabs amplified for both targets; however, 4/14 (28.57 %) FFPE tissue samples failed to amplify at the 214 bp target. Therefore, while seemingly more DNA was observed from FFPE tissue compared to buccal swabs, its ability to be amplified was substantially less.

Overall, statistically significant differences were observed between sample types when quantified using (Figure 5.9). Further, significantly less DNA was quantified when using qPCR compared to spectrophotometry for each sample type (buccal swabs: $p < 0.0001$; FFPE tissue: $p < 0.0001$) (Figure 5.9).

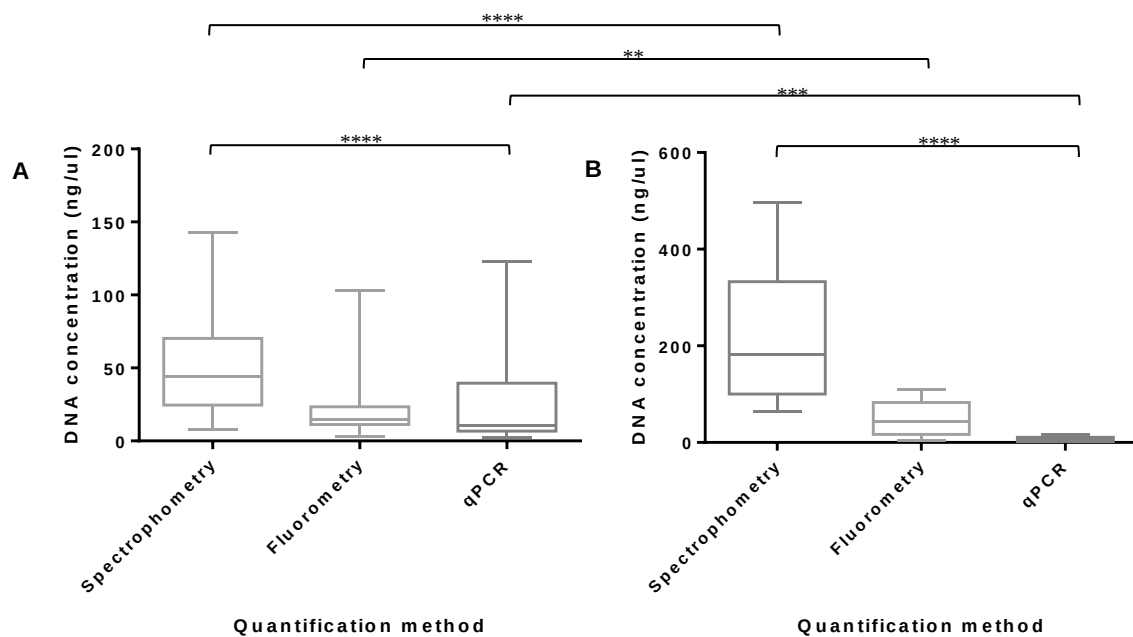


Figure 5.9: DNA concentration obtained from (A) buccal swabs and (B) FFPE tissue samples using three different quantification methods.

The DNA concentrations obtained were significantly different between quantification methods and sample type.

The assessment of DNA degradation indicated that DNA obtained from blood samples was not degraded, and was significantly less degraded than both buccal swabs ($p < 0.0001$) and FFPE tissue samples ($p < 0.0001$) (Figure 5.10).

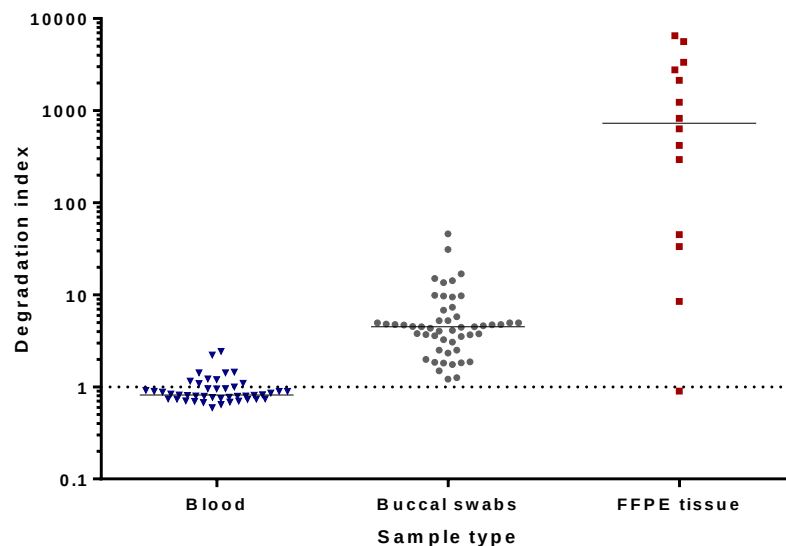


Figure 5.10: Degradation index scores for blood, buccal swab and FFPE tissue samples.

The y-axis is represented as a log scale. The dotted line at $y = 1$ represents where DNA is not degraded. DNA from FFPE tissue was significantly more degraded than that from buccal swabs ($p < 0.001$), both of which were significantly more degraded than blood samples ($p < 0.001$).

The QFI of each sample type was also analysed, whereby DNA from all blood samples had a $QFI > 49$, and had significantly higher QFI scores compared to buccal swabs ($p < 0.0001$) and FFPE tissue samples ($p < 0.0001$) (Figure 5.11). DNA from buccal swabs also had a significantly higher QFI scores compared to FFPE tissue samples ($p < 0.0001$), indicating that buccal swabs are better suited for next generation sequencing compared to FFPE tissue.

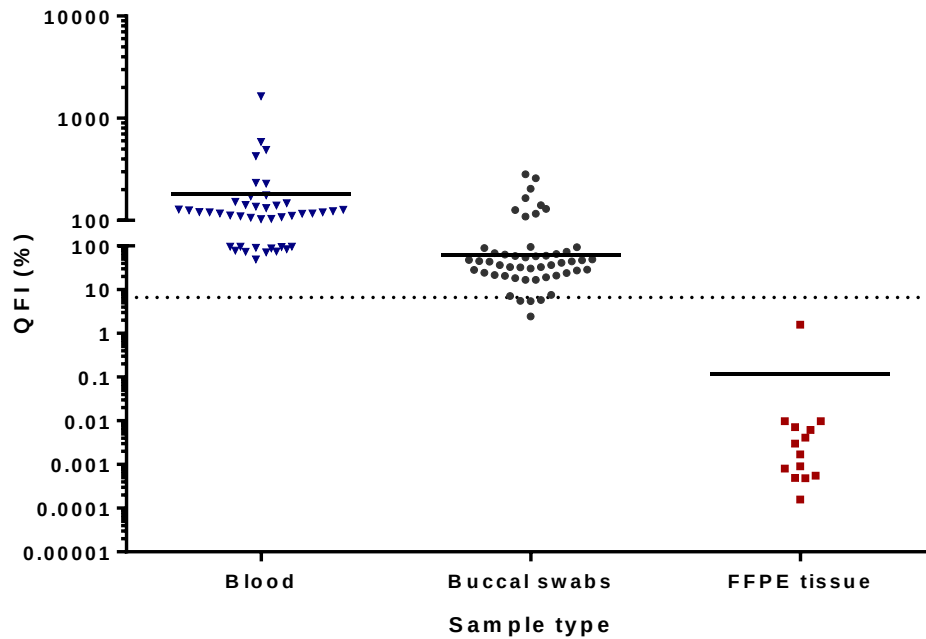


Figure 5.11: The quantitative functional index (QFI) for blood, buccal swab and FFPE tissue samples.

The y-axis is represented as a log scale. The dotted line represents the 6.6 % threshold, whereby samples above this line are deemed suitable for next generation sequencing. DNA from FFPE tissue had significantly lower QFI scores compared to that obtained from buccal swabs ($p < 0.001$), and both of these sample types had significantly lower QFI scores compared to blood samples ($p < 0.001$).

5.3.4 DNA profiling

Forensic DNA profiling was performed to assess if DNA contamination was present in the samples. Overall, no forensic DNA profile showed a mixed profile, thus indicating that DNA samples were each from a single source. The forensic DNA profiling methods and results were analysed further as some results showed partial profiles. Initially, the amount of input DNA template added to the PCR was calculated based on the DNA concentration of the respective samples as measured by spectrophotometry. While full forensic DNA profiles were obtained for all DNA samples extracted from blood, only 31.58 % full forensic DNA profiles were obtained from DNA extracted from buccal swabs (Figure 5.12).

It was hypothesised that the amount of input DNA was not optimal as spectrophotometry quantifies total nucleic acid, regardless of its integrity or species. DNA profiling was therefore repeated on DNA extracted from buccal swabs, but using the concentration of DNA as measured by qPCR to calculate the amount of input DNA required for PCR. Upon repetition, full forensic DNA profiles were obtained in 84.21 % samples, a 2.6-fold increased performance (Figure 5.12).

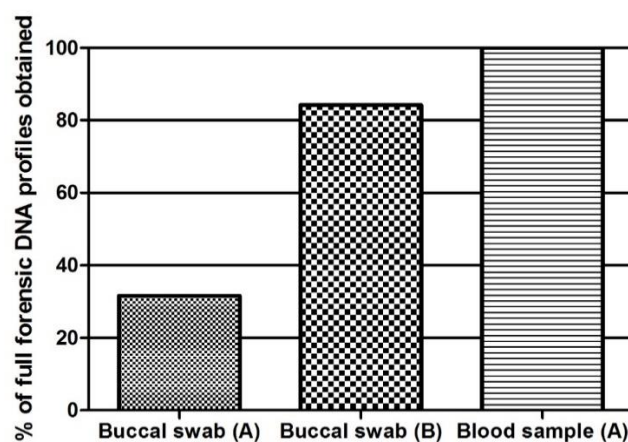


Figure 5.12: Percentage of full forensic DNA profiles obtained from buccal and blood samples quantified using different DNA quantification methods: (A) spectrophotometry and (B) qPCR.

Blood samples produced full forensic DNA profiles in all cases, while DNA extracted from buccal swabs produced DNA profiles from 84.21 % of samples when DNA was quantified using qPCR.

The remaining buccal samples all yielded partial profiles upon quantification using qPCR, all of which had alleles above the analytical threshold at ≥ 8 out of 16 markers. Figure 5.13 depicts one channel of the forensic DNA profile obtained from the buccal swab from one individual, and under the different input DNA quantification methods. The samples which generated partial profiles, when quantified using qPCR, all had degradation indices higher than 4.0 (range 4.041 – 9.77).

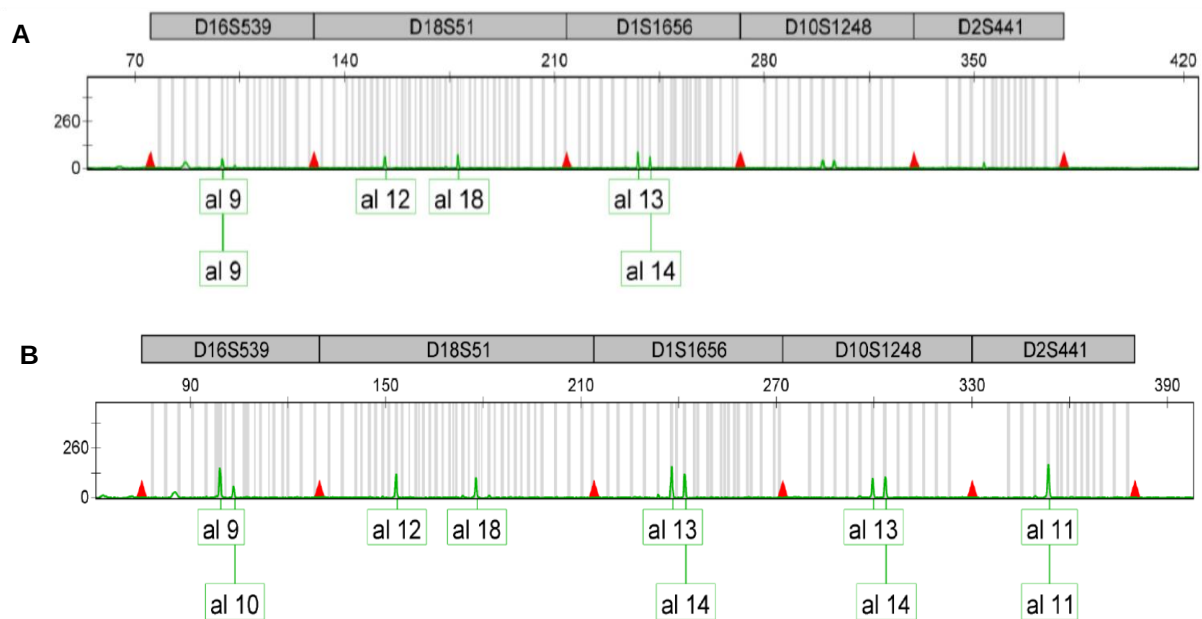


Figure 5.13 One channel from the electropherogram representing forensic DNA profiles obtained for a buccal DNA swab using (A) spectrophotometry or (B) qPCR to determine the amount of input DNA. When DNA was quantified using spectrophotometry and its concentration used for dilutions prior to DNA profiling, only a partial profile was obtained.

Following the determination of degradation index, it was postulated that the degree of degradation may be due to the time between death and DNA extraction. However, no significant association was found between time and degradation index for both buccal swabs ($p = 0.179$) and blood samples ($p = 0.296$). When plotting degradation index versus days between death and DNA extraction, it was found that no difference in the slope of the best fit line existed between the two sample types (Figure 5.14).

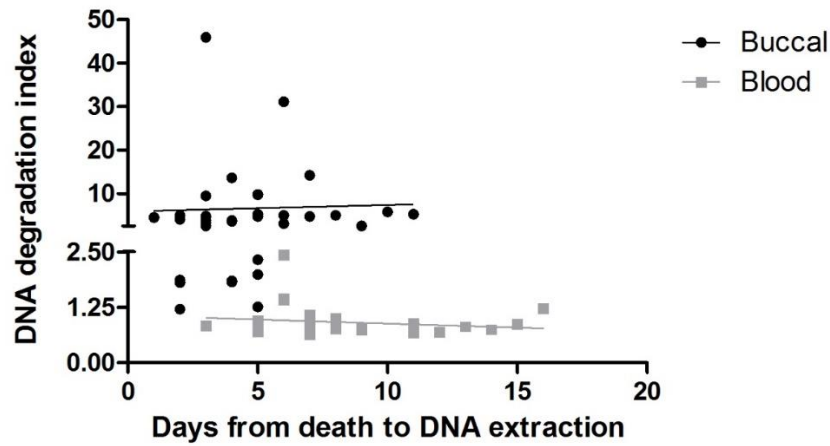


Figure 5.14: Degradation index of DNA obtained from blood and buccal swabs compared to days between infant death and DNA extraction.

No significant association was found between time and degradation index for both buccal swabs ($p = 0.179$) and blood samples ($p = 0.296$).

The degradation index of FFPE tissue appeared to exponentially increase as the number of days between sample collection and DNA extraction increased (Figure 5.15), however, this was not statistically significant.

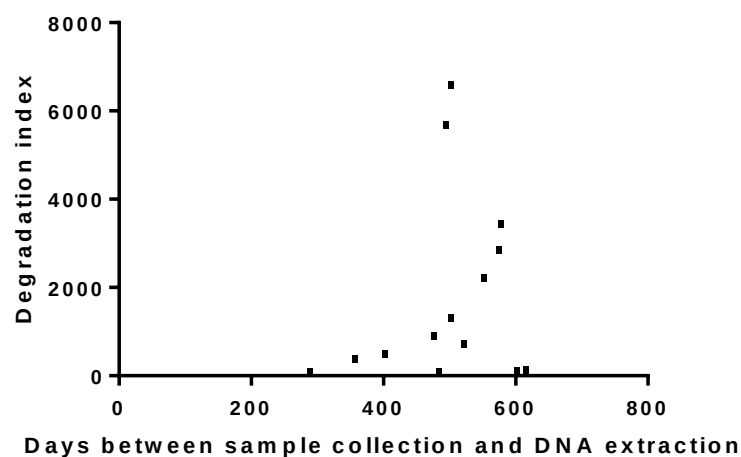


Figure 5.15: Degradation index of DNA from FFPE tissue compared to the number of days between sample collection and DNA extraction.

No significant association was found between time and degradation index for FFPE tissue.

5.3.5 PCR and Sanger sequencing

PCR and Sanger sequencing were successful on DNA extracted from all 48 blood samples (Figure 5.16A for an example). For buccal swabs, the sequencing signal was initially very low (Figure 5.16B), but after extensive optimisation involving secondary electropherograms of good quality were obtained for all samples (Figure 5.16C). For FFPE tissue however, only 1 out of 14 samples was successfully sequenced, which was the sample with the lowest degradation index (Figure 5.16D). Further optimisation for FFPE tissue samples did not improve results and failure to sequence was attributed to degradation of DNA.

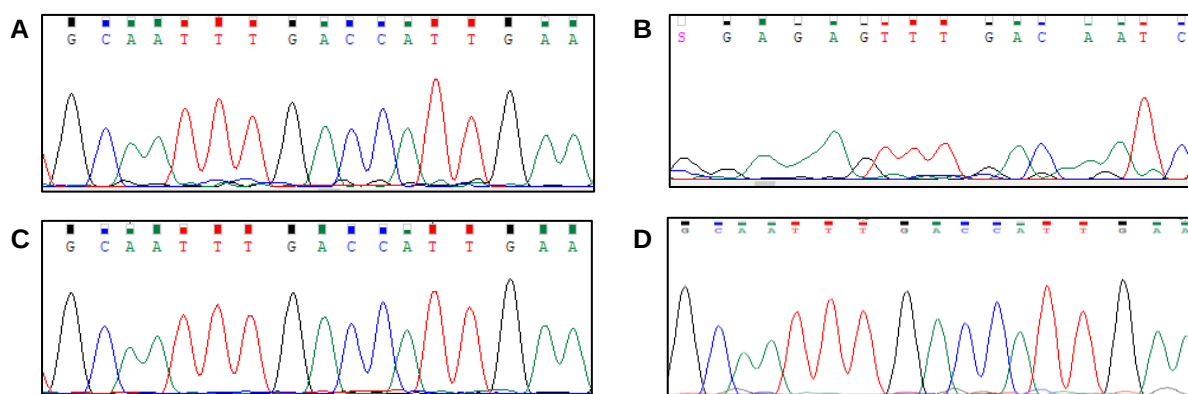


Figure 5.16 Electropherograms showing Sanger sequencing results from DNA from (A) blood samples, (B) buccal swabs prior to optimisation (C) buccal swabs subsequent to optimisation and (D) FFPE tissue. Sequencing was successful on DNA from all blood samples, all buccal swabs but only after optimisation and one out of fourteen FFPE tissue samples.

5.4 Discussion

5.4.1 FFPE tissue

During the optimisation process, it was clear that two sections of tissue, each at 10 microns in thickness, produced more DNA compared to one section of tissue at 10 microns ($p = 0.0006$). This was true independent of tissue type, deparaffinisation method (Figure 5.5) and DNA extraction kit. In future work, additional sections of FFPE tissue should be investigated in an attempt to further improve DNA yields. Several studies have used between two and seven sections of FFPE tissue at thicknesses of 5 or 10 microns [180,182,194], while Dietrich *et al.* (2013) used 50 sections of placenta and prostate FFPE tissue and were successful in PCR-based applications [195].

Three different DNA extraction kits were investigated in this study, all of which were silica-membrane based. The QIAamp[®] DNA FFPE Tissue kit (Qiagen, Hilden, Germany) produced slightly better DNA concentrations than the other two kits, but was the only kit which produced DNA which had 260/230 absorbance ratios of > 1.8 for all samples (Figure 5.8). As such, this kit was used to process the remaining samples. While no published study to date has compared the three kits investigated here, many studies have been performed which assessed different DNA extraction methods and many concluded that DNA extraction method played a large role in the recovery of DNA from FFPE tissue [165,180]. However, the studies are difficult to compare as not only were different DNA extraction methods used, but different tissue preparation parameters and DNA assessment methods were followed. Overall, the phenol-chloroform DNA extraction method, as well as the QIAamp[®] DNA FFPE Tissue kit (Qiagen, Hilden, Germany) seem to have produced DNA with better yields, purity and amplification ability [165,177,180,194,196,197].

The number of tissue sections and the DNA extraction kit were the only two variables which seemed to substantially impact DNA quantity and quality results, as tissue type and deparaffinisation method seemed to vary between cases. Lung tissue prepared with deparaffinisation solution was slightly better than the other variable combinations, however, this was not statistically significant, perhaps due to only four cases being tested. Further, lung tissue prepared with deparaffinisation solution also had the most variability in DNA concentration (Figure 5.6A) and DNA degradation (Figure 5.6B) measurements between cases, and therefore, it could have been by chance that these parameters appeared better.

For the majority of optimisation, only two cases were used. Consistently, the lung tissue from the first case appeared to be the better tissue type, whereas heart tissue from the second case appeared to be better (regardless of deparaffinisation method). This may have been attributed to the cell density in each of these tissue sections; as lung tissue from case 1 (Figure 5.3A) had more cells than heart tissue from the same case (Figure 5.3B); and the converse was true for case 2. Thus, the number of cells in the tissues could perhaps explain the difference in results with regards to tissue type, and going forward it is recommended that the corresponding H&E stained slides are reviewed first to determine the tissue type with the most cells. The more cells, the more nuclei available, and thus the potential for higher DNA yields.

Upon reviewing the post-mortem report of the cases used in optimisation, it was noted that case 1 had a LRTI, which may be the reason for the increased number of macrophages in the tissue. While more white blood cells would mean more human DNA, it may also mean that bacterial DNA was present. This was observed in this study as the overall DNA concentration as per spectrophotometry (154.5 ng/μl) as higher than that obtained via qPCR; the latter which quantifies human DNA only (3.42 ng/μl). The difference in quantification results is perhaps

partially due to bacterial DNA being present, which could be investigated in the future using a 16S ribosomal RNA approach [198].

Other reasons for the difference in quantification results include: (i) fragmented DNA and therefore no intact template available for qPCR amplification, and (ii) PCR inhibition from FFPE tissue. Fragmentation of DNA has been attributed to the acidic nature of unbuffered formalin, in which hydrolysis of purines occurs and cleavage of strands takes place [170]. Further, the cross-linking of DNA to proteins makes DNA from FFPE an unfavourable template for PCR as it is challenging for DNA polymerase to bind [169]. Varying success rates in PCR amplification have been reported, with many studies recommending that PCR primers should be designed to amplify fragments < 300 bp in size [175,197].

Dietrich *et al.* (2013) showed that the initial starting amount of FFPE tissue is not necessarily problematic, but rather the amount of input DNA (from FFPE tissue) into PCR [195]. While fragmented DNA from FFPE tissue is usually observed, these authors demonstrated that intact DNA was also present (as indicated on agarose gel electrophoresis), and poor PCR amplification was partly due to inhibition of the DNA polymerase by the fragmented DNA itself. The authors showed that by adding the standard 1 µg of DNA template (obtained from fifty 10 micron tissue sections) but increasing the DNA polymerase 4-fold (4 units), fragments of 600 bp could be amplified. This was in contrast to < 300 bp fragments being amplified with more DNA (5 ng) and 4 units of DNA polymerase. They suggested that the small fragments of DNA, combined with the formalin modifications of DNA, inhibit the activity of DNA polymerase to bind to the template during PCR and adding more reagents seemed to overcome the inhibition, at least partially [195].

5.4.2 DNA quantity and quality assessment

As expected, blood samples produced DNA of high yield and good quality in all instances. DNA concentration and overall yield from blood samples was significantly higher than that obtained from buccal swabs ($p < 0.0001$) and FFPE tissue samples ($p < 0.0001$). Further, DNA from blood samples was eluted into 300 μ l of TE buffer, as compared to 50 μ l of elution buffer for the other sample types. The increased volume of extracted DNA from blood, in addition to its higher concentration and quality, makes blood the sample of choice for DNA analysis. However, in 6 out of 60 cases, blood could not be obtained due to the scope of autopsy (only a buccal swab was obtained) and in a further 6 cases, no samples were obtained due to forensic pathologists' time constraints. Therefore, while DNA from buccal swabs yielded significantly less DNA compared to blood samples, it was a valuable sample to collect when no other sample could be obtained.

When quantifying the DNA from 52 buccal swabs using different methods, NanodropTM spectrophotometry overestimated the amount of double stranded DNA by 3 times (as measured by Qubit[®] fluorometry) and human amplifiable DNA also by 3 times (as measured by qPCR). This infers that while single stranded DNA was present in the total sample, most of the double stranded DNA in the sample was amplifiable. However, when quantifying the DNA from FFPE tissue from all 14 cases, NanodropTM spectrophotometry overestimated the amount of double stranded DNA by 6.5 times (as measured by Qubit[®] fluorometry) and human amplifiable DNA by 24 times (as measured by qPCR). Kapp *et al.* (2014) reported a similar observation whereby DNA concentrations from FFPE tissue measured using NanodropTM spectrophotometry were a median 5 times more compared to measurements obtained from Qubit[®] fluorometry [199]. This infers that the samples from FFPE tissue contained single stranded, degraded DNA and PCR inhibition was possibly present.

While not a primary objective of this study, investigation into the possible reasons for the degradation of sample types were performed, focusing on the time between death and DNA extraction. Here it was hypothesised that the longer the interval, the greater the degradation index; however, no significant association was found between the interval and degradation index for blood and buccal swab samples. However, for FFPE tissue samples, a trend was noticed whereby degradation index seemed to exponentially increase with time since collection. Watanabe *et al.* (2017) also reported that aging of tissue between 6 months and 3 years was significantly associated with DNA degradation when using the same DNA extraction kit as in this study [194].

It is important to note, that the investigation of degradation in this study was limited on a number of accounts. Firstly, since it was not an objective of the study, the time periods were not controlled and predefined, but rather, blood and buccal samples were processed as soon as possible. The FFPE tissue samples were processed approximately 12-18 months after collection, but did not vary greatly within this time. Should this variable of time be studied in the future, a longitudinal study design should be considered, ensuring sufficient repeats of sampling at each time interval. Further, a realistic time interval should be considered, as the processing of blood and buccal swab samples within nine days (as was the situation in this study) is not always feasible in an operational environment, particularly when dealing with vast caseloads. Feigelson *et al.* (2001) recommended processing buccal swabs within five days to maximise the quantity of human DNA obtained [200].

However, whilst looking towards the literature for guidance on the variable of time between death and DNA extraction, it emerged that its influence on forensic DNA profiling is under-researched and is a possible area for further research.

5.4.3 Forensic DNA profiling

Forensic DNA profiling confirmed that all blood sample and buccal swabs were single source, and thus no contamination had been introduced during the collection procedures. This was an important observation as variants observed in DNA from infants can confidently be attributed to the infants and not from an external source. Unfortunately, forensic DNA profiling was not attempted on DNA from FFPE tissue, due to their severe degradation (with a median degradation index of 729). A previous study found that samples with a degradation index of greater than 10 were severely degraded and failed to obtain full DNA profiles [192]. The expense of forensic DNA profiling on these samples was therefore not justified.

While forensic DNA profiling was used in this study to investigate possible DNA contamination, it also has legal implications for deceased infants who remain unidentified or abandoned. Firstly, obtaining a full forensic DNA profile of the infant would enable the linking of the baby to the mother (potential suspect) who in turn could be prosecuted (where appropriate). When a suspect is identified, perhaps by police or by the community, the forensic DNA profile obtained from the deceased infant can be compared to the forensic DNA profile of the suspect, through the use of kinship or familial testing [201,202]. Secondly, if the mother of an abandoned infant claimed that the infant was the product of sexual assault, the forensic DNA profile of the infant could also be compared to that of the alleged father [203]. In both instances the use of DNA profile has potential in aiding the legal proceedings against the accused. It is thus of great importance that forensic DNA profiles are able to be obtained from deceased infants, and as such, that the sample collection method is optimal.

In this study, all blood samples were able to generate full forensic DNA profiles, while this was true for only 84 % of buccal swabs. NanodropTM spectrophotometry as a method for

quantifying DNA obtained from buccal swabs, has a high likelihood of overestimating the amount of human DNA [204,205]. The reason for this observation could be attributed to the open nature of the oral cavity to the external environment, increasing the probability of bacterial colonisation, particularly post-mortem [206]. Differences in peri- and post-mortem microflora populations has been noted in various studies [207,208], where Guo *et al.* (2016) showed that *Proteobacteria* species population size increased in the oral cavities of rat cadavers [207]. This observation was noted in this study, as the spectrophotometric DNA concentration of buccal swabs were 1.8-fold higher than when quantified using qPCR, indicating the presence of non-human DNA in the buccal swabs.

As such, spectrophotometric methods of quantification are not routinely used to quantify DNA from buccal cells for forensic DNA profiling. Despite this, the manufacturer's protocol suggested initially performing forensic DNA profiling using the concentration of DNA obtained spectrophotometrically [209], as a basis for calculating the amount of input DNA. While this was sufficient for good quality DNA from blood samples, it was insufficient for DNA from buccal swabs, particularly those samples which had a DNA degradation index greater than 4. This index is in accordance with a previous study by Vernarecci *et al.* (2015) who found that when using the same Quantifiler[®] Trio Kit, a degradation index of 4 – 10 indicated a degraded DNA sample and resulted in the failure to obtain full DNA profiles [192].

When performing forensic DNA profiling in this study, the input DNA concentration, as measured spectrophotometrically, resulted in a DNA dilution carrying too little human amplifiable DNA, resulting in only 32 % full forensic DNA profiles from buccal swabs. However, when using qPCR as the means of measurement for input DNA, full forensic DNA profiles were obtained for 84 % of samples and partial profiles with alleles at ≥ 8 markers were

obtained for the remaining samples. These results show that the use of buccal swabs to non-invasively collect DNA samples may not be ideal but is perhaps better than no sample being collected at all [162,193,210]. Further, while resources are often constrained in low and middle income countries, quantification of samples using qPCR is strongly justified and motivations for sensitive and PCR-based quantification instrumentation should be made.

Further improvement to the forensic DNA profiles, was not observed when cycling conditions or amount of input DNA were adjusted. Rather, allele dropout was noted in the larger loci (> 270 bp) indicating the presence of degradation in the DNA extracted from buccal swabs. This was in accordance with the degradation index scores obtained using the Quantifiler[®] Trio Kit, wherein the large target fragment of 214 bp was not amplified in degraded samples. Comparatively, there was negligible degradation noted in the forensic DNA profiles obtained from blood samples. This was quantitatively shown through the calculation of the degradation index. The significant difference observed in degradation index between blood and buccal swabs could be attributed to several reasons: the sample type itself and its location within the deceased body could play a role, as blood is contained in vessels as opposed to buccal cells being exposed. Additionally, due to the open nature of the oral cavity, it is expected that more bacteria would be observed here than in blood, and the presence of bacteria is known to have adverse effects on the DNA quality of the host [193]. Further, blood was collected into a vial containing EDTA, whereas no preservatives were used in conjunction with the cotton swab. EDTA is known to prevent coagulation of the blood, without the risk of compromising the DNA [211]. In addition to the prevention of coagulation, EDTA also assists in diminishing the degradation effect of MgCl₂ and also allows for the long term storage of un-extracted blood samples [211]. While it is known that swabs provide an effective and non-invasive opportunity to collect DNA samples from individuals [193], a lack of consensus remains regarding the best

swab option. One study ranked different swab types according to their sampling ability as well as to yield sufficient DNA for molecular analyses, and found that different swabs were suited for different substrates and surfaces [212]. For example, cotton swabs were better at collecting biological stains from smooth nonporous surfaces, whereas polyester swabs were better at collecting touch DNA from the same surface type. Another study showed that nylon flocked swabs yielded more DNA compared to cotton swabs, but also yielded DNA which was less pure [213].

Storage of buccal swabs has been reported to be successful for up to four years, provided the cotton swab was placed directly into lysis buffer immediately after collection [214]. To further elongate the time period over which buccal samples can be stored and still yield sufficient amounts of DNA, the use of an FTA card has been supported [162]. FTA cards are suggested to allow the storage of buccal or blood samples for over a decade, due to the presence of antimicrobial reagents as well as the ability to reduce enzymatic degradation [193]. For these reasons it is recommended that future studies investigate the efficiency of different swab types, preservatives and additional sampling methods to optimise the success of molecular testing from swabs.

5.4.4 Conclusion

While blood is indeed the sample type of choice for post-mortem molecular autopsies, the need to investigate both non-invasive and retrospective sample types is critical. Non-invasive sample collection is important because the majority of SUDI cases investigated in a forensic pathology setting in South African (*e.g.* at Salt River Mortuary) will more than likely undergo an external autopsy only, and with high caseloads, it is not always feasible to partially dissect an infant to obtain blood for an additional analysis. Retrospective samples, in the form of FFPE tissue,

often provides the only stored biological sample for many SUDI cases where cause of death remains undetermined, and such cases would benefit enormously from a genetic analysis which can have the potential to resolve cause of death.

As such, blood and buccal swabs were collected prospectively from the same infants and the quantity and quality of DNA was assessed. To collect buccal cells, cotton swabs were used, as the Whatman® EasiCollect™ device can be challenging when collecting buccal cells from deceased infants due to the size of the foam applicator tip. Before this study, cotton swabs to collect buccal cells from deceased infants for DNA analysis had not been investigated. In addition, FFPE tissue was available for a subset of infants and was included in the comparison, after optimisation of the DNA extraction.

Optimisation of DNA extraction from FFPE tissue revealed that two sections of 10 microns FFPE tissue yielded significantly more DNA as compared to one section of tissue. The QIAamp® DNA FFPE Tissue kit (Qiagen, Hilden, German) also outperformed the other two kits investigated (ZR FFPE DNA MiniPrep™ Kit (Zymo Research, USA) and the Nucleospin® DNA FFPE XS Kit (Macherey Nagel, Germany) in terms of DNA purity. Lung tissue prepared with deparaffinisation solution was a slightly better combination than heart tissue and mineral oil. DNA obtained from FFPE tissue was significantly lower in yield compared to that obtained from blood and buccal swabs, and in addition, was severely degraded. Buccal swab DNA was also significantly lower in yield and quality compared to that obtained from blood.

In an attempt to assess potential contamination of samples, forensic DNA profiling was used, which indicated no external DNA was present in the blood or buccal swabs. This technique is also a valuable tool in legal proceedings for the identification of an individual and also for

linking suspects to a crime. In abandonment of infants, forensic DNA profiling may be used to link the suspected mother to the infant, so that the necessary criminal charges can be laid. Forensic DNA profiles obtained from deceased infants indicated that blood samples should preferentially be collected as they yielded a 100 % success rate. If this is not achievable (as in the case of severely dehydrated, premature and/or scavenged infants), cotton swabs can be used to obtain buccal cell, however, in these situations, calculations of input DNA must be performed using qPCR as a DNA quantification method, as opposed to spectrophotometry based methods. Even then, there is a chance that a full forensic DNA profile may not be obtained, but the alleles at the remaining markers may provide enough discriminatory value for identification.

The chances of obtaining a partial profile was significantly associated with the degradation of the sample, but it was found that degradation of DNA from buccal swabs not associated with time elapsed between death and DNA extraction. The degradation of FFPE tissue samples however, seemed to be exponentially linked with time since death, but further research should be conducted to investigate this, using a controlled time frame and a larger sample size.

Despite the relatively poor quality of DNA obtained from buccal swabs and FFPE tissue, PCR and Sanger sequencing of a 217 bp fragment was successful on all buccal swab DNA following extensive optimisation, as well as one of the fourteen FFPE tissue samples. These results confirm that blood is the sample of choice for molecular analysis, and show that in the absence of obtaining blood samples, buccal swabs should be obtained. In addition, the DNA extraction procedure from FFPE tissue shows potential, and increasing the number of sections as well as altering PCR conditions should be explored further.

Chapter 6

MOLECULAR ANALYSIS OF SUDI CASES: INFECTIONS

The screening of candidate variants associated with infection in SUDI cases from Salt River Mortuary (Cape Town)

6.1 Introduction

In South Africa, the majority of SUDI cases are attributed to infectious causes, with respiratory tract infections being disproportionately high in Cape Town [4,119,215–217]. As reported in Chapter 2, microbiology and virology testing is seldom carried out to identify a possible causative pathogen in SUDI cases, and histology is only performed in a subset of cases at Salt River Mortuary. Thus, our understanding of infections and pathogens in SUDI cases in a local context remains limited.

The role of infection in SUDI has been previously investigated by various researchers, and in 1987, the common bacterial toxin hypothesis was proposed by Morris *et al.* [218]. They suggested that bacterial growth in the infant's respiratory tract could initiate a toxic-induced cytokine release which could act on neural or myocardial membranes or decrease sleep arousal; ultimately leading to sudden and unexpected death of an infant [219,220]. Further, the bacterial growth in the nasopharyngeal area could develop as a result of viral infection, which is sometimes difficult to detect during autopsy [218,219].

The hypothesis is consistent with the age distribution of SUDI, which peaks generally during the second to fourth month of life [221,222]. This is explained taking into account that during the first month of life, the infant is relatively protected by maternal IgG; and that the subsequent drop of IgG levels associates inversely with the risk of an infant contracting an infection. As growing infants are exposed to infection, they develop their own immunity, which is consistent with the drop of SUDI rates after six months of age [218].

It was further hypothesised that the prone sleeping position would decrease the natural drainage and consequent accumulation of mucosal secretions, comprising of bacteria and toxins, in infants who slept in this position [218]. An experiment which supported this hypothesis, showed that nasal swabs from adults who lay prone contained more bacteria than when they lay in a supine position [86]. This is additional evidence of the prone sleeping position being a risk factor for SUDI [44,105,223].

Not only do bacterial infections play a role in SUDI cases, but viral infections have also been observed [224,225]. These include, but are not limited to, human adenovirus [226], enterovirus [227], cytomegalovirus [228,229], Epstein-Barr virus [224], influenza virus [230,231], parainfluenza virus [232], respiratory syncytial virus (RSV) [233], rhinovirus [234,235], rotavirus [236] and parvovirus B19 [237,238]; and more are still being discovered [235]. However, the role that viruses play in contributing to death is inconclusive, because viruses have also been observed at similar or higher frequencies in living infants and infants who have died accidentally from a traumatic injury [239].

Viral infections could play a role in initiation of the common bacterial toxin cascade, or enhance cytokine activity, which might contribute to sudden death [127,240]. For example,

interleukin 10 (IL-10), an anti-inflammatory cytokine, was found to be significantly lower in infants than in adults [241], suggesting that infants may be less capable of offsetting the damaging effects of pro-inflammatory cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF- α) and interferon-gamma (INF- γ) [240,242]. If such pro-inflammatory mediators are left uncontrolled, septic shock could follow, thereby contributing to infant death [241,242]. It follows then that genetic variants in pro-inflammatory or anti-inflammatory mediators may further predispose or protect an infant to/from death in the presence of an infection [243]. This is because the optimal functioning of the immune system is largely controlled by the expression and regulation of many genes which are activated in response to pathogens.

Dashash *et al.* (2006) investigated genetic variants in several anti-inflammatory and pro-inflammatory cytokines in 25 SIDS cases and found a significant association of a promoter region variant in *IL-6* (c.-174G>C, rs1800795) compared to 136 controls [244]. This variant has previously been associated with respiratory infection, sepsis [245], and cardiovascular disease [246]. IL-6 is pro-inflammatory cytokine, secreted by T lymphocytes and macrophages, and is activated after interaction with pathogen-associated molecular patterns (PAMPs) [247]. It is involved in the mediation of an acute immune response, as well as in the differentiation of mature B cells into plasma cells that secrete antibodies. It has also been shown that increased IL-6 production inhibited that of IFN- γ , the latter which is needed for the response against *Mycobacterium tuberculosis* infections [248]. Therefore, the G allele of the rs1800795 variant [249], which is associated with increased IL-6 production, can reduce the ability to overcome *M. tuberculosis* infections. This finding was corroborated in a study performed on a South Brazilian population where the C allele showed a protective role against pulmonary tuberculosis [250].

Tuberculosis is a burden worldwide and is currently the ninth leading cause of death, globally [251]. The WHO estimated that the global tuberculosis rate is more than 125 cases per 100 000 live births, while in South Africa, this rate is a remarkable 834 per 100 000 live births [251]. In Cape Town, 13.7 % of the total tuberculosis burden was documented in children under the age of 13 years, of which more than half (52.3 %) was in children under than of three years [252]. A case report in the Western Cape has previously documented primary pulmonary tuberculosis as the cause of death in a four month old SUDI case [253]. The diagnosis was only detectable after a full internal autopsy was done; thus Dempers *et al.* (2011) advocated for thorough investigations to be performed in all SUDI cases to elucidate other possible cases of tuberculosis. Such a cause of death has direct importance for the ongoing health management of the family and the exposure of tuberculosis to the community [253].

Other variants have also been associated with increased susceptibility to pulmonary tuberculosis. An example is the variant rs1800629, which is downstream from the *lymphotoxin alpha* (*LTA*) gene and upstream from *TNF- α* (c.-488G>A). While a meta-analysis study in 2012 showed that this variant did not confer a risk for pulmonary tuberculosis in a Caucasian cohort [254], the opposite was found in an African Mozambican population in 2015 [255]. The associated risk for pulmonary tuberculosis with the rs1800629 variant was further increased with tobacco smoke exposure [256].

Another important set of genes in the immune response pathway is the *toll-like receptor* (*TLR*) genes, which are responsible for detecting pathogens and initiating the innate immune response [257]. Genetic variants in *TLR* genes have previously been associated with infections [247,258–261], and are thought to increase the chances of contracting an infection due to a suboptimal response in detection. The variant in *TLR4* (c.896A>G, rs4986790) has been

associated with RSV in young children and infants [262], and thus may be of interest in the context of SUDI investigations in South Africa. While associations have been made with this variant and infections, it has not yet been investigated in a cohort of SUDI cases; with or without infection.

Therefore, the aim of this study was to begin an on-going research project to explore the relationship between selected genetic variants and SUDI cases in South Africa. Given the complexity of the immune response pathways and the numbers of genes involved, this particular investigation was considered the first of potentially many more studies. This study was limited to three variants which were previously associated with infection; namely *IL-6* rs1800795; *TNF- α* rs1800629 and *TLR4* rs4986790. The exploratory nature of this research meant that the results obtained would hopefully lay a foundation to guide research in larger cohorts of SUDI in South Africa in the future.

6.2 Cohort, materials and methods

6.2.1 Cohort and samples

The cohort comprised SUDI cases who were recruited from Salt River Mortuary (Cape Town, South Africa) between 1 March 2016 and 30 September 2018. Recruitment of deceased infants was conducted in accordance with institutional ethics approval (HREC: 445/2015) and in-house guidelines which have subsequently been published [32]. Informed consent from next of kin was obtained (Appendix 6). The sample size of the cohort was guided by a power calculation, which indicated that 162 infants were required for a confidence interval of 95 % (Appendix 1).

Infants were recruited for this study if they were between 1 day and 12 months old and admitted to Salt River Mortuary under the circumstances that their death was sudden and unexpected, thus requiring medico-legal investigation. Initial inclusion was independent of their eventual cause of death, which was unknown at time of recruitment. This is because recruitment and informed consent was done before the autopsy in order to know if a biological sample should be taken for this study. As such, more than 162 SUDI cases were initially recruited, to account for possible exclusion of cases which had either had poor quality DNA or those which turned out to be due to unnatural causes. Therefore a total of 201 SUDI cases were recruited using these criteria.

Post-mortem examination of these cases included a full history from the caregiver (Appendix 4) as per international infant death protocols (Centers for Disease Control and Prevention; publicly accessible: <https://www.cdc.gov/sids/index.htm>), a full body Lodox[®] Xmplar-dr scan, and full external examination of the body. Depending on the case context, a macro dissection of the lungs or a full autopsy may have been performed, and samples may have been collected for ancillary investigations.

Blood samples (1 - 4 ml) were also obtained from the infants for the purposes of this study. Blood was collected into purple-capped EDTA blood vials (BD Vacutainer, New Jersey, USA) and stored at 4 °C until sample processing. In eight cases, blood collection was not possible due to a combination of factors, including the dehydration of the infant, the time interval between death and autopsy as well as the scope of the autopsy being external. In these cases, buccal cells were obtained from the infant using a cotton swab (Copan, Italy). In another ten cases, all blood samples obtained were used for the medico-legal investigation, but FFPE tissue was available for the study.

6.2.2 DNA extraction and quantification

DNA was isolated from biological samples as described in Chapter 5. DNA was subsequently quantified using qPCR using the Quantifiler[®] Trio qPCR kit and 7500 real time thermal cycler (Applied Biosystems, Foster City, USA), as previously described [192]. Data from qPCR was used to assess both concentration and degradation of DNA [192].

6.2.3 Genotyping

Three variants in three genes were selected for genotyping in the cohort of SUDI cases (Table 6.1). This selection was guided by the literature which indicated previous associations with these variants and infections. Since infections play a role in SUDI in South Africa, it was hypothesised that they may also be significantly associated with SUDI in this study cohort. Each region of interest was amplified using specifically designed primers (Table 6.1 and Appendix 7). DNA (50 ng) from each infant was amplified using the PCR setup and cycling conditions described in Chapter 5. The optimal annealing temperature (T_a) for each region was determined by means of temperature gradients which resulted in the following T_a for each region: *IL6* and *TNF α* multiplexed at 53 °C; and *TLR4* at 54 °C.

PCR products were visualised on 1 % (w/v) agarose gels as described in Chapter 5. The *TLR4* fragment (10 μ l) was then subjected to restriction enzyme digestion using 1 unit of *Bcc1* and 2 μ l of CutSmart[®] Buffer (New England Biolabs, Ipswich, USA) in a total volume of 30 μ l. The digestion was visualised on 3 % (w/v) agarose gels, electrophoresed at the same conditions as described above, but also alongside an undigested PCR product.

Table 6.1: Variants associated with infection alongside primers that were designed for the amplification of target regions containing SNPs of interest as well as for SNaPshot® PCR.

Primer properties were assessed using OligoAnalyzer 3.1 (publicly accessible: <https://eu.idtdna.com/site/account/login?returnurl=/calc/analyzer>, date accessed 18/06/2016 and 27/04/2017).

F: Forward; R: Reverse; I: Internal, whereby the underlined sequence represents a tail.

Gene	rs number	Type of variant	cDNA nucleotide change	Primer sequence (5' - 3')
<i>IL-6</i>	rs1800795	5' upstream	c.-174C>G	F: CAGCATTACTCTTTGTCAAGAC R: GCAGAATGAGCCTCAGAC I: <u>TTGTTGTTGTTGTTGTTG</u> GTCTTTAGCA
<i>TNF-α</i>	rs1800629	5' upstream	c.-488G>A	F: AGCATTTCCCTCCAACC R: CTCTGCTGTCCTTGCTG I: AGGCAATAGGTTTTGAGGGGCAT
<i>TLR-4</i>	rs4986790	Missense	c.896A>G	F: GTCTGGCTGGTTTAGAAGTC R: TCAATAGTCACACTCACCAGG

The *IL6* and *TNFα* PCR products were cleaned using the ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. Cleaned PCR products underwent mini-sequencing using the SNaPshot® Multiplex Ready Reaction Mix (Applied Biosystems, Foster City, USA) using standard methods [192] and internal primers in a ratio of 1:10 for *IL6:TNFα* (Table 6.1). SNaPshot® PCR products were added to HiDi™ formamide and GeneScan™ 120 LIZ® Size Standard (Applied Biosystems, Foster City, USA) and capillary electrophoresis was carried out on the ABI PRISM® 3130xl Genetic Analyser (Applied Biosystems, Foster City, USA). A subset of cases which showed variants at the loci of interest also underwent Sanger sequencing as a means of confirmation. This was achieved using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) as described in Chapter 5.

6.2.4 Data analysis

GeneMapper™ v3.0 (Applied Biosystems, Foster City, USA) software was used to collect and analyse raw data from the Genetic Analyser, as well as to genotype the SNaPshot® PCR results. Chromas Lite version 2.6 (publicly accessible: www.technelysium.com.au) was used to view sequencing data, and BioEdit Sequence Alignment Editor version 7.0.5.2 [263] was used to align sequence data from SUDI cases to the respective reference sequences. Reference sequences for each gene were downloaded from Ensembl Genome Browser build 84 (publicly accessible: <https://www.ensembl.org/index.html>, date accessed 18/06/2016).

Allele frequencies for each variant were calculated and Hardy Weinberg Equilibrium was assessed. Data were then stratified according to cause of death in the following categories: (i) infection-related (n = 116); (ii) infection-unrelated (*e.g.* unnatural, congenital abnormalities) (n = 31); and (iii) unknown (n = 34); and allele frequencies compared between groups using a Fishers Exact test. Lastly, allele frequency data from infection-related SUDI cases were compared to background data from the Genome Aggregation Database (gnomAD) (publicly accessible: <http://gnomad.broadinstitute.org>, date accessed 20/11/2018) using the Chi-squared test and Yate's correction. Unfortunately, due to the small number of controls with information pertaining to both genetic data and risk factors, it was not possible to statistically assess the associations of risk factors (*e.g.* sleeping position and exposure to tobacco smoke) in combination with genetic data on the outcome of cause of death.

6.3 Results

The demographics of the cohort (n = 201) mirrored those of SUDI cases in general at Salt River Mortuary (Chapter 2), with a slight preponderance of males (50.8 %). The majority of infants

were from the indigenous African population group (60.5 %) while the remainder were of Mixed Ancestry (39.5 %) (Figure 6.1).

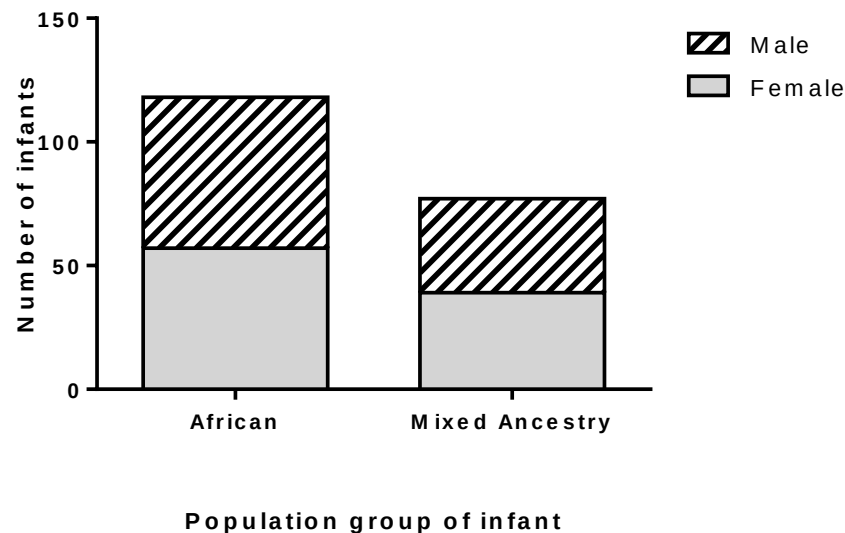


Figure 6.1: Sex and population groups of the 201 SUDI cases included in this study.

These demographics were representative of the general demographic trends of SUDI cases admitted to Salt River Mortuary.

Infants ranged from 0 to 12 months of age (median: 2 months) with 61.5 % of infants being three months old or younger (Figure 6.2). A Chi-square test showed no significant difference between the age of the infant at death and their sex ($p = 0.9651$), thus the graph does not present sex separately for each age category.

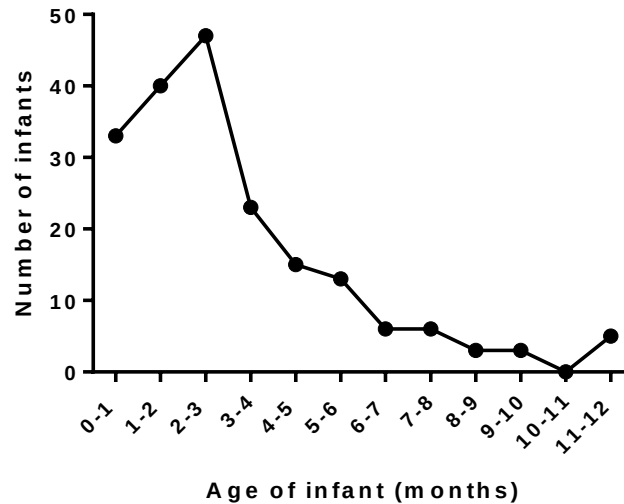


Figure 6.2: The age of infants at death ranges from 1 day to 12 months, with a peak at 2-3 months old.

The majority of SUDI cases were less than 3 months old at the time of their death.

A full autopsy was performed in 34.69 % of cohort; 3.06 % received a partial autopsy, while the remainder (62.25 %) had external autopsies only. The time between death and autopsy was between 0 and 17 days, with a median of 4 ± 2.49 days. Samples were collected for histology in 65 cases; for microbiology and/or virology in 44 cases and toxicology in 21 cases. For virology, 6 of the 21 requested tests detected a pathogen; the most common was adenovirus ($n = 4$) and in two of these cases, human bocavirus was also detected. Cytomegalovirus and coronavirus were each found in one SUDI case. The percentage of positive results was greater for microbiology cultures, where 14 out of 37 cases had a pathogen identified: *Staphylococcus aureus* ($n = 3$), *Enterobacter cloacae* complex ($n = 1$); *Streptococcus pneumoniae* ($n = 1$); *Serratia fonticola* ($n = 1$); *Escherichia coli* ($n = 1$); *Haemophilus influenzae* ($n = 1$); *Proteus vulgaris* ($n = 1$); *Klebsiella pneumonia* ($n = 1$); *Neisseria meningitides* ($n = 1$); *M. tuberculosis* ($n = 1$); *Streptococcus pyogenes* ($n = 1$) and coagulase negative *staphylococcus*, with doubtful significance ($n = 1$). The sample type as well as the number of days between death and post-mortem is provided for each positive microbiology result in Appendix 8. All toxicology results were either negative or still pending.

Following post-mortem investigation, several cases were found to be due to unnatural causes (n = 8) or complications from previous medical procedures (n = 3) and were kept aside as controls for this study. Eight cases were excluded as it was determined at autopsy that either the infants were stillborn (n = 3), that they demised within hours of their birth (n = 2) or they were older than 12 months of age (n = 3).

A further twelve cases were excluded as their DNA yield was of insufficient quantity and/or quality for molecular analysis. The cohort also comprised one set of monozygotic twins which were both included in genetic analysis, but data from only one of the individuals was included in statistical analysis to avoid over-representation. This resulted in 170 SUDI cases (including one set of twins) and 11 controls in this study (Figure 6.3). The control data for genetic variants was supplemented using that in publicly available online repositories.

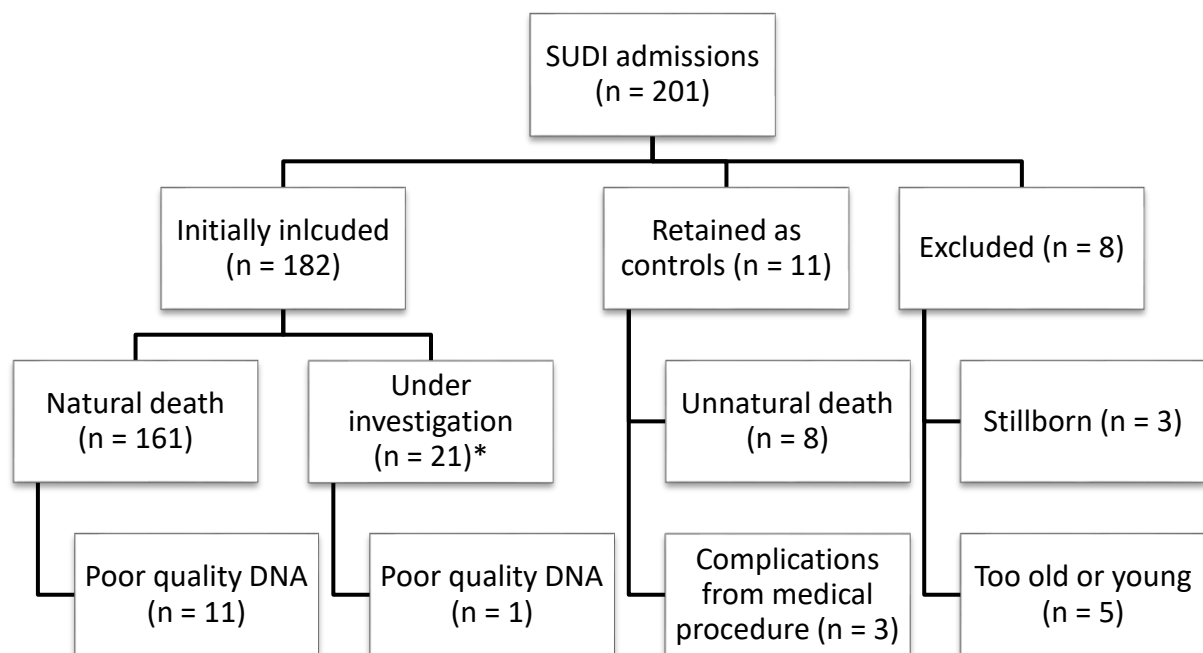


Figure 6.3: Flow diagram indicating 201 SUDI admissions recruited for this study and subsequent inclusions and exclusions.

Overall, 182 cases were initially included, but 12 had poor quality DNA. This resulted in 170 SUDI cases being included, which had DNA suitable for molecular analysis. Of these, 20 cases were still under investigation which included one set of monozygotic twins*.

Out of the included 170 SUDI cases, 150 had natural causes of death and 20 were still under investigation. The cases with natural causes of death were attributed to: respiratory tract infection (n = 98); gastroenteritis (n = 11), sepsis (n = 5), meningitis (n = 2), non-infectious natural causes (*e.g.* congenital abnormalities) (n = 20) unspecified natural causes (n = 14).

Assessment of DNA yield indicated a range of 438 ng to 1 236.17 µg with a median of 138.64 ± 226.45 µg. DNA was not degraded in the majority of samples, as indicated by a median degradation index of 0.775 ± 0.25. Genotyping was performed on DNA samples using SNaPshot® PCR for *IL-6* and *TNF-α*, and restriction enzyme digestion for *TLR4*. Allele frequencies were calculated (Table 6.2) and it was found that the SUDI population was in Hardy Weinberg Equilibrium [264].

Table 6.2: Minor allele frequencies (MAF) for three variants in this study cohort as well as from the background population. The parentheses indicate the number of times the allele was observed.

MAF from this study are represented according to whether the death was infection-related or not, as well as according population group. Non-infection related deaths comprised those which had specific natural causes but were not infectious (*e.g.* congenital abnormalities) and those which were unnatural (*e.g.* aspiration, asphyxia). Background MAF, from the African population group as well as the global data, were obtained from the genome Aggregation Database (gnomAD)* (publicly accessible: <http://gnomad.broadinstitute.org>, date accessed 20/11/2018) which contained 31 386 genomes and 250 890 exomes at the time of study.

	<i>IL-6</i> rs1800795 C	<i>TNF-α</i> rs1800629 A	<i>TLR-4</i> rs4986790 G
MAF in infection-related deaths	0.03670 (8)	0.19725 (43)	0.03043 (7)
<i>MAF in Black African population</i>	0.00714 (1)	0.23571 (33)	0.02703 (4)
<i>MAF in Mixed Ancestry population</i>	0.08974 (7)	0.12820 (10)	0.03659 (3)
MAF in infection-unrelated deaths	0.01667 (1)	0.16667 (10)	0.05 (3)
Background MAF* (African)	0.0730 (684)	0.1223 (2 034)	0.07371 (1 839)
Background MAF* (Global)	0.3170 (21 362)	0.1216 (22 159)	0.06121 (17 279)

Due to the purported functional roles of the variants in immune response pathways, the allele frequency data was stratified according to whether the infant had an infection-related cause of death or not. SUDI cases where it was unknown whether their death was infection-related or not were excluded for this statistical analysis. When comparing the allele frequencies for the three variants between the infection-related and infection-unrelated deaths within the SUDI cohort, none were significantly different.

However, the MAF for *IL-6* rs1800795 was significantly different between the infection-related SUDI cases in this cohort and the global MAF reported in gnomAD ($p < 0.0001$). Further, when matched for population group, the infection-related SUDI cases in this cohort who were Black African, had significantly different MAF compared to the African population group on gnomAD ($p = 0.0304$). Similarly, the MAF for *TNF- α* rs1800629 was significantly different between infection-related SUDI cases and the global background population ($p = 0.0009$); as well as between infection-related SUDI cases from the Black African population group and the African population group on gnomAD ($p < 0.0001$). For *TLR4* rs4986790, there was only a significant difference between Black African infection-related SUDI cases from this study and African population group on gnomAD ($p = 0.0704$). All of these differences remained significant after Yate's correction.

6.4 Discussion

Infections have recurrently emerged as role players in SUDI [106], and have also been implicated in SIDS aetiology [258]. The definition of SIDS specifically deals with infections, and explains that the presence of mild infections which are insufficient to explain death are acceptable to diagnose SIDS [2,41]. Mild infection has been reported in many SUDI cases at the time of death, both internationally and in South Africa [47,265]. It is of utmost importance

for the pathologist to determine the contribution (or lack thereof) of the infection to the death [80]. This is a challenging task due to the absence of universally-accepted and objective biomarkers, and thus typically requires an interdisciplinary approach, encompassing pathology, histology, microbiology and virology. More recently, genetics has become recognised due to its role in the regulation of immune response.

In this study, three variants which have formerly been associated with infection, were genotyped in a cohort of SUDI cases from Salt River Mortuary. One of these variants, *IL-6* rs1800795, had previously been associated with SIDS in a British cohort [244], while the other two had only been associated with infections and not SUDI or SIDS. Indeed, Feng *et al.* (2015) found that while *TNF- α* rs1800629 increased risk for pneumonia-induced septic shock compared to individuals with sepsis alone, no significant difference was observed between survivors and non-survivors [245].

Within the study cohort, allele frequencies were stratified according to whether the infant had an infection-related death ($n = 116$) or not ($n = 30$) but no significant differences were observed for the three variants using Fisher's Exact tests. Due to the relatively small sample size of the control cohort, Chi-squared tests were done to assess differences between infection-related SUDI cases in this cohort and background frequencies available on the online repository, gnomAD, which contained data from 31 386 genomes and 250 890 exomes, at the time of study. After Yate's correction, the *IL-6* rs1800795 and *TNF- α* rs1800629 variants were significantly different between the infection-related SUDI cases in this cohort compared to the global background frequencies on gnomAD. This statistical difference remained significant when population groups were matched. This suggests that there may be an association between these two variants and infection-related SUDI in a South African cohort. However, this needs

to be confirmed with a larger cohort of appropriate South African SUDI controls with infection-unrelated death.

Based on these preliminary findings, the minor C allele at *IL-6* rs1800795 has lower frequencies in African populations compared to other populations on gnomAD, and even lower frequencies in this SUDI cohort. It has been suggested that the minor C allele at *IL-6* rs1800795 plays a protective role against pulmonary tuberculosis [250], which suggests that the SUDI cases recruited in this study may have been at greater risk for tuberculosis infection.

Children are thought to account for 10 % of all new tuberculosis cases each year, with infants being most at risk for active tuberculosis infection [266]. A meta-review showed that young age was a major factor associated with extrapulmonary dissemination of tuberculosis [267]. Children of African ancestry who were exposed to tuberculosis have been highlighted as high risk for infection [268], and one hypothesis is that this might be related to genetic variants in immune-regulation pathways. Unfortunately, large proportions of tuberculosis are undiagnosed in infants [269], including SUDI cases admitted to medico-legal facilities. In a clinical setting, this may be attributed to a lack of resources for routine screening, whereas in a forensic setting, this sort of testing does not fall within the legal mandate of the forensic pathologist, which is to investigate *unnatural* death.

The gold standard for tuberculosis detection in a clinical setting is the testing of a sputum sample using a technique called GeneXpert MTB/RIF, which is a qPCR-based method. In a forensic setting, it is not possible to obtain a sputum sample via the patient coughing, and as such, a section of pulmonary tissue must be collected. This sample type is invasive and time-consuming to collect, particularly if an external autopsy was considered sufficient to determine

cause of death in the South African forensic pathology setting. Therefore, it is unclear what proportion of SUDI cases have tuberculosis and if these infections contributed towards their death. The lack of this baseline information is a limitation for this study, but also for the public health sector; as the true burden of tuberculosis mortality is not fully being investigated nor documented accurately. The benefits of this information strongly motivates for the allocation of resources towards this major health crisis in sub-Saharan Africa. This stark gap in the unknown number of SUDI cases with tuberculosis has prompted a research project linked with this one, which aims to optimise the detection of tuberculosis in SUDI cases in the least invasive way possible. Using this method, the objective will be to establish baseline rates of tuberculosis in SUDI cases admitted to Salt River Mortuary.

For the *TLR4* rs4986790 variant, allele frequencies were only significantly different between matched populations; *i.e.* between Black African infection-related SUDI cases and background frequencies for the African population on gnomAD. The weaker evidence for the association between *TLR4* rs4986790 and infection-related SUDI in this study is in agreement with previous studies which investigated the association of this variant with RSV [262,270]. Awomoyi *et al.* (2007) found that the variant allele was associated with RSV in infants [262], while Kresfelder *et al.* (2011) could not replicate these findings in a South African population [270]. However, it is unclear how many of SUDI cases in this study had RSV or other viral pathogens. This was a limitation of this investigation, in that the pathogen responsible for the infection-related death was only known in the minority of SUDI cases.

In South Africa, the National Health Laboratory Service (NHLS) typically conduct a shell vial culture to identify viruses from post-mortem samples; however, this analysis is not routine for the investigation of SUDI in most, if any, South African mortuaries [124,271]. This may be

attributed to the methodology utilised by most NHLS laboratories, whereby the shell vial culture technique has shown to not detect all viruses, when compared to the detection rate of common viruses by qPCR and immunohistochemistry [217]. This was further supported by la Grange *et al.* (2014) who reported that out of 148 SUDI cases at Tygerberg Medico-legal Laboratory (Cape Town) over a one year period, qPCR detected that 88 cases (59.5 %) had viruses, in comparison to shell vial culture which detected virus in only 9.5 % of the same cases [216]. In this study, 21 virology tests requested specifically using the qPCR method, yet only 5 (23.81 %) returned positive results. Further, 14 pathogens were detected via bacterial culturing out of 37 cases. It must be stressed though, that if a virus or bacteria is merely detected, it does not necessarily imply that it caused the death of the infant.

A systematic retrospective case review by Weber *et al.* (2008) of 546 deceased infants between 1996 and 2005 showed that although many post-mortem cultures showed organisms were present in SUDI cases, most could not be related to the death. However, the authors proposed that *S. aureus* and *E. coli* might be associated with SUDI as these bacteria were detected more commonly in cases of unexplained SUDI compared to those that were explained [265]. The interaction of toxins from *S. aureus* and other bacteria, such as *E. coli*, could have an increased negative effect on an infant [219,272]. This, combined with nicotine exposure, has been shown to be lethal in animal models. In chick embryos, nicotine increased the lethal effect of toxins which were injected into the embryos, which is also consistent with smoking being a risk factor for SUDI [148].

Blackwell (2008) supported Weber *et al.* (2008) and Morris *et al.* (1987 and 1999), indicating that *S. aureus* is a candidate organism, consistent with the common bacterial toxin hypothesis [273]. This is because of the inflammatory response that the toxins may be lethal if

uncontrolled. The toxic-induced cytokine response can contribute to lack of arousal, hypoxia, arrhythmia and hyperthermia, which have also been reported to be causes of, or contributory to SUDI [127,272,273]. Blackwell (2008) also proposed that the toxins are unlikely to be from post-mortem contamination as the bacteria generally grow optimally between 37 °C and 40 °C, while cadavers are stored at 4 °C [273]. However, not all bodies of the deceased are refrigerated immediately after death, suggesting that caution should be exercised when interpreting post-mortem results [272]. There are other limitations with such interpretations, such as the effects of post-mortem changes as well as the possible post-mortem contamination between organs or from an outside source during autopsy [135,274–276].

Overall, the contribution of infection to SUDI is challenging to interpret, but is nevertheless important; especially in South Africa where infection-related death is among the highest in the world. While the identification of pathogens in a medico-legal forensic setting is not legally mandated, it would provide valuable insight into underlying mechanisms of death. One organism which should be prioritised in this regard is *M. tuberculosis*, and research has been initiated in response to this important topic. Further, the analysis of pathogen data in SUDI cases alongside the geographical region of the death may also identify hotspots for certain infections which may prompt public health interventions in these areas.

Salt River Mortuary investigates more than 250 SUDI cases per annum, with the majority being attributed to natural causes of death. The majority of these cases are diagnosed as infection-related, based on external autopsies (Chapter 2). The role of genetic variants in immune response genes in SUDI cases in South Africa has not yet been explored; as such, this study genotyped three variants previously associated with infections in a prospective cohort of SUDI cases from Salt River Mortuary. These findings suggest that there may be associations between

these variants and infection-related SUDI cases from the Black African population group and this needs to be confirmed with a greater number of control samples. This study achieved its aim of laying the foundation for SUDI recruitment from Salt River Mortuary, as well as setting up genetic assays to test for these variants. Using this framework, this study can be expanded in the future to investigate more variants in more SUDI cases in South Africa.

Chapter 7

MOLECULAR ANALYSIS OF SUDI CASES: INHERITED METABOLIC DISEASES

The screening of candidate variants associated with metabolic diseases in SUDI cases from Salt River Mortuary (Cape Town)

7.1 Introduction

Inborn errors of metabolism are a group of rare, genetic disorders whereby some food types cannot be metabolised properly [277]. This is due to genetic variations in the genes coding for enzymes which are responsible for the conversion of food into various compounds, which the body then uses to function [278]. This suboptimal metabolism can cause an accumulation of metabolic intermediates in the body, which can result in various symptoms, and may even be fatal if uncontrolled. The collective incidence of inborn errors of metabolism may be as high as 1 in 800 live births, with variation in different conditions and between populations [278]. Metabolic disorders can be classified into several different groups, including, but not limited to, disorders of fatty acid oxidation and disorders of amino acid metabolism [278]. The focus of this chapter will be on three metabolic disorders, namely, (i) medium-chain acyl-CoA dehydrogenase (MCAD) deficiency; (ii) glutaric aciduria type 1; and (iii) galactosaemia.

In developed countries, candidate variants in *medium-chain acyl-CoA dehydrogenase* (*ACADM*; previously known as *MCAD*) have been reported to account for the cause of 1 % of SUDI cases [27,47,279]. MCAD is an enzyme that dehydrogenates fatty acids during beta-oxidation to produce energy for the body once its stores of glucose and glycogen are depleted [280]. This usually occurs during fasting, and is compounded by an increased demand for energy, such as during illness [280]. The gene, *ACADM*, encodes the MCAD enzyme and thus a molecular basis for MCAD deficiency has been proposed.

The investigation of the MCAD deficiency in SUDI cases was based on seminal work by Howat *et al.* (1985) who reviewed 200 infant cases of unexplained death (aged 1 month – 2 years) from the Children's Hospital, Sheffield, and observed that 7 % of cases (n = 14) showed signs of fatty acid oxidation deficiency. Biochemical enzymatic analysis was performed in cases where tissue was available, and two out of five cases revealed a defect in MCAD. The authors suggested then, that such fatty acid oxidation deficiencies should be investigated in SIDS cases, which may help to resolve cases with an unknown cause of death [281].

Research into genetic variations causative of MCAD-deficiency revealed the first reported variant in *ACADM*: c.985A>G (NM_001127328.2:c.997A>G (p.Lys333Glu)); which, in 1990-1991, accounted for 85 % (n = 112), 89.1 % (n = 55) and 91 % (n = 17) of MCAD deficiency in separate Caucasian cohorts of patients [282–284]. The c.985A>G variant follows an autosomal recessive inheritance pattern and its phenotype is influenced by the environment; for example, the presence of fasting influences the presence of symptoms, which could include hypoketotic hypoglycaemia and liver dysfunction [280].

However, numerous studies in the 1990s showed little support for this variant (c.985A>G) being responsible for sudden infant death syndrome (SIDS). To reiterate, SIDS is defined as the sudden and unexpected death of an infant less than one year of age, which remains unexplained after thorough and exhaustive post-mortem investigation [2]. Out of 67 SIDS cases in Monroe County (New York State, USA), only three were heterozygous, and none were homozygous for the c.985A>G variant [284]. In an investigation of 233 SIDS victims in Scotland, the carrier frequency (1 in 74) was not significantly different from that observed in 552 healthy controls (1 in 276) , and none of the deceased were homozygous for the c.985A>G variant either[285]. A similar observation was reported in Normandy (France) where only one infant out of 225 SIDS cases was heterozygous for the c.985A>G variant [286]. A limitation of these studies was that they did not appear to screen for other variants in the same gene to assess the possibility of compound heterozygotes. Given the recessive nature of MCAD deficiency, it is possible that that infants heterozygous for c.985A>G, may have indeed been affected via a compound heterozygote mechanism.

In Australia, 708 SIDS cases were screened for the c.985A>G variant, with seven infants being heterozygotes. In a subset of these cases (n = 414), testing for a second variant (c.583G>A) in the same *ACADM* gene proved negative. However, in another study, the c.583G>A variants was observed as a compound heterozygote with c.985A>G in four individuals (two unrelated families). In these individuals, MCAD deficiency presented in the first week of life [287]. The authors reported that the expressed protein from the allele containing c.583G>A displayed no enzyme activity, and that this variant was associated with SUDI [287].

Screening for MCAD deficiency in newborns has become routine in several developed countries and the results from these tests have contributed to the reporting of numerous

additional variants in MCAD which could also underlie the deficiency on a molecular level [288]. In 2006, Rhead sent out questionnaires to northern European, North American and Australian laboratories to gather the results of their newborn screens, and this information was collated with already published data in the literature. The proposed global incidence of MCAD-deficiency in newborns ($n \approx 8.2$ million) was reported to be 1 in 14 600. The c.985A>G variant was found to be the most common among these newborns and accounted for 54 % to 90 % of disease alleles (47 % to 80 % of MCAD deficiency cases) [288].

There is a paucity of literature documenting the genotype or allele frequency of the c.985A>G variant in South Africans. ‘1000 Genome’ data does not report the c.985A>G (rs77931234G) variant in Africans (publicly accessible: <http://www.internationalgenome.org/>; accessed 26/01/2016), but considering the admixed nature of the South African population, it would be worthwhile establishing baseline frequencies of this variant for epidemiological reasons. Further, metabolic screening is not routine for newborns in South Africa, and seldom forms part of post-mortem examinations. Very little is therefore known about the variability of genes involved in metabolic disease in South African infants. There have been no reports indicating any candidate variants which should be prioritised for post-mortem testing in SUDI cases in a South Africa setting. However, local research studies at Red Cross Children’s Hospital (Cape Town) could provide some guidance in this regard [289], [290].

A newborn screening study at Red Cross Children’s Hospital (Cape Town) showed a high carrier frequency of the c.877G>A/T variant in *glutaryl-CoA dehydrogenase* (*GCDH*) [290]. Variants in *GCDH* can cause glutaric aciduria type 1, which is an autosomal recessive disorder of amino acid metabolism, characterised by the inability to fully metabolise the amino acids lysine, hydroxylysine and tryptophan [291]. The accumulation these amino acids can be

damaging to the brain, where symptoms manifest as developmental delay, intellectual impairment and difficulty in regulating movement. Symptoms are exacerbated under stress, and can be triggered by infections. Onset during neonatal or infancy periods is usually fatal [291].

The variant c.877G>A/T was screened in 750 unrelated Black African newborns after eleven Black African children (3 days – 5 years old) with glutaric aciduria type 1 were found to be homozygous A/A for this variant. The carrier frequency was a remarkable 1 in 36 unrelated South African Black newborns [290]. Given the absence of metabolic testing at birth and at post-mortem examinations, it is plausible that these patients are not diagnosed and thus do not survive. It is thus hypothesised that at least some infants admitted for medico-legal investigation as SUDI in South Africa are affected by glutaric aciduria type 1, and it may even be the cause of their death.

In another newborn screening at Red Cross Children's Hospital (Cape Town), two variants in the *galactose-1-phosphate uridylyltransferase (GALT)* gene were screened for in nine infants with galactosaemia: c.404C>G/T and c.563A>G [289]. Four Black African infants and two infants of Mixed Ancestry were homozygous T/T for the c.404C>G/T variant, while three White patients were homozygous for c.563A>G. Galactosaemia is an autosomal recessive disorder, whereby galactose cannot be metabolised [292]. The background carrier frequency of the c.404C>G/T variant G was reported to be 1 in 60 in Black African newborns, yielding an overall incidence of 1 in 14 400 for this population group [289]. The authors suggest that many patients go undetected as this incidence is much higher than the current detection rate [289,293,294]. A similar hypothesis for that described for glutaric aciduria type 1 applies here, and these South African studies highlight the need to investigate these locally relevant variants

in SUDI cases. The aim of this study was to therefore investigate if variants in *ACADM*, *GCHD* and *GALT* were present in a South African cohort of SUDI cases.

7.2 Cohort, Materials and Methods

7.2.1 Cohort

A total of 201 SUDI cases were recruited from Salt River Mortuary (Cape Town, South Africa) between 1 March 2016 and 30 September 2018 in accordance with institutional ethics approval (HREC: 445/2015). Several cases which were admitted as SUDI were subsequently excluded due to infants not meeting the inclusion criteria ($n = 19$) or insufficient DNA quality for molecular analysis ($n = 12$), as described in Chapter 6. Further, among those included, two cases were a set of identical twins and thus the data from only one infant was included in genetic data analysis to avoid over-representation. Therefore, the 169 cases were included in this genetic investigation, of which 150 had natural causes of death and the remaining 19 cases were still under investigation. This sample size exceeded the 161 infants required for statistical power. Majority of infants were Black African (61.2 %) with the rest being of Mixed Ancestry; males accounted for 50.6 % of this cohort. Other demographics and post-mortem details of these cases are described in Chapter 6.

7.2.2 DNA analysis

As previously described, biological samples were obtained from infants at autopsy, following informed consent from next of kin. DNA was isolated from samples and quantified (Chapter 5 and 6). Four variants in three genes were selected for genotyping in the cohort (Table 7.1). This selection was guided by the literature as well as *in silico* pathogenicity assessment using Alamut Visual Software v2.1 (Interactive Biosoftware, Rouen, France). This software

integrates the results from various bioinformatic tools, clinical phenotypes, minor allele frequencies, four missense prediction tools and four splicing site prediction tools. All four variants were pathogenic, supported by entries on ClinVar (publicly accessible: <https://www.ncbi.nlm.nih.gov/clinvar/>).

Each region of interest was amplified using primers which were designed for this study (Table 7.1 and Appendix 7). PCR setup and cycling conditions were the same as described in Chapter 5 and 6. The optimal T_a for each region was determined as: *ACADM*: 54.5 °C; *GCDH*: 57 °C; *GALT*: 60 °C. Quality checks on agarose gels and post-PCR clean ups were done, as described in Chapter 5 and 6. SNaPshot® PCR was used to genotype the *GCDH* variant with the intention of expanding the assay for multiplex genotyping in the future, while the remaining fragments underwent Sanger sequencing (Table 7.1). All PCRs and incubations in this study were performed in a T100 thermal cycler (BioRad, Hercules, USA). Capillary electrophoresis was carried out on the ABI PRISM® 3130xl Genetic Analyser (Applied Biosystems, Foster City, USA). In cases where heterozygous genotypes were observed, the rest of the gene was also sequenced, to investigate the possibility of compound heterozygotes.

Table 7.1: Details of the four variants genotyped in this study alongside designed primer sequences.

F: Forward; R: Reverse; I: Internal. Underlined region indicates a tail.

Gene	rs number	cDNA nucleotide change	Amino acid change	Genotyping method	Primers (5' – 3')
<i>ACADM</i>	rs121434278	c.583G>A	G195R	Sequencing	F: ATGTAGCTGGTATAAAGACC R: TCCAGTTCCACAAACATACC
<i>GCDH</i>	rs121434371	c.877G>A/T	A293T	SNaPshot® PCR	F: TGGTTCTGCATAGGCCCTC R: CCCATGGTATAGAGAGTGAAACCC I: <u>AGAGAG</u> GCTGCCTGAACAAC
<i>GALT</i>	rs111033690	c.404C>G/T	S135L	Sequencing	F: GTAGCACAGCCAAGCCCTA
	rs75391579	c.563A>G	Q188R	Sequencing	R: CCATTAATCCTGTTCCCATGTCCAC

7.2.3 Data analysis

The software and genome tools utilised in Chapter 6 were also used for data analysis in this chapter; this included GeneMapper™ v3.0 (Applied Biosystems, Foster City, USA), Chromas Lite version 2.6 (publicly accessible: www.technelysium.com.au); BioEdit Sequence Alignment Editor version 7.0.5.2 [263] and Ensembl Genome Browser build 84 (publicly accessible: <https://www.ensembl.org/index.html>, date accessed 18/06/2016). Lastly, the medico-legal case files of case(s) where pathogenic variants were found were reviewed.

7.3 Results

Among the 169 SUDI cases, no variants were observed at two of the four loci investigated (c.985A>G in *ACADM* and c.563A>G in *GALT*) but variations were observed at the other two loci (Table 7.2). There were also three additional heterozygous variants in *GALT* in five SUDI cases, but all were ranked as ‘benign’ (Appendix 9).

Table 7.2: Details of the variants observed in SUDI cases

MAF: minor allele frequency. Global minor allele frequency was obtained from the genome aggregation database (gnomAD) which comprised 15496 genomes and 123136 exomes at the time of study

Gene	rs number	cDNA nucleotide change	Number of observations	MAF	Global MAF(number)
<i>ACADM</i>	rs121434278	c.583G>A	0	A: 0	A: 3.65595e-05 (9)
<i>GCDH</i>	rs121434371	c.877G>A/T	4 A/T heterozygotes (carriers)	A: 0.02367	A: 6.11232e-05 (15) T: 4.07488e-06 (1)
<i>GALT</i>	rs111033690	c.404C>G/T	1 G/G homozygote (affected)	T: 0.01183	T: 2.47696e-04 (61) G: 4.06058e-06 (1)
	rs75391579	c.563A>G	0	G: 0	G: 0.001 (351)
	rs12000481	c.508-17G>A	3 G/A heterozygotes (benign)	A: 0.01775	A: 0.00309 (760)
	rs2277202	c.507+62G>A	1 G/A heterozygote (benign)	A: 0.00592	A: 0.0480 (1486)
	rs41274867	c.508-24G>A	1 G/A heterozygote (benign)	A: 0.00592	A: 0.05453 (13428)

Four infants were heterozygous G/A for c.877G>A/T in *GCDH*, but did not have another variant in the rest of the *GCDH* gene, and were therefore unaffected. Two of these infants were Black African and two were of Mixed Ancestry (Table 7.3).

Table 7.3: Demographics of infants who were found to be heterozygous G/A for the c.877G>A/T variant in *GCDH*, and therefore carriers of glutaric aciduria type 1

Two infants were male and two were female; their age at death ranged from 3 weeks to 7 months.

	Population group	Sex	Age
1	Mixed Ancestry	Female	3 weeks
2	Mixed Ancestry	Male	3 months
3	Black African	Female	5 months
4	Black African	Male	7 months

Lastly, at the c.404C>G/T locus in *GALT*, no infant was heterozygous, but one infant was homozygous T/T (Figure 7.1). This infant was a Black African female who was 3 months old at the time of death. The case was admitted to Salt River Mortuary with a history that the mother noticed that her baby was not breathing well. She took the baby to the clinic, but on route, the baby stopped breathing and was declared dead on arrival at 12h50. In the absence of other medical history, the clinic referred the case to Salt River Mortuary as a sudden unexpected death. Since the admission to Salt River Mortuary was from a clinic, there was no investigation of the death scene or the home where the baby lived.

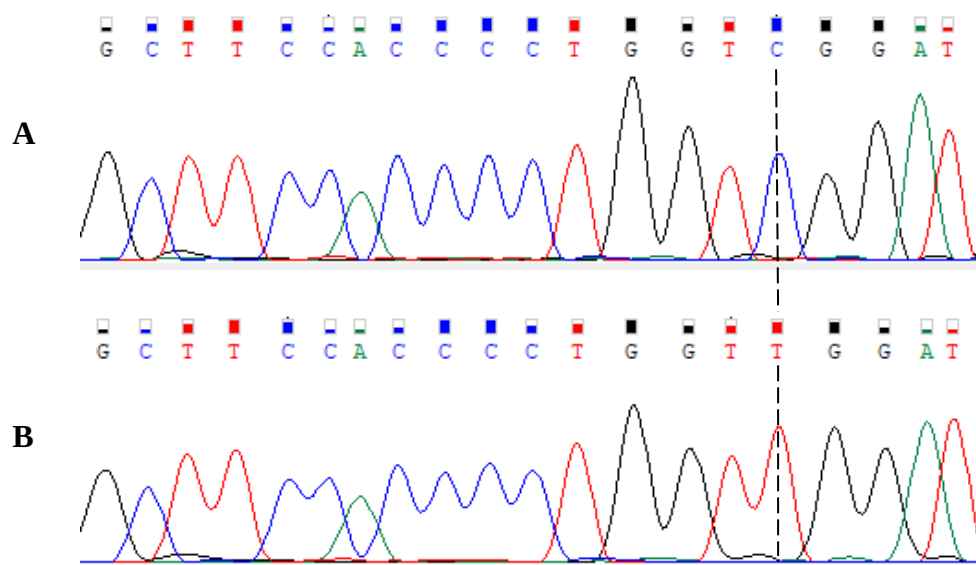


Figure 7.1: Electropherogram of the GALT region
(A) C/C genotype and (B) T/T genotype for the c.404C>G/T variant

The events leading up to death (*i.e.* shortness of breath prior to death) prompted a review of the infant's clinical history, which showed that the infant was born at 36 weeks gestation by caesarean section and was fully up to date with vaccines. The mother, who was 39 years old at the time of pregnancy, received antenatal care, and was tested HIV positive before pregnancy. It was documented that the mother received antiretroviral medication and the infant was put on Nevirapine at birth. At 10 weeks old, a PCR showed that the infant was HIV negative and Nevirapine treatment was terminated.

During post-mortem investigation, a Lodox[®] Xmplar-dr scan (Figure 7.2) showed signs of mild respiratory tract infection, and there were no signs of neglect, undernourishment or unnatural death. Based on the external autopsy, it was concluded that the death was natural, consistent with respiratory tract infection. Governed by the National Health Act No 61 of 2003 [36], the Forensic Pathologist is not mandated to further investigate cases of natural death and hence the forensic investigation of this SUDI case was concluded.

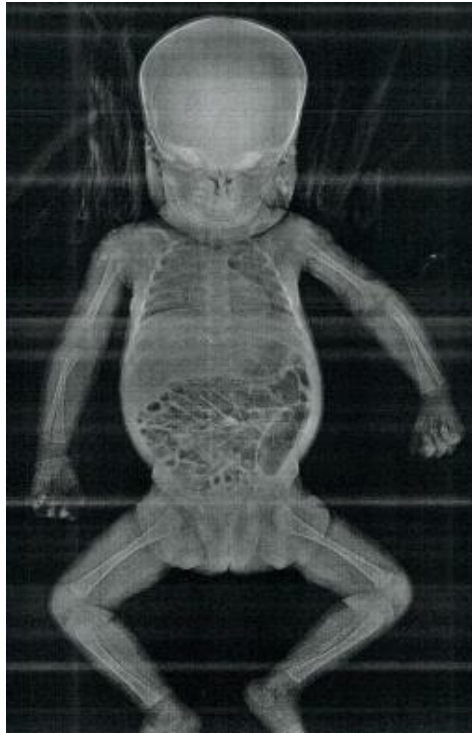


Figure 7.2: Lodox® Xmplar-dr scan of infant who was homozygous T/T for the *GALT* c.404C>G/T variant.

The scan showed signs of mild respiratory tract infection.

Analysis of the interview conducted with the family after the infant's death showed that the family, comprising three adults and three children, lived in a small informal dwelling. No one reported smoking cigarettes in the household and the mother did not drink alcohol or take drugs. The mother was single and unemployed, with a grade 8 education. The infant slept on her back and co-slept with her mother and sister, and was fed both with breastmilk and bottle formula. Of importance however, it emerged that the deceased infant was an identical twin. Given this information, together with the genetic results, contact was made with the family in attempts to refer them for genetic counselling. This process was intended to also include a validation of these results in the twin, as no second sample could be obtained from the deceased infant to replicate these results. It was envisaged that if the twin was also homozygous T/T for the *GALT* c.404C>G/T variant, that the results would be of value to the family, particularly the twin, as galactosaemia is a treatable condition.

A meeting was set up with the mother, as well as the mother's brother, to provide some feedback about the study in general, but to also inform them that they had been referred for a genetic counselling session. The mother then revealed that the other twin had subsequently passed away while they were travelling in the Eastern Cape, South Africa. She shared that she had accepted the deaths of her twins, and as a consequence, she did not want to take up her genetic counselling session, as knowing more information (*e.g.* genetic details) would not change anything. She also shared that she is no longer with the father of the twins, and given her age, she was not planning on having more children.

7.4 Discussion

The aim of this study was to investigate if a cohort of South African SUDI cases had genetic variants associated with three metabolic disorders. This was motivated by reports that 1 % of SUDI cases in developed countries are due to MCAD-deficiency [27,47,279] and that metabolic testing is not routinely performed at birth or at autopsy in South Africa. It was hypothesised that less than 1 % of SUDI cases would be due to this metabolic disorder in this study, as local SUDI cases are predominantly from communities of African and/or Mixed ancestries, where carrier frequencies of *ACADM* variants are low. The results from this study supported this hypothesis, whereby no SUDI case exhibited the variant of interest in *ACADM*.

Indeed, not all population ethnicities internationally were reported to have such a high prevalence of *ACADM* variants. Out of 64 MCAD-deficient infants in the UK who were homozygous for c.985A>G, 60 infants were White and no infants were of Asian or African ethnicity [295]. A meta-regression analysis in 2013 of more than 10 million individuals with the c.985A>G variant, showed significant regional differences (mostly within Europe) with

regards to the frequency of the variant [296]. These studies support the notion of a founder effect from north west Europe, which was first proposed in 1993 [297].

The c.563A>G variant in *GALT*, which had previously been observed in three of nine South African White galactosaemia patients [289] was not present in any of the 187 SUDI cases genotyped in this study. This is likely due to the different population groups of the SUDI cases in the two studies. This highlights the importance of selecting population-specific and locally relevant variants to screen during molecular analysis of SUDI cases, particularly in a resource-constrained setting, such as Salt River Mortuary (or perhaps across South Africa).

At post-mortem examination, the infant with the homozygous variant in *GALT* had an external examination and the full body X-ray showed bilateral granular pulmonary infiltrates with air-bronchograms. This observation is consistent with a LRTI. Patients with galactosaemia have previously been reported to present with infections, such as *Escherichia coli* sepsis [298–302], fungal sepsis [303] and pneumococcal infection [304]; overall suggesting a strong association between galactosaemia and serious infections in infants [300]. Infections are the leading cause of death in SUDI cases admitted to Salt River Mortuary [121], which means that other SUDI cases with infectious causes of death could also have underlying galactosaemia.

Galactosaemia is a treatable metabolic disorder, but can be fatal if undiagnosed [292]. Due to the heritability of this disease, such results are thought to be of significant value to living relatives and to parents who might plan on having additional children in the future [31,156]. In this study, a Black African infant was found to be homozygous T/T for the c.404C>G/T variant in *GALT*. Upon review of the case file, it was noted that the infant was an identical twin, thus increasing the potential value of these genetic results to the family. Unfortunately by the time

the genetic results were available, the second twin had also demised. This example demonstrates the need for the prioritisation of genetic testing of metabolic disorders in SUDI cases involving twins (or where additional children may be at risk), as a quicker turn-around time of results may save a child's life.

In this study, an effort was made to link the affected family with genetic counselling. It was not anticipated that the mother would decline this opportunity, but it is also fully understandable, given her personal circumstances. At the time of recruitment, the mother still had another twin, for which these results may have been helpful, and may have been the reason why she consented in the first place. However, since the passing of the second twin, this study held little personal value for her anymore. In conversing with her, she indicated that she did not regret participating in this study, as these results may have been helpful for other families. This admirable approach certainly has shed light on the importance of genetic testing for metabolic disorders in SUDI cases, particularly when the SUDI is part of a set of twins.

It was also of interest to note that the c.404C>G/T variant in *GALT* was not observed as a heterozygote in the entire tested cohort. This was unexpected given its carrier frequency of 1 in 60 in Black African newborns [289]. As such, one to two SUDI cases of African ancestry were expected to be carriers in this cohort. This misalignment between previously reported carrier frequencies in African newborns and the results of this study was also observed for the c.877G>A/T variant in *GCDH*, where four infants were heterozygous G/A. Two were of Black African ancestry and two were of Mixed Ancestry, which translated to a carrier frequency of 1 in 50.5, and 1 in 31.5, respectively. The carrier frequency rate for the Black African population was lower compared to the reported 1 in 36 in Black newborns [290]. Reasons for these

differences may be attributed to the relatively small sample size of this cohort or that the carrier frequency is lower in SUDI cases compared to the background population.

Overall, these findings demonstrate the value of molecular genetic testing in a forensic autopsy setting to elucidate underlying inherited disorders which may go undiagnosed by a conventional autopsy. Until metabolic screening becomes routine in South Africa, this important phenomenon may go undetected in SUDI cases. Not only is this information important to incorporate into cause of death outcomes from an epidemiological point of view, metabolic testing is also a requirement for the diagnosis of SIDS [2]. Moreover, genetic results are thought to be meaningful to families, for closure and acceptance of death, as well as for learning about diseases for which parents might be carriers. Further research is required to investigate other genetic variants associated with these and other metabolic disorders, which are relevant to SUDI cases in the South African setting.

Chapter 8

MOLECULAR ANALYSIS OF SUDI CASES: INHERITED CARDIAC DISEASES

*Analysis of 43 candidate genes associated with arrhythmia in a
selected SUDI cohort from Salt River Mortuary (Cape Town)*

8.1 Introduction

Cardiovascular disease is global burden and is the leading cause of premature death in the world [305]. While lifestyle and environmental factors play a large role in the aetiology of these diseases [306–308], there is a subset of cardiovascular diseases which are largely genetic-based [309]. Inherited cardiac disorders can be life-threatening and sometimes the first symptoms are in the form of sudden death [310–313]. While these deaths may ultimately be due to natural causes, in many countries they are referred for forensic post-mortem investigation due to the sudden and unexpected nature of demise [36]. In general, many sudden unexpected deaths are attributed to natural or even unnatural causes following autopsy, but some remain unexplained.

It has been hypothesised that at least some of the deaths that remain unresolved by a conventional autopsy are due to inherited cardiac disorders which present with a structurally normal heart [314]. These specifically include cardiac channelopathies and cardiomyopathies [309,310]. Channelopathies are a group of disorders which affect the electrical functioning of

the heart and can lead to fatal arrhythmias, and examples include long QT syndrome (LQTS), Brugada syndrome (BrS) and catecholaminergic polymorphic ventricular tachycardia (CPVT) [309]. Cardiomyopathies however are characterised by the thickening of the cardiac muscles, which affect the pumping action of the heart and can also lead to fatal arrhythmias [315]. These include hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM) and arrhythmogenic (right) ventricular cardiomyopathy (ARVC) [309,310,315,316].

As reviewed by various authors, many mutations and variants have been linked to inherited cardiac disorders [309,317], and some of these are included as medical diagnostic testing for at-risk individuals [318]. More importantly in the forensic context, the molecular analysis of DNA from sudden unexpected death cases which remained unexplained after autopsy, have subsequently been resolved through the testing of the candidate mutations [31,110,319]. Due to the growing body of associated variants, as well as the possibility of novel variants contributing towards disease, a next generation sequencing approach is often used to simultaneously sequence several candidate genes [30,319].

It is also important to note that molecular autopsies may not always have a high diagnostic yield in sudden unexpected death cases: Doolan *et al.* (2008) showed that none of the 59 sudden unexpected deaths cases were resolved by testing for mutations in two genes linked to LQTS (*i.e. potassium voltage-gated channel subfamily Q member 1 (KCNQ1)* and *sodium voltage-gated channel alpha subunit 5 (SCN5A)*) [320], while Glengarry *et al.* (2014) showed that only three out of 71 (4 %) of unselected SIDS cases were resolved through genetic testing of 102 genes linked to LQTS [26]. Both studies suggested that a more selected approach should be utilised for choosing cases for molecular autopsies, and should preferably include individuals who had a family history of inherited cardiac disorders [26,320].

In South Africa, as in many countries, the investigation of sudden unexpected death is governed by legislation [1,36], whereby such deaths are referred for medico-legal investigation as they may be due to unnatural causes. If an unnatural death is ruled out at autopsy, the forensic pathologist is not mandated to determine the specific natural cause of death. As a result, many cases which are admitted as ‘sudden unexpected death’ have *unspecified* natural causes – therefore, there exists the possibility that some of these cases (in addition to those which remained unexplained) may be due to inherited cardiac disorders.

The diagnosis of inherited cardiac disorders in sudden unexpected death cases is important for many reasons. From the family’s perspective, it provides an explanation as to why their relative passed away, which is essential for closure and acceptance of the death [187]. A molecular diagnosis also allows for a targeted genetic screen in blood relatives who may carry the same mutation(s) and be at risk for sudden death themselves [311,321]. In the case of sudden infant death, this may help parents plan for future pregnancies and better manage the associated risks. In addition to the social and clinical applications, the understanding of contributors towards mortality has value for preventative healthcare, whereby identifying genetic risk factors and associated diseases underlying sudden unexpected death may aid in targeted educational interventions [322,323].

Infant mortality is particularly high in sub-Saharan Africa, and statistics from the MDGs have shown that the proportion of child death in the first month of life is increasing [323]. In attempts to reduce infant mortality, it is important to understand the specific causes of mortality and to this end, a molecular autopsy assay would be of value in revealing molecular causes of death. However, it is currently unclear what proportion of sudden infant deaths cases in South Africa exhibit underlying inherited cardiac diseases. This information is critical in the designing and

setting up of a routine molecular autopsy which is relevant to the South African population. As such, the aim of this study was to investigate if a selected cohort of SUDI cases from Salt River Mortuary had variants associated with inherited cardiac disorders which were likely to contribute towards their demise.

8.2 Subjects, materials and methods

8.2.1 Cohort and sample collection

A subset of infants whose cause of death remained undetermined after post-mortem investigation were prioritised for next generation sequencing. These infants did not show any of the four metabolic mutations in the screening phase (Chapter 7). This selected cohort included potential SIDS cases ($n = 4$); infants whose manner of death was natural, but the exact cause was unknown ($n = 11$) and infants whose cause of death was natural, but who were either a twin or who had a previous SUDI in the family ($n = 4$). These infants were admitted to Salt River Mortuary for medico-legal investigation between 1 March 2016 and 31 October 2018, and the post-mortem details have been described previously in Chapter 6.

This cohort subset thus consisted of 19 infants from indigenous African ($n = 4$; 21.1 %) and Mixed ($n = 15$; 78.9 %) Ancestry population groups, with 57.9 % of infants being female. Infants ranged from 2 days to 5 months of age at the time of death (median: 2 months; standard deviation 1.2 months) with 89.5 % of infants being three months old or younger (Figure 8.1).

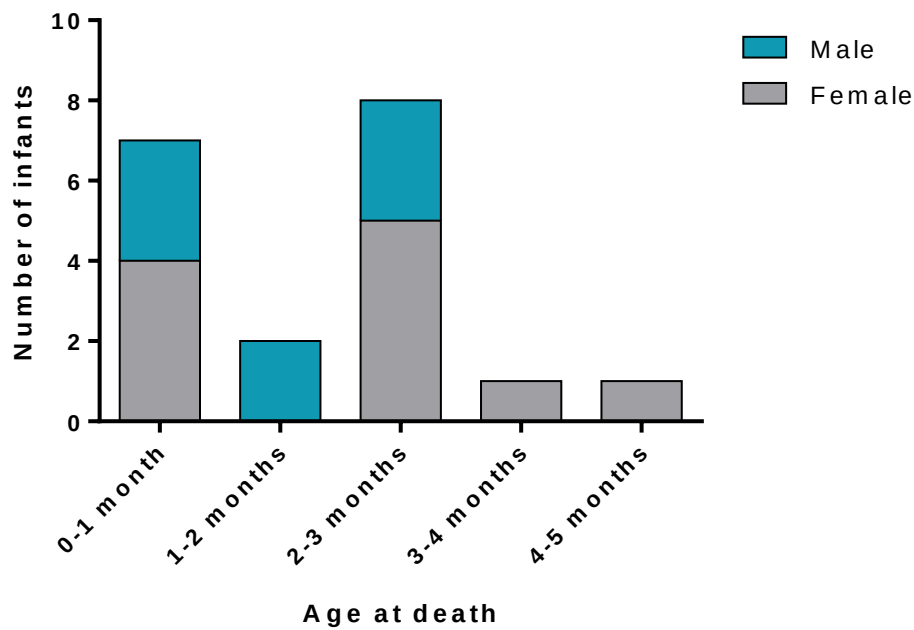


Figure 8.1: Demographic representation of infants included for next generation sequencing: sex and age of infants at death.

A total of 19 infants were included in this part of the study, of which 57.9 % were female and 42.1 % were male.

The majority of infants were (89.5 %) were younger than three months old at the time of death.

Exhaustive attempts were made to contact each of these 19 families to obtain DNA samples from biological parents; this was successful for three of the families. Informed consent was provided by parents for inclusion of their samples in the study. Family pedigrees and full family histories from these three families were also obtained by a genetic counsellor. Thereafter, 5 ml of blood from each parent ($n = 6$) was collected into a purple-capped EDTA collection vial (BDVacutainer Systems, Plymouth, UK) and stored at 4 °C until DNA extraction. Overall, a total of 19 infant samples and 6 parental samples ($n = 25$) were included for next generation sequencing of candidate genes associated with sudden and unexpected death.

8.2.2 DNA extraction and quantification

DNA was already extracted from blood samples from infants as described in previous chapters.

DNA was extracted from parental blood samples using the QIAamp® DNA Investigator Kit

(QIAGEN, Hilden, Germany) according to the manufacturer's protocol and eluted into 50 µl of ATE elution buffer [324].

All 25 DNA samples were quantified fluorometrically using the QuantiFluor® dsDNA System Protocol on the GloMax®-Multi detection system (Promega, Madison, USA). Standards were prepared in by diluting the Lambda DNA standard (100 µl/ml) (Promega, Madison, USA) to result in the following concentrations of DNA: 0 ng/µl, 25 ng/µl, 50 ng/µl, 100 ng/µl, 300 ng/µl, 500 ng/µl, 700 ng/µl and 1 000 ng/µl. Approximately 200 ng - 300 ng of each DNA sample, as quantified by Nanodrop™ spectrophotometry (Thermo Fisher Scientific, Waltham, USA) was diluted 100-fold in 1 x TE buffer. A volume of 50 µl of each standard and each diluted DNA sample was then added to 96-well micro-centrifuge plate in duplicate containing 50 µl QuantiFluor® dsDNA Dye (Promega, Madison, USA). The plate was loaded into the GloMax®-Multi detection system where fluoro-detection automatically took place. Using the standard curve generated from the set of DNA standards, the concentration of double stranded DNA within samples was calculated, upon which DNA samples were diluted to 5 ng/µl.

8.2.3 Library preparation

DNA was prepared for next generation sequencing using the HaloPlex Target Enrichment System for Illumina Sequencing according to the manufacturer's protocol with no deviations (Agilent Technologies, Santa Clara, USA). The specific kit used was developed by the National Health Service (NHS), Churchill Hospital UK, which entailed the design of probes to target specific genes for next generation sequencing. In this instance, the probes targeted 43 genes known to be involved in arrhythmia (Appendix 10). This kit is routinely used as a molecular autopsy to investigate cases of SUD in the UK, and was thus chosen for this study.

In brief, library preparation entailed restriction enzyme digestion of the DNA (5 ng/μl) at 37 °C for 30 minutes; hybridisation of specific probes and unique indices (or barcodes) to DNA fragments at 95 °C for 10 minutes followed by 54 °C for 3 hours; the capturing of target DNA using HaloPlex Magnetic Beads (Agilent Technologies, Santa Clara, USA) at 46 °C for 10 minutes; ligating the captured DNA targets with DNA Ligase at 55 °C for 10 minutes; eluting of the DNA from the magnetic beads using freshly prepared 20 mM NaOH; amplification of the target libraries using PCR; and duplicate purification of amplified libraries using AMPure XP beads (Beckman Coulter, Brea, USA).

For the PCR step, each PCR contained 5 X Herculanase II Reaction buffer, 25 μM of the forward and reverse primers, 2 M acetic acid, 100 nM dNTPs, 0.5 X Herculanase II Fusion DNA polymerase and 20 μl of target library in a total volume of 50μl. All PCR reagents were available within the Herculanase II Fusion PCR kit specifically designed for the Haloplex library preparation method (Agilent Technologies, Santa Clara, USA). Amplification followed typical cycling conditions: initial denaturation at 98 °C for 2 minutes; 18 cycles of 98 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1 minute; followed by a final extension at 72 °C for 10 minutes. Amplification and all incubations were carried out in the DNA Engine Dyad™ Peltier Thermal Cycler (MJ Research Inc., Waltham, USA).

Each purified library was then quantified by microfluidic analysis using the High Sensitivity DNA Assay kit (Agilent Technologies, Santa Clara, USA). A volume of 2 μl of each library sample was added to 2 μl of High Sensitivity DNA buffer (Agilent Technologies, Santa Clara, USA) into a 96-well micro-centrifuge plate, shaken and then loaded onto the 2200 TapeStation Instrument (Agilent Technologies, Santa Clara, USA). Data were analysed on the TapeStation

Controller Software (Agilent Technologies, Santa Clara, USA) to ensure fragments within each library were of the correct size.

Each purified library sample was also quantified fluorometrically using the GloMax[®]-Multi detection system (Promega, Madison, USA), as described above; however, this time standards were prepared to result in the following DNA concentrations: 0 ng/μl, 5 ng/μl, 10 ng/μl, 20 ng/μl, 50 ng/μl, 70 ng/μl, 100 ng/μl and 200 ng/μl. Concentrations of individual libraries were analysed in order to normalise the pooling of the libraries; different volumes of each sample's library were then pooled such that equal yields of libraries per individual were present. The pooled library was then stored for approximately two weeks at -20 °C until sequencing.

8.2.4 Sequencing using the MiSeq[™] System

The pooled library was thawed and then quantified fluorometrically using the GloMax[®]-Multi detection system (Promega, Madison, USA), and with standards of the following DNA concentrations: 0 ng/μl, 1 000 ng/μl, 250 ng/μl, 62.5 ng/μl, 16 ng/μl, 3.9 ng/μl, 1 ng/μl and 0.2 ng/μl. While standards were measured in duplicate as described previously, the pooled library was quantified in a replicate of four. In addition, one control pooled library, which had previously been sequenced successfully using the methods described here, was also quantified in a replicate of four, in order to account for any loss in library due to the freeze-thaw cycle.

Using the mean fluorometric concentration of the pooled library, and taking into account potential loss, the pooled library sample was diluted to 4 nM. To denature the DNA into single strands, 5 μl of fresh 0.2 N NaOH was added to 5 μl of diluted library, mixed, centrifuged at 300 x g for 1 minute in the 5415 D Centrifuge (Eppendorf, Hamburg, Germany), and left to incubate at 5 minutes at room temperature. A volume of 990 μl of pre-chilled HT1 buffer

(Illumina, Cambridge, UK) was added, resulting in a 20 pM denatured library in 1 mM NaOH. The 20 pM library was then diluted further to 6 pM using HT1 buffer (Illumina, Cambridge, UK), and then spiked with 1 % 20 pM PhiX (Illumina, Cambridge, UK).

The cartridge within the MiSeq™ Reagent Kit v3 600 cycles PE (Illumina, Cambridge, UK) was thawed and 600 µl of prepared library was loaded into the Load Sample Reservoir. The flow cell (Illumina, Cambridge, UK) was cleaned thoroughly with 18.2Ω water and lint-free paper towel. The cartridge, clean flow cell and Incorporation Buffer (Illumina, Cambridge, UK) were loaded into their respective compartments in the MiSeq™ System (Illumina, Cambridge, UK), after which cluster generation and sequencing by synthesis took place.

8.2.5 Bioinformatics and data analysis

The FASTQ files from the MiSeq™ System (Illumina, Cambridge, UK) were subjected to a series of bioinformatic analyses to assist with data analysis, with the overall aim of producing a list of variants, alongside associated nomenclature, for each sample that was sequenced. Firstly, Cutadapt [325] was used to remove sequencing adapters and then a custom Python script was used to trim 5 bp from both ends of each read, to account for the restriction enzyme footprint. Low quality reads were removed using the FASTX toolkit (available at http://hannonlab.cshl.edu/fastx_toolkit/, in the public domain); reads were only kept if at least 95 % of the bases within that read had an average coverage of 30 X or more (Q-score ≥ 30). A custom Python script was also used to remove ‘orphan’ reads, to ensure that every read in the forward direction had a matching reverse read, and vice versa.

Sequencing reads were aligned to the human reference sequence (GRCH37) using Burrows-Wheeler Alignment (BWA) tool to produce a ‘sequence alignment/map’ (SAM) file [326].

SAMtools was used to convert the SAM file into a 'binary alignment/map' (BAM) file [327], which was then sorted by co-ordinate using Picard (available at <https://broadinstitute.github.io/picard/>, in the public domain) and indexed using SAMtools [327]. Variant calling and local realignment was achieved using Platypus to generate a VCF file [328], after which variants were annotated using a combination of a Perl script, ANNOVAR [329] and Mutalyzer [330] to generate a mutation report. Within this process, variants were grouped into (i) 'polymorphisms' which have been observed and previously investigated by the NHS and were known to be not pathogenic; and (ii) 'unknown variants' which have not been previously investigated by the NHS, and thus their significance was unknown. Lastly, GATK [331] was used to generate statistics pertaining to sequencing coverage of each base, fragment and gene, as well as a summary of regions which failed coverage thresholds.

8.2.6 Prioritisation of variants

The polymorphisms previously investigated by the NHS and known to not cause disease were not analysed further in this study; whereas the 'unknown variants' were each explored individually using Alamut-Visual 2.11.0. For each variant, their effect on altering splicing was analysed using four prediction tools (SpliceSiteFinder-like, MaxEntScan, NNSPLICE and GeneSplicer); and their frequency in the background population was recorded from gnomAD and 1000 Genomes. Literature was also searched for each variant, with particular emphasis on functional studies. For nonsynonymous variants, prediction of pathogenicity was calculated using four tools (SIFT, PolyPhen-2, MutationTaster and Align GVGD), and the amino acid change was investigated with regards to its conservativeness across species, the physiochemical difference between the amino acids. Assessment was also made regarding the

variant's position within the protein (*e.g.* in a functional domain) and this was interpreted in the context of the mechanism of disease associated with the gene.

Assessment of each variant was done on a case-by-case basis, but overall, variants were prioritised if they met the criteria below. Scoring of variants was based on the guidelines of the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) [332], where by class 1 indicated not pathogenic and class 5 indicated pathogenic. In order to assess the likely effect of the variant on the infant's demise, other variants in the same genes were taken into consideration.

Pathogenicity criteria:

- For all variants:
 - The frequency had to be rare: observed less than 20 times globally and had a frequency of less than 0.0001 % any population. This was critical due to frequency of arrhythmia diseases in the population.
 - Literature supported the variant's functional role in the disease, and/or co-segregation studies had been completed to support the consistent inheritance of the variant and disease OR the variant was novel.
 - The arrhythmia-related disease associated with the gene under study was identified and the deceased infant ideally showed other symptoms pertaining to that disease (*e.g.* sinus infection).
 - There had to be sufficient coverage of the variant itself (as opposed to coverage of the base, which was filtered for previously), and it had to be sequenced in multiple different read fragments.

- For splice variants: at least three of the four tools had to predict the creation or destruction of a donor or acceptor splice site, which was not in close proximity to an existing splice site, and this change had to be in accordance with the disease mechanism; *e.g.* if a splice site was created and a loss of gene function was predicted, then disease mechanism also had to be via loss of function. This was important since some arrhythmia-related diseases have very specific mechanisms; further, some genes are associated with more than arrhythmia-related disease, but via different mechanisms.
- For nonsynonymous variants: at least three of the four tools had to predict that the variant was pathogenic; the nucleotide and amino acid had to be highly conserved across species and there had to be a large physiochemical difference between the amino acids. The variant also had to be in a functional domain of the protein and the predicted altered protein function had to be in accordance with the disease mechanism. The latter point particularly applied to nonsense variants, whereby the disease mechanism associated with that gene had to be ‘loss of function’.
- For insertion/deletions (indels): the indel had to cause a frameshift, and the resulting amino acid sequence had to be different to the original sequence, or produce a premature stop codon. The loss of function of the protein had to be in accordance with a ‘loss of function’ disease mechanism.

Taking all available information into account, an interpretation was made regarding the contribution of the variant to the demise of the infant, which was categorised as: probably, possibly and unlikely. If there was too little information, it remains a VUS.

8.2.7 Confirmation of variants by Sanger sequencing

Unknown variants which met the criteria above and all novel variants were confirmed using Sanger sequencing. This was achieved using in-house primer sets provided by the NHS (available on request and approval from NHS), and involved the typical process of PCR, verification of amplification on agarose gel electrophoresis, sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) and capillary electrophoresis using the ABI Genetic Analyser 3130xl.

8.3 Results

8.3.1 All variants observed

Sequencing data was subjected to bioinformatic analysis, which revealed a total of 1 775 variants in the 19 infants; each infant exhibited between 67 and 111 variants within the 43 genes analysed (Table 8.1). Most of the variants observed were known polymorphisms, leaving 123 different ‘unknown variants’ among the cohort for further investigation. Each infant had between two and 18 unknown variants (Table 8.1).

Table 8.1: Number of variants observed in the 43 genes among the 19 infants studied.

A total of 1775 variants were found, of which 1603 were known polymorphisms. There were 123 unique unknown variants which were analysed further to assess their pathogenicity.

Infant ID	Total number of variants	Number of known polymorphisms	Number of unknown variants			
			Missense	Sense	Intronic	Indel
1	109	97	5	6	0	1
2	105	93	2	7	3	0
3	96	87	3	5	0	1
4	75	67	2	5	0	1
5	108	95	6	4	2	1
6	96	78	7	8	2	1
7	111	95	5	9	2	0
8	95	87	2	3	1	2
9	110	98	2	5	5	0
10	91	89	0	2	0	0
11	67	61	0	5	0	1
12	94	86	4	3	0	1
13	93	88	1	2	2	0
14	61	57	0	2	0	2
15	97	91	2	3	1	0
16	82	73	2	6	0	1
17	101	90	3	6	1	1
18	101	93	1	5	2	0
19	83	78	0	3	1	1
Total	1 775	1 603	47	89	22	14
Total unique unknown variants (n = 123)			40	59	22	2

8.3.2 Prioritised and novel variants

Variants were investigated using bioinformatic tools to assess their pathogenicity, and then individually assessed in terms of the literature and within their respective case contexts. Overall, none of the unknown variants met all the criteria of being pathogenic (class 5), as outlined in the methods, mainly due to the absence of functional and co-segregation studies. However, based on bioinformatic tools and background frequencies, ten missense variants in nine infants were prioritised for further investigation, as they were putatively pathogenic from *in silico* analyses (Table 8.2). Of these ten variants, the evidence for pathogenicity was slightly weaker for three variants due to slightly higher background frequencies than expected (n = 2) or only one out of three prediction tools indicating pathogenicity (n = 1) (Table 8.2).

A total of four novel unknown variants were observed in the cohort (Table 8.3). Interestingly, they occurred in four infants who also each had a prioritised variant. While there was slight evidence for two of these novel variants affecting splicing, none of them were convincing enough to explore further. Of all the 123 unknown variants, and where parents were available (three families), no variant occurred *de novo*.

Despite weaker evidence for three of ten the variants, each of the prioritised variants were studied in more detail in relation to the literature and case context (Table 8.4 and Section 8.4). It must be noted that the ‘cardiac diseases’ listed in Table 8.4 are not diagnosed diseases in the infants, nor are they necessarily being caused by the observed variants in this study – they are rather indicating the cardiac disease previously associated with *other* mutations in the *same* gene as being investigated here.

Overall, it appeared that one variant *probably* contributed towards one infant’s demise, three *possibly* contributed towards two cases (one case had two variants), four were *unlikely* to contribute towards demise, and two remained VUS (Table 8.4).

Table 8.2: List of unknown variants after prioritisation

A total of ten variants in nine infants were prioritised in terms of likely pathogenicity, of which the first seven were more convincing. All variants below were missense variants, none appeared to affect splicing, and none had supporting literature regarding function or co-segregation. One infant was homozygous for one of the unknown variants. MAF: minor allele frequency; obtained from gnomAD (<http://gnomad.broadinstitute.org>; date accessed 21/04/2018)

Gene	Accession number	Variant (cDNA)	rs number	Amino acid change	Conserved across species	Physiochemical difference between amino acids	Number of tools (missense) which predict pathogenic	Global MAF (number of observations)	Highest MAF in any population	Occurrences (infant study number)
Variants of unknown significance (Putative pathogenic)										
<i>AKAP9</i>	NM_005751.4	c.5638C>G	rs761946454	p.Arg1880Gly	High	Large	4 out of 4	0.000020 (5)	0.000182 (1)	1 (#5)
<i>ANK2</i>	NM_020977.3	c.3421C>T	rs772003205	p.Arg1141Cys	High	Large	3 out of 4	0.000012 (3)	0.000045 (2)	1 (#4)
<i>HCN4</i>	NM_005477.2	c.2069A>G	rs776033369	p.Asn690Ser	High	Small	4 out of 4	0.000002 (6)	0.000089 (3)	1 (#6)
<i>LMNA</i>	NM_170707.2	c.1279C>T	rs373584456	p.Arg427Cys	Moderate	Large	3 out of 3	0.000025 (5)	0.000058 (1)	1 (#9)
<i>PKP2</i>	NM_004572.3	c.2612G>A	rs372263407	p.Arg871Gln	High	Small	4 out of 4	0.000061 (15)	0.000259 (8)	1 (#8)
<i>SCN10A</i>	NM_006514.2	c.40C>T	rs750771811	p.Arg14Cys	High	Large	3 out of 3	0.000020 (5)	0.000065 (1)	1 (#1)
<i>SCN10A</i>	NM_006514.2	c.1522C>T	rs112774699	p.Arg508Trp	High	Moderate	3 out of 3	0.000040 (9)	0.000197 (6)	1 (#2)
Variants of unknown significance (Putative pathogenic, but evidence is weaker and/or conflicting)										
<i>CACNA1C</i>	NM_199460.2	c.5075C>T	rs376642156	p.Ser1692Phe	Moderate	Large	1 out of 3	0.000004 (1)	0.000058 (1)	1 (#6)
<i>DSP</i>	NM_004415.2	c.2773C>T	rs145933612	p.Arg925Trp	Moderate	Moderate	4 out of 4	0.000398 (98)	0.004000 (64)	1 (#7)
<i>SCN3B</i>	NM_018400.3	c.328G>A	rs147205617	p.Val110Ile	High	Small	4 out of 4	0.000288 (71)	0.002000 (42)	1 (#3)

Table 8.3: Novel variants observed in the 43 genes among the 19 infants

A total of four novel variants were identified in the cohort, although none of them were predicted to be pathogenic.

Gene	Accession number	Variant (cDNA)	Amino acid change	Conserved across species	Physiochemical difference between amino acids	Number of tools which predict splice site alteration	Number of tools (missense) which predict pathogenic	Pathogenicity	Occurrences (infant study number)
<i>AKAP9</i>	NM_005751.4	c.11069G>A	p.Ser3690Asn	Weak	Small	0 out of 4	0 out of 4	Not pathogenic	1 (#4)
<i>RYR2</i>	NM_001035.2	c.166A>C	p.Lys56Gln	Moderate	Small	3 out of 4	1 out of 4	Unlikely	1 (#5)
<i>SCN10A</i>	NM_006514.2	c.1226A>G	p.Asp409Gly	Moderate	Small	1 out of 4	0 out of 4	Unlikely	1 (#6)
<i>TMEM43</i>	NM_024334.2	c.396G>C	p.Glu132Asp	Moderate	Small	3 out of 4	1 out of 4	Unlikely	1 (#7)

Table 8.4: Infant details where a prioritised unknown variants was observed, alongside associated autopsy details and disease associated with the variant.

One homozygous variant in *SCN10A* probably contributed towards infant demise; one heterozygous variant in *SCN3B* possibly contributed towards infant demise; and two variants in the same infant possibly acted together, and with other factors, to contribute towards demise. Six prioritised variants which were putatively pathogenic and rare were unlikely to have contributed towards cardiac disease in these infants. BrS: Brugada syndrome; LQTS: long QT syndrome; ARVC: arrhythmogenic right ventricular cardiomyopathy; DCM: dilated cardiomyopathy; VUS: variant of unknown significance.

Case	Infant				Autopsy details		Variants and diseases associated with genes			Contribution to SUDI
	Ancestry	Sex	Age at death	Previous SUDI or stillborn	Autopsy extent	Cause of death	Variant(s) observed	Disease associated with gene	Mechanism if disease	
1	African	Male	2 months	No	Partial	Natural causes	<i>SCN10A</i> p.Arg14Cys (homozygous)	BrS	Loss of function	Probably
2	Mixed	Female	2 months	Yes	External	Natural causes	<i>SCN10A</i> p.Arg508Trp	BrS	Loss of function	VUS
3	Mixed	Female	2 months	No	External	Natural causes	<i>SCN3B</i> p.Val110Ile	BrS	Loss of function	Possibly
4	Mixed	Female	2 days	Yes	External	Natural causes	<i>ANK2</i> p.Arg1141Cys	LQTS	Loss of function	Unlikely
5	Mixed	Female	3 months	No	External	Natural causes	<i>AKAP9</i> p.Arg1880Gly	LQTS	Loss of function	Unlikely
6	Mixed	Female	5 months	No	External	Natural causes	<i>CACNA1C</i> p.Ser1692Phe	Timothy syndrome	Gain of function	Possibly
							<i>HCN4</i> p.Asn690Ser	Sick sinus syndrome	Loss of function	
7	Mixed	Female	2 weeks	No	Partial	Under investigation	<i>DSP</i> p.Arg925Trp	ARVC	Haploinsufficiency	Unlikely
8	African	Male	2 months	No	Full	Under investigation	<i>PKP2</i> p.Arg871Gln	ARVC	Loss of function	VUS Possibly
9	African	Male	3 weeks	Yes	External	Natural causes	<i>LMNA</i> p.Arg427Cys	DCM	Loss of function	Unlikely

8.4 Interpretation of variants in case contexts

Case 1

A homozygous variant *SCN10A* c.40C>T (p.Arg14Cys) was observed in a two month old male of African ancestry. The *SCN10A* gene has been previously linked to BrS [333,334]; however, this association has been widely debated [335–338]. Signs of respiratory tract infection and a structurally normal heart was observed at autopsy, and review of clinical history showed signs of fever prior to death. Amin *et al.* (2008) documented that fever was the triggering factor for approximately 18 % of the cardiac arrests in BrS patients [339].

Some studies have demonstrated the functional effect of *SCN10A* on *SCN5A* [334,340,341], with the latter gene having considerably more causal evidence for channelopathies [342,343]. For example, Bezzina *et al.* (2013) showed that variants in *SCN10A* contributed to BrS by altering the function of the ‘Nav1.5’ protein, which is encoded by *SCN5A* [333]. However, Behr *et al.* (2015) showed that while the minority of rare variants were significantly associated with BrS, a SNP-set (Sequence) Kernel Association Test (SKAT) indicated that overall, the presence of rare variations in *SCN10A* was not significantly different between in cases and controls, particularly in *SCN5A*-negative cases [344]. However, more recently Okamura *et al.* (2017) showed that variants in *SCN10A* may decrease *SCN5A* expression through DNA methylation in patients without *SCN5A* exonic mutations [341].

Hu *et al.* (2014) identified 17 variants in *SCN10A* in 25 BrS patients (cohort n = 150), and found that patients with these variants were more symptomatic than those without; and that 16 of the 17 variants were missense. A p.Arg14Leu variant was identified in four unrelated patients, and heterologous co-expression in HEK cells reduced *SCN5A* activity by 79 % [345]. In this study, the variant in case 1 (p.Arg14Cys) also affected amino acid number 14 but instead

altered the arginine to cytosine. Considering that the p.Arg14Cys was very rare, it was predicted to be pathogenic *in silico* as well as the functional importance of arginine at residue 14, this variant was considered to *probably* play a role in the infant's demise. This was further supported by the homozygosity of the variant, which has not been reported before, and particularly that this residue functionally affects *SCN5A* activity which is well-linked with channelopathies [346].

Although it was not apparent from medico-legal documentation pertaining to the infant death; it appeared that the parents of the infant may have been somewhat related, as about 36 % of all observed variants in this infant were homozygous. This was slightly higher than the number of homozygous variants observed in other cases (median = 30 %). Interestingly, however, another variant towards the end of *SCN10A* was observed in this infant (c.3421G>A; p.Val1141Met), but was heterozygous, and thus a recombination event must have occurred within the gene.

Case 2

A second unknown variant in the same gene, *SCN10A*, was also prioritised for further investigation. The heterozygous *SCN10A* c.1522C>T (p.Arg508Trp) variant was observed in a two month old female of Mixed ancestry where signs of gastroenteritis was observed at autopsy. This infant was included for next generation sequencing analysis as there had been a previous SUDI in the family, but unfortunately biological material was not retained from the sibling and the parents were lost to follow up.

This variant has been previously observed during large-scale sequencing studies, of which two of the nine reported were in the 'Bushman' (or southern African San) population (<http://gnomad.broadinstitute.org/>; accessed on 02/03/2018). The variant seems to not have

previously been observed in relation to SUDI or BrS, but this may be due to the population groups previously investigated in such studies. This infant belonged to the Mixed Ancestry population group which is largely underrepresented in genetic studies, and may thus be the reason for this variant being observed in a SUDI case for the first time.

While this variant was rare and predicted to be pathogenic *in silico*, there was no functional study or co-segregation reports to support that this variant contributed towards the demise of the infant. The residue 508 has not been functionally tested nor reported in other SUDI cases. In the absence of this important information, there is substantially less evidence that *SCN10A* c.1522C>T (p.Arg508Trp) played a similar role to that in case 1. This variant therefore remained a VUS.

Case 3

The final prioritised variant in a sodium voltage-gated channel gene was *SCN3B* c.328G>A (p.Val110Ile), and occurred in an infant (female, 2 months old) for whom parent DNA samples were available. The infant apparently demised during sleep, and a chest X-ray scan at autopsy showed signs of LRTI. The infant was heterozygous for the variant. Limited co-segregation analysis found that the variant was inherited from her father (aged 33 years at the time of study), who was also heterozygous for the variant, and who did not have documented clinical history of arrhythmia or any heart-related disease. However, his maternal half-sister's daughter has had four miscarriages.

In silico tools consistently predicted that the variant was pathogenic, and functional studies showed that cells with this specific variant had significantly reduced sodium currents through

the loss of a subunit in Nav1.5 trafficking to the plasma membrane [347]. The functional effect of the variant was consistent with the loss of function disease mechanism apparent in BrS.

The variant has been associated with BrS (ClinVar; <https://www.ncbi.nlm.nih.gov/clinvar/>; accessed 02/03/2018), and it was present in three out of 178 Japanese patients with BrS, and zero out of 480 Japanese controls [347]. It was also identified in one SUDI case (male, 1 month, African-American) at Harris County Institute of Forensic Sciences (Houston, Texas) via next generation sequencing in 2016, and was considered a reportable genetic mutation by a multi-disciplinary team of experts [25]. However, the variant had a 0.0297 % frequency in the background population (<http://gnomad.broadinstitute.org/>; accessed on 02/03/2018), which raises doubt regarding the pathogenicity of this variant - as not this many individuals in the background population have BrS, let alone caused by this single variant. Alternatively, this may suggest reduced penetrance of this variant.

Overall, there is some evidence suggesting that this variant contributed to the demise of this infant, however, its occurrence in 0.0297 % of the background population suggests that there may be other factors (genetic or environmental) which may have acted alongside the variant to fully explain the death - in this instance, perhaps the mild respiratory tract infection. This theory is in line with the triple-risk hypothesis, which describes a predisposed infant (in this case the *SCN10A* c.1522C>T (p.Arg508Trp) variant); at a critical stage of development (two months old); and a trigger event (possibly the respiratory tract infection), which occurred simultaneously and together results in death [13]. Therefore, it was concluded that this variant *possibly* contributed towards infant death.

Case 4

The heterozygous variant *ANK2* c.3421C>T (p.Arg1141Cys) was detected in a three month old female of Mixed Ancestry. This variant is in exon 30 of *ANK2*, which Cunha *et al.* (2008) showed was absent in cardiac tissue [348]. Previously it was shown that exon 30 would disrupt the spectrin-binding domain which is encoded by surrounding exons [349]. Since the variant is within an exon that is not present in cardiac tissue, it is very *unlikely* that this variant contributed towards infant death.

Case 5

AKAP9 c.5638C>G (p.Arg1880Gly) was observed in a two day old female infant of Mixed Ancestry (heterozygous), and signs of respiratory tract infection were noted at autopsy. Within this family, a previous SUDI had been reported, but unfortunately, the family was lost to follow up and were not contactable to request parental DNA samples. Biological material was not previously retained from the sibling, and an unspecified natural cause of death was documented in the post-mortem report.

AKAP9 encodes at least two isoforms, which interact with various signalling proteins to assist with spatial arrangement and temporal signalling [350,351]. AKAP proteins (to which *AKAP9* belongs) often join opposing regulatory molecules to establish localised temporal regulation of signal transduction [350,351]. In the heart, *KCNQ1* and *KCNE1* form a slow potassium ion channel (I_{Ks} channel) and generate the I_{Ks} current which is essential for the late phase of repolarisation of the cardiac action potential [352]. The I_{Ks} channel responds to depolarisation and is regulated by the sympathetic nervous system via the β -adrenergic receptor/Gs/cAMP/PKA pathway [352]. If the I_{Ks} channel is inhibited, it will cause prolonged action potential duration and thus LQTS [353].

Yotiao 9 is the smallest isoform of AKAP9 and brings together various proteins, including PKA, PPI, AC9, PDE4D3 and KCNQ1, which each play a role in the signal transduction pathway [354]. Overall, this complex regulates phosphorylation of KCNQ1 at Ser27 and therefore the activity of the I_{Ks} channel [354]. Different regions of Yotiao bind each of these proteins; thus mutations which disrupt this binding underlie LQTS [355,356]. The variant observed in this study, however, was at residue position 1880 which is not involved in binding any of these other proteins; thus is it *unlikely* that this variant contributed towards LQTS in this infant.

Case 6

This five month old female infant of Mixed Ancestry had two prioritised variants (i) *HCN4* c.2069A>G (p.Asn690Ser) and (ii) *CACNA1C* c.5075C>T (p.Ser1692Phe). A large heart and patchy lungs with discharge were observed via a Lodox[®] scan during autopsy. The post-mortem report indicated natural causes of death, but the exact cause was unspecified.

HCN4 is mainly expressed in the sinus node cells and forms the pacemaker current (I_F) [357]. Variants in *HCN4* have been associated with BrS as well as Sick Sinus Syndrome type 2, the latter which is characterised by a lack of heart rate response to stress [358,359]. The *HCN4* channel is modulated by cAMP and mutations in the C terminus of the protein have made the protein unresponsive to cAMP [360]. Another functional study showed that mouse embryos were not compatible with life, when they were homozygous for p.Arg669Gln, which is a missense variant in the C terminus of the protein, nine residues after the one observed in this study [361]. The same functional study also showed that the role of *HCN4* as a pacemaker in adult heterozygous mice was less important, but rather prevented sinus pauses during and after

exercise. Overall, their results highlighted the necessity of cAMP binding for HCN4 function, and thus demonstrated the importance of the C terminus domain [361].

The variant observed in this study was in the C terminus of the protein and it is plausible that it contributed towards the lack of cAMP binding. Although this has not been confirmed through functional studies, this variant may have a similar effect to other missense variants in the C terminus which result in sinus bradycardia and eventually chronotropic incompetence.

The second variant was in exon 42, in the C terminus, of *CACNA1C*. Other variants in this gene have previously been associated with LQTS8 or Timothy syndrome (TS), which is very rare and usually leads to death in children who are two to three years old [362,363]. If no other symptoms manifest, then ‘cardiac only Timothy syndrome’ (COTS) may be present, which is via a gain of function mechanism. However, Boczek (2015) noted that affected COTS individuals with the p.Arg518Cys/His variants also had congenital heart defects, and one affected family member died suddenly and unexpectedly at age 30 [359].

However, in TS, in addition to the typical prolonged QT interval, other symptoms include immune deficiency, facial dysmorphisms, bone formation abnormalities and syndactyly [364]. None of these features were noted at autopsy, although patchy lungs in the absence of a confirmed pathogen may be suggestive of immune deficiency. Further, this infant had a heterozygous variant (c.-488G>A) in the promoter region of *TNF α* (Chapter 6), which is a proinflammatory cytokine, and polymorphisms in this regulatory promoter region have been linked with sudden infant death [109,131,365,366].

The protein domain in which *CACNA1C* c.5075C>T (p.Ser1692Phe) falls (IQ domain isoleucine-glutamine motif; residues 1607-1681) regulates two self-regulatory calcium-dependent feedback mechanisms: calcium-dependent inactivation (CDI) and calcium-dependent facilitation (CDF) [367]. Therefore, variants in the C terminus (such as this variant) have the potential to affect this regulation, which would lead to a gain of *CACNA1C* function. However, there are no functional studies pertaining to the specific variant observed in this study, and it has not been previously observed in a patient with TS or COTS.

While each of these variants alone may not have contributed significantly towards death, it may be plausible that each of the three variants observed in this infant partially contributed towards an overall genetic predisposition, in an infant who was undergoing critical stages in their development, and who had signs of respiratory pathogens - it is therefore *possible* that together these factors may have all contributed towards infant's demise. This notion of 'polygenic risk scoring' has been interrogated in other complex multifactorial disorders (*e.g.* for bipolar and schizophrenia), whereby there may be an additive effect of multiple 'seemingly straightforward' variants, each of little 'pathogenic' effect - but may collectively produce a 'below threshold functioning' of pathways [368,369]. This possible explanation for this case is merely a hypothesis which would warrant further testing.

Case 7

This infant had a variant in *DSP*, whose desmosomal protein interacts with desmin intermediate filaments in cardiomyocytes to form strong adhesions between cells [370]. Suboptimal adhesion between cells can give rise to ARVC, which is characterised by fibro-fatty replacement of myocardium [370]. The mechanism of disease associated with *DSP* is via haploinsufficiency, by frameshift and nonsense mutations prior to the final exon [371]. The

variant observed in this study was a missense variant (*DSP* c.2773C>T p.Arg925Trp) and therefore was *unlikely* to have contributed towards the demise of the two week old female infant of Mixed Ancestry.

Case 8

The heterozygous variant *PKP2* c.2612G>A (p.Arg871Gln) was observed in a two month old indigenous African male. A full autopsy was conducted, yet the case was undetermined by autopsy alone and is currently still under investigation.

PKP2 encodes for a desmosomal protein which acts as a physical link between cardiomyocytes and thus facilitates communication between cells. Sato *et al.* (2009) showed that a loss of *PKP2* function reduced the sodium channel activity in cardiomyocytes [372]. Loss of the desmosomal protein leads to ARVC which manifests with fibro-fatty infiltration of the myocardium. This can be life-threatening and variants in *PKP2* have presented with early onset ventricular fibrillation and sudden cardiac death in the young, particularly when cardiomyopathy is not yet obvious [373].

More than 120 putatively pathogenic variants in this *PKP2* have previously been associated with ARVC [374]. Alcade *et al.* (2015) showed that most pathogenic variants in *PKP2* were radical frameshift or nonsense variants. Out of 48 missense variants which were reported in *PKP2*, only 57 % were predicted to be pathogenic *in silico* [374].

The variant observed in this infant as a missense variant in the final exon of the gene. The variant appeared to be present after the armadillo repeats, which are involved in binding to other proteins [375]. ClinVar reported the variant to be associated with ARVC but with

uncertain significance. The absence of functional studies of the specific domain including this variant makes it challenging to assess its functional influence. In the absence of this information, the variant remains of unknown significance.

Case 9

The *LMNA* c.1279C>T (p.Arg427Cys) heterozygous variant was observed in a three week old male infant of indigenous African ancestry. Signs of bilateral lung infiltrates were noted at autopsy. While the mother of this infant has not had a previous SUDI, she has had a previous stillborn, and some studies suggest that risk factors for sudden death in infancy and stillborn babies overlap [376–378].

Lamin proteins act as structural filaments in the inner nuclear membrane and various changes to the gene may give rise to various diseases [379,380]. Of relevance to this study however is that variants in *LMNA* have been associated with DCM and sudden death [312]. Missense, splicing and nonsense variants throughout the gene have been reported as pathogenic, with little evidence for ‘hotspot’ areas for cardiac diseases [380]. The variant was in exon 7, part of the lamin tail protein domain. Different phenotypes have been observed within families with the same mutation, which demonstrates the heterogeneity of the disease, but also suggests that other factors may also play a role in disease (*e.g.* other variants in other genes which may modulate function) [380,381]. The age of onset of DCM is typically in adulthood, and sudden death is often the result of progressive disease [312,379,382], thus making it overall *unlikely* that this infant had DCM from this variant.

8.5 Discussion

The purpose of this study was to investigate 43 arrhythmia-related genes in a selected cohort of SUDI cases to determine if any case could be explained through genetic analysis, or to identify novel or unknown variants which may warrant further exploration in the future. Next generation sequencing combined with bioinformatic analysis revealed 123 different ‘unknown variants’ in nineteen infants, of which ten were putatively pathogenic (*in silico*) and another four were novel. The four novel variants were not predicted to be pathogenic, but two showed some signs of affecting splice sites. Testing this hypothesis functionally was not part of the scope of this study, but may be worthwhile investigating in future work.

One of the factors which affected the complexity of interpretation of next generation sequencing data in this study was lack of available clinical history for infants. While many individuals in South Africa cannot afford specialist clinical testing (*e.g.* echocardiogram) it is unlikely that this would be performed within the first few months of life; hence comprehensive and informative clinical history is rarely available for SUDI cases. This contributed to the difficulty in assessing the role of rare, putatively pathogenic variants in cases which presented as sudden unexpected death, where a potential inherited cardiac disease should be considered.

The 10 putatively pathogenic variants were thus subjected to further scrutiny to assess if they may have contributed towards the death of the infant. After careful consideration, together with a conservative approach, one of the ten prioritised putatively pathogenic variants (homozygous) was concluded to have probably contributed towards the death of the infant (case 1) in the form of BrS. In a further two cases (case 3 and 6), it was concluded that the variants observed possibly contributed towards death, but other factors probably added to the demise. This notion is consistent with the triple risk hypothesis which Filiano and Kinney first

proposed in 1994 [13]: in these cases, the infants were at a critical stage of their development (2 and 5 months old), the trigger event may have been the contraction of an infection, and the predisposition may have been genetic variants. The simultaneous occurrence of these events may have resulted in a cardiac arrhythmia ultimately leading to death.

The majority of the deceased infants who are admitted for medico-legal investigation at Salt River Mortuary under the category that their death was sudden and unexpected [36], appear to have signs of respiratory tract infection at autopsy [215,383]. Even if these signs are mild, and no signs of unnatural death are observed, the case is considered natural, and unfortunately, forensic resources are not available to investigate such cases further – as it is the mandate of the FPS to investigate cases of *unnatural* death [1]. In addition to this reality (which is not unique to South Africa), there are difficulties in establishing whether mild infections are really causative of death – first because no empirical tests exist to assess this, but also, symptoms of fever can trigger more serious events such as a cardiac arrest in individuals with BrS [339]. As such, it is plausible that at least a subset of the SUDI cases with mild infections at Salt River Mortuary have underlying genetic variants, which, combined with infection, would result in death. This hypothesis was indeed supported with three of the nineteen selected cases in this study (approximately 15 % of cohort) having signs of respiratory tract infection, but moreover, had genetic variants which either possibly or probably contributed towards their demise.

While one infant's death could be attributed to an inherited cardiac disorder (5.26 % resolution), the molecular autopsy added substantial value to three (15.79 %) of nineteen selected cases. This percentage resolution was higher than the median resolution documented in other studies involving SUDI cases from non-African population groups (median resolution = 4) (Chapter 3). What was different in this study to other molecular autopsy studies, was that

no prioritised variant was observed in the ‘classic’ genes typically associated with channelopathies and sudden death (*e.g.* *RYR2*, *KCNQ1*, *KCNH2* and *SCN5A*). While one variant *SCN3B* c.328G>A (p.Val110Ile) (case 3) had previously been associated with a sudden infant death case in the USA [25], this study was the first to associate (i) *SCN10A* c.40C>T (p.Arg14Cys) (case 1); (ii) *HCN4* c.2069A>G (p.Asn690Ser) and (iii) *CACNA1C* c.5075C>T (p.Ser1692Phe) (case 6) variants with sudden infant death. One of the next steps in this larger ongoing study would be to investigate if these variants are also present in other SUDI cases admitted to Salt River Mortuary, particularly those with signs of mild infection.

Sudden unexpected death of infants or adults may be attributed to a host of different diseases, only one of which is inherited cardiac disorders. Exploring the possibilities of many diseases in such a deceased individual brings about its many challenges, especially when clinical history is absent. As such, it is imperative that an open mind is kept about the disease(s), disease mechanism(s) and gene(s) which may be associated with the deceased individual. Most of the inherited cardiac disorders suggested in this study turned out to have a dominant inheritance pattern, therefore analysis of the proband’s DNA was sufficient. However, this may not always be the case, as inherited disorders might be recessive and could present as a result of compound heterozygous mutations (*i.e.* two different mutations on different alleles). In these cases, parental samples become increasingly important for co-segregation analyses and interpretation of genetic results. Since it is unknown what disease and inheritance pattern may transpire at the time death and recruitment into research, every effort should be made to collect parental DNA samples for the purposes of assessing recessive disorders as well as *de novo* mutations. While this did not impede the investigation in this study (no inherited recessive disorders were suggested), it did limit the analysis of *de novo* mutations in most infants.

In this study, parental samples were available for three infants, which created the valuable opportunity to assess if these specific infants had a *de novo* mutation. A *de novo* mutation, particularly in the mitochondrial DNA (not assessed here), is hypothesised to be a particularly plausible mechanism for sudden death, as it would explain the survival of parents and not the infant [29,115,384,385]. While no *de novo* mutation was observed in this study, it must be noted that only 43 genes were investigated, and such a mutation could exist in a gene not investigated here. One of the infants with parental samples (case 3) had the variant *SCN3B* c.328G>A (p.Val110Ile), which possibly contributed towards death. However, the remaining two infants where parental samples were available were not resolved with this molecular autopsy. The investigation of other genes associated with other diseases (*e.g.* inborn metabolism errors, primary immunodeficiency diseases), is strongly motivated for in these cases, due to the availability of parental samples.

Overall, 43 genes associated with arrhythmia were sequenced in nineteen selected SUDI cases and ten putatively pathogenic variants were identified in nine infants. The importance of interpreting these results in the context of functional studies and case context was demonstrated, as only four of these ten variants remained convincing enough to have either probably or possibly contributed towards death. The molecular autopsy added value to the post-mortem investigation of 15.79 % of case, which is higher than that reported in similar studies on other populations. In future work, these four variants should be screened for in other SUDI cases and the homozygous variant observed in case 1 (*SCN10A* c.40C>T p.Arg14Cys) should be considered for inclusion in a functional study to assess if it has a similar effect as p.Arg14Leu on *SCN5A* activity [345]. Only then should this genetic result be considered for feedback to the family of case 1. Additional genetic testing should be performed on the DNA from these cases to investigate other inherited diseases which may have contributed towards

the infants' demise. Cases where a previous SUDI occurred in the family and those for which parental DNA samples are available should be prioritised, due to the purported interpretational value that these samples bring to the investigation.

Chapter 9

MOLECULAR AUTOPSY AND THE CLINICAL EXOME

Clinical exome sequencing of sudden unexpected death in infants: two case reports

9.1 Background

SUDI cases form a substantial portion of the already high caseload experienced in forensic mortuaries in South Africa [33,124]. While most of these SUDI cases are diagnosed with infectious causes of death following post-mortem investigation, a subset of cases remain unexplained. At Salt River Mortuary, these include cases which do not fully meet the criteria for SIDS, mainly because some of the analyses required for the diagnosis of SIDS are not common practice or readily accessible within this resource-limited setting (Chapter 2). This includes metabolic screening, which is also not offered routinely at birth, as well as toxicology testing.

Since SIDS is the leading cause of death in developed countries [18,20,59] there has been much effort to further elucidate underlying causes. One of these has been the recognition of genetic variants contributing towards death [34]. This prompted a research study at the University of Cape Town, which is linked with Salt River Mortuary, to prospectively investigate genetic variants in South African SUDI cases [32]. To this end, 201 SUDI cases were recruited over a 31 month period, of which 170 cases met the inclusion criteria (Chapter 6). These cases

underwent screening of four mutations causative of metabolic diseases (Chapter 7), and a subset of cases then underwent sequencing of 43 genes associated with inherited cardiac arrhythmias (Chapter 8). For three of the infants, parental samples were also available for genetic analysis, and sequencing analysis identified a candidate variant in *SCN3B* in one of the families (Chapter 8). Thus, the remaining two trios (infant and parents) were prioritised for further genetic testing, and these results are presented as case reports here.

9.2 Case histories

The first infant was a one month old South African female, from the Mixed Ancestry population group. She was born premature at 32 weeks via an emergency caesarean section, with a low birth weight at 2135 g. The mother received iron supplements during pregnancy and occasionally smoked. She reported that she did not drink alcohol or use drugs. The baby was exclusively breastfed and was reported to be healthy.

The baby lived in a ‘Wendy house’ (wooden shed) with her parents and her two siblings, who were one and two years old respectively, at the time of the demise of the proband. All five family members co-slept on one large adult bed, with the infant usually sleeping on her side. These are common sleeping practices within the culture (Chapter 2). One night, the mother woke up and felt that her baby was cold. The baby was found on her back with her face up with no items covering her face. The mother reached to put the infant on her chest to keep her warm, when she realised that the baby was unresponsive.

The second case was also a female South African infant from the Mixed Ancestry population group, and was two months old at the time of her demise. She was born premature, at 37 weeks gestation, via natural delivery. The mother received antenatal care but smoked 3-5 cigarettes

per day during pregnancy. At the time of the interview with FPS, she acknowledged that she knew smoking harms unborn babies, and she denied drinking alcohol and using drugs.

The premature baby received kangaroo mother care (KMC) and was fed a combination of breastmilk and formula. The infant was fully up to date with vaccinations and was apparently well. On the night of her demise, the baby was put to sleep (co-slept with parents), and in the early hours of the morning, the mother woke up to her baby coughing blood. The baby was on her side, with one eye open and one eye closed. The mother described that she saw the baby turn blue as she rushed her to their neighbour for help.

9.3 Post-mortem examination

The infants were admitted to Salt River Mortuary as SUDI cases for determination of cause of death. A death scene investigation was performed in both cases by FPS, using the FPS006(b) form as a guide (Appendix 4). Full clinical history was available for both infants in the form of the 'Road to Health' Clinic Card (Appendix 3).

For the first case, a full body X-ray was first performed using the Lodox[®] Xmpar-dr which showed no evidence of bone fractures. After a full autopsy was performed, there was no evidence of injury to the body and there were no remarkable signs of disease. Of note, there was some blood in the left nostril, the lips and gingiva were blue in colour, and the lungs were firm, showing congestions and oedema. Everything else was normal. Tissue sections were retained for future histological examination, only if ancillary tests indicated this would be necessary. Various samples were obtained for biochemistry, virology, microbiology and toxicology, all of which tested negative. The preliminary post-mortem report indicated that the infant demised from natural causes.

The second infant had an external autopsy, whereby the Lodox[®] Xmplar-dr scan showed signs of LRTI in the right lung. This finding was considered sufficient evidence that the death was due to natural causes, which meant that this case was no longer obligated to be investigated by the FPS, under the National Health Act No. 61 of 2003 [36]; therefore the specific cause of natural death was not investigated further.

Following informed consent from both sets of parents, a 4 ml blood sample was collected from each the infants at autopsy for the purposes of the research study. Later, blood samples from the parents were also collected for analysis. A full family history was also obtained from both families with the aid of a genetic counsellor.

9.4 Genetic analysis

DNA was extracted from the blood samples and subjected to various genetic tests as described in Chapters 6 – 8. Despite these analyses, no genetic variants were identified which likely contributed towards the infants' death. Therefore clinical exome sequencing was performed on the DNA from the two probands and their respective biological parents. This entailed the simultaneous sequencing of 4811 genes which are known to be associated with a broad range of clinical phenotypes.

DNA was quantified using Qubit[®] fluorometry (Thermo Fisher Scientific, Waltham, USA) and then diluted to 5 ng/μl using a two-step approach. Clinical exome libraries were prepared using the TruSight One Sequencing Panel Series (Illumina, Cambridge, UK) according to the manufacturer's Reference Guide [386]. Each trio's samples were prepared and sequenced together (*i.e.* two multiplexes of three samples each) to ensure adequate coverage. The pooled

library was quantified using Qubit[®] fluorometry and then diluted to 6 pM using pre-chilled HT1 buffer (Illumina, Cambridge, UK) in a three-step approach. This was spiked with 1 % 20 pM PhiX control (Illumina, Cambridge, UK) and placed into the MiSeq[™] Reagent Kit v3 600 cycles cartridge (Illumina, Cambridge, UK). Loading of the MiSeq[™] FGx instrument (Illumina, Cambridge, UK) as well as primary bioinformatics (*i.e.* conversion of FASTQ files to VCF files) was done as described in Chapter 8. VCF files were then uploaded onto Illumina BaseSpace Variant Interpreter (publicly accessible: <https://basespace.illumina.com/dashboard>; date accessed 26/11/2018). Variants were called using GAKT version 1.6 [331], annotated using BaseSpace Annotation Engine 1.6.2.0 and were filtered according to predicted pathogenicity and prioritised according to clinical significance.

Candidate variants were explored using ClinVar (publicly accessible: <https://www.ncbi.nlm.nih.gov/clinvar/>) and peer-reviewed literature to assess the functional significance of the variants within the case contexts. The infants' DNA sequences were the focus of the analysis, whereas the sequences from the parents were used only to assess the presence of *de novo* variants (*i.e.* those which occur in the infant but neither of the parents) as well as to assess the segregation of variants found in the infants.

9.5 Results and discussion

9.5.1 Case 1

A total of 10722 variants were identified in the first infant, of which three were prioritised for further investigation (Table 9.1). The prioritised variants were all heterozygous in the infant and in at least one of the parents (*i.e.* none was *de novo*). When the dataset was filtered for *de novo* variants specifically, 19 were identified: one was a heterozygous frameshift insertion/deletion (indel) (c.320delG) in the gene *rod outer segment protein 1 (ROM1)* and

predicted to be pathogenic *in silico*, however, this gene was associated with retinitis pigmentosa (inherited blindness) and thus unlikely contributed towards death. The other *de novo* variants were not rare and/or were predicted to be benign or likely benign. Hence these were not prioritised.

The prioritised variants had previously been reported in patients with diagnosed diseases (Table 9.1). This does *not* imply that the variant was causative of the disease in the patient, but rather that the variant was observed in a patient diagnosed with a particular disease. The clinical significance of these three variants were classified by ClinVar as pathogenic (n = 1) and VUS (n = 2).

The variant classified as pathogenic in *adenosine monophosphate deaminase (AMPD1)* (rs34526199) was associated with muscle AMP deaminase deficiency (MMDD) (ClinVar submission accession number SCV000109808.7). MMDD is an autosomal recessive disorder characterised by muscle weakening and exercise-induced muscle pain. Some homozygous individuals may be predisposed to heart disease [387] but many are asymptomatic [388]. The rest of the gene was analysed in the infant, which showed no variants, and this excluded the possibility of the infant being affected by a compound heterozygote mechanism [389,390]. However, since both parents were carriers, a sibling or a possible future baby will have a 25 % chance of being homozygous for this variant, and affected by MMDD. As such, the family will be referred for genetic counselling in this regard to inform them of this finding and to suggest screening of siblings for this variant.

A VUS (rs28934588) was found in *fucosyltransferase 1 (H blood group) (FUT1)*. This gene is involved in the synthesis of the precursor of the H antigen in blood, and thus mutations in this

gene can cause the H-Bombay blood type by a homozygous or compound heterozygous mechanism. However, affected individuals are not affected unless given blood which is incompatible with their blood type [391]. There was no other variant identified in the same gene, and since this infant did not receive a blood transfusion, it was unlikely that this variant contributed towards death.

A second heterozygous VUS (rs148175795) was identified in *collagen type VI alpha 3 chain* (*COL6A3*), which has previously been identified in a patient with Bethlem myopathy 1 (BTHLM1) (ClinVar submission accession number SCV000820320.1). The *COL6A3* gene encodes the largest of the three alpha chains of collagen type VI which is an essential filament in most connective tissue. The amino terminal globular domain of the chain binds extracellular matrix protein, thereby providing vital structure and organisation of the cell components. Mutations in *COL6A3* have been associated with a range of muscular disorders with different degrees of severity including BTHLM1 and Ullrich congenital muscular dystrophy (CMD). BTHLM1 is an autosomal dominant disease, characterised by proximal muscle weakness and contractures, with early prenatal or neonatal onset. Ullrich CMD has an autosomal recessive inheritance pattern, and is a more severe form with later childhood onset, which can also affect respiratory function more critically.

At autopsy, no contractures or other associated symptoms (*e.g.* hypotonia or torticollis) were noted in the case study, which suggests that BTHLM1 was unlikely. Further, the mother was also heterozygous for the same variant, but was asymptomatic. The involvement of the cardiovascular system in BTHLM1 has also been proposed, but a review showed that the heart is only mildly affected in the minority of patients and perhaps rather due to co-morbidities [392]. However, exome sequencing in a case-control study of neonates revealed 14 candidate

genes that were significantly associated with bronchopulmonary dysplasia, one of which was *COL6A3* [393]. Bronchopulmonary dysplasia is a chronic lung disease, typically in premature infants, which manifests by respiratory impairment, and can result in respiratory arrest and death [394].

Gene ontology (GO) analysis showed that the candidate genes clustered in certain pathways, including collagen fibril organisation, morphogenesis of embryonic epithelium and regulation of Wnt signalling [393]. Suboptimal functioning of these pathways have previously been proposed to predispose premature infants to develop bronchopulmonary dysplasia [395].

Li *et al.* (2015) hypothesised that since bronchopulmonary dysplasia does not follow typical Mendelian inheritance, and many affected patients were heterozygous for variants in the identified candidate genes, variant alleles with loss of function contribute towards the disease phenotype [393]. The authors went on to show that candidate genes in patients with bronchopulmonary dysplasia were indeed significantly more haploinsufficient compared to the other protein-coding genes in the genome, which was not the circumstance in controls [393]. Analysis of gene expression in pulmonary tissue of patients showed an upregulation of candidate genes, and functional analysis in rates exposed to a hyperoxic environment shown elevated expression of the candidate genes in comparison to controls [393]. Lastly, a significant association was found between infants with bronchopulmonary dysplasia and SIDS in 1982, where the incidence of sudden death was seven times greater in patients than controls [396].

These findings therefore suggest evidence that the SUDI case admitted to Salt River Mortuary, with the heterozygous variant rs148175795 in *COL6A3* may have been predisposed to an undiagnosed bronchopulmonary dysplasia. This is supported by the autopsy findings of blue

lips, blood in nose and firm lungs, which are all consistent with a hypoxic event. To further investigate this hypothesis, the other thirteen candidate genes that were significantly associated with bronchopulmonary dysplasia in the Li *et al.* (2015) study, were assessed in the infant. Analysis showed that there was a heterozygous variant in *dentin matrix acidic phosphoprotein 1 (DMP1)* (rs142880465), which was also predicted to be pathogenic *in silico*, was rare (MAF = 0.000289; gnomAD) and was inherited from the father. Given the dosage sensitivity model for bronchopulmonary dysplasia, the two heterozygous variants present in two candidate genes (one inherited from each parent) in a premature infant, provides support for the hypothesis that respiratory arrest as a mechanism for the sudden death of the infant. Histology analysis of the pulmonary tissue was available for assessment and is currently being reviewed by the forensic pathologist.

9.5.2 Case 2

The second infant had 11 230 variants and three variants were also prioritised for further investigation (Table 9.1). Similarly, none of the prioritised variants occurred *de novo*. The first was a heterozygous variant (rs535816514) in *CDK5 regulatory subunit associated protein 2 (CDK5RAP2)* which has been observed in a patient with autosomal recessive primary microcephaly (MCPH). This condition is characterised by an abnormally small brain and head which also causes intellectual disability. The SUDI cases was unlikely to have this condition as the clinical record documented a normal head circumference at birth and no signs of MCPH were observed at birth or at autopsy. Further, the infant was heterozygous for this variant, with no other variants in the same gene; as such, she would not have been affected by this autosomal recessive condition.

A heterozygous variant (rs75134564) was identified in the infant, which was in the gene *3-oxoacid CoA-transferase 1 (OXCT1)*. The variant has previously been found in patients with succinyl-CoA acetoacetate transferase deficiency (SCOT), which is when the body cannot metabolise ketones effectively. This disorder presents with ketoacidotic attacks, with symptoms of lethargy, loss of appetite and vomiting, which are aggravated by infections, fevers and periods of fasting. It has an early onset, with many individuals experiencing their first attack within their first few days of life.

Although this particular variant observed in the SUDI case was predicted to be pathogenic *in silico*, the consensus on ClinVar from multiple entries was that this variant is benign on its own (publicly accessible: <https://www.ncbi.nlm.nih.gov/clinvar/variation/242800/>; date accessed 28/11/2018). Rather, it was found to contribute towards pathogenicity when in a compound heterozygous state with another variant rs267606930 (NM_000436.3:c.398T>A; p.Val133Glu) in the same gene [397]. However, this SUDI case did not have this second variant, or any other variant in *OXCT1*, and therefore was unlikely to have been affected by SCOT.

Lastly, one homozygous variant (rs201340753) was found in the infant, which was heterozygous in both parents and which met the criteria for pathogenicity for prioritisation. This variant was in the *mannan binding lectin serine peptidase 1 (MASP1)* gene which encodes a serine protease which contributes towards the activation of the lectin complement system [398]. Within the innate and adaptive immune response, complement activation is critical to identify and remove pathogens, as well as to promote inflammation. The mannose-binding lectin (MBL) protein functions within this pathway by binding to various clinically relevant

pathogens [399] and it is thought to also be involved in coagulation [400]. Loss of function of this gene has shown to be inhibitory to the complement pathway [401].

Genetic variants have been associated with MBL deficiency in serum [402] and MBL deficiency has been associated with increased infections, particularly respiratory tract infections [403,404]. It has also been associated with recurrent pregnancy loss [405], which is interesting within this case study where the mother had a previous stillbirth, as miscarriages, stillbirths and SUDI have many overlapping risk factors and it has been suggested that may form part of a continuum spectrum [406,407]. Kilpatrick et al. (1998) also showed that SIDS cases who were MBL deficient had significantly greater MBL values compared to controls and suggested that some SUDI cases are precipitated by bacterial infections [408].

While it is no longer possible to test for MBL deficiency in the deceased infant due to unavailability of appropriate samples, it is plausible the homozygous variant in *MASP1* may have contributed towards an MBL deficiency. Within the context of the triple-risk model for SIDS [13], the genetic variant may have contributed towards the predisposition or vulnerability component in the infant; and combined with the infant being in a critical stage of development (2 months old) and signs of respiratory tract infection (trigger), may act together to adequately explain a possible mechanism for death. This may have been further exacerbated by maternal smoking.

Table 9.1: List of prioritised variants from clinical exome sequencing

Three variants in infant 1 and three variants in infant 2 were prioritised. They were all predicted to be pathogenic *in silico*, were rare and had good quality scores from sequencing. All prioritised variants were missense variants; none were *de novo*. MAF: minor allele frequency; obtained from gnomAD (<http://gnomad.broadinstitute.org>; date accessed 26/11/2018)

Gene	Accession number	Variant (cDNA)	rs number	Amino acid change	MAF (number of observations)		ClinVar: Phenotype of patient with variant		Inheritance in family
					Global	African	Disease	Inheritance pattern	
Case 1									
AMPD1	NM_000036.2	c.959A>T	rs34526199	p.Lys320Ile	0.02875 (8126)	0.004748 (118)	Muscle AMP deaminase deficiency (pathogenic)	Autosomal recessive	
FUT1	NM_000148.3	c.725T>G	rs28934588	p.Leu242Arg	0.0002760 (69)	0 (0)	Bombay phenotype (VUS)	Autosomal recessive	
COL6A3	NM_004369.3	c.3199G>A	rs148175795	p.Val1067Met	0.00009960 (28)	0.0008024 (20)	Bethlem myopathy (VUS)	Autosomal dominant	
Case 2									
CDK5RAP2	NM_018249.5	c.506A>C	rs535816514	p.Lys169Thr	None reported	None reported	Autosomal recessive primary microcephaly (uncertain)	Autosomal recessive	
OXCT1	NM_000436.3	c.173C>T	rs75134564	p.Thr58Met	0.001273 (360)	0.0003206 (8)	Succinyl-CoA acetoacetate transferase deficiency (conflicting)	Autosomal recessive	
MASP1	NM_001879.5	c.2029C>T	rs201340753	p.Arg677Cys	0.000007075 (2)	0.00008012 (2)	None	N/A	

9.6 Conclusion

Clinical exome sequencing was conducted in two probands of SUDI cases, alongside their biological parents, in attempts to better understand specific causes of death and underlying mechanisms for SUDI. The molecular autopsies elucidated two variants which may be worthwhile investigating in larger cohorts of SUDI with similar circumstances at death. The first was a heterozygous variant (rs148175795) in *COL6A3*, which suggested a bronchopulmonary dysplasia phenotype. This hypothesis led to finding of a second candidate variant in *DMP1* (rs142880465), which may contribute towards a polygenic mechanism of a more severe phenotype. This has prompted a detailed histological analysis of the pulmonary tissue which is currently underway. In the second infant, a homozygous variant (rs201340753) was identified in *MASP1*, which was heterozygous in each parent. While MBL deficiency could not be assessed, it is plausible that this variant may have acted in combination with other risk factors within the triple-risk model to result in sudden death; however, this requires further research where appropriate samples are obtained at autopsy for immunological analysis.

Immunology tests are not a routine ancillary investigation in the forensic pathology setting in South Africa, and perhaps more attention should be given to this important component in future research and SUDI investigations. While these genetic findings do not change the overall manner of death as being natural, they do provide valuable insight into two SUDI cases, which may have genetic importance for family members. To this end, genetic counsellors have been engaged to converse with these families and explain these findings within the contexts of their respective family histories.

Overall, these two case reports demonstrate the value of including parental samples in the genetic analysis, to assist with co-segregation analysis of recessive disorders as well as to assess

polygenic conditions, where variants in different genes may each contribute towards an overall affected phenotype. These findings also represent possible new candidate variants to assess in the future – both functionally as well as in case-control SUDI cases, to better understand their pathogenicity and potential role. These emerging avenues of molecular research may be more locally relevant for SUDI cases in South Africa, which are not only under-studied, but also succumb to abnormally high rates of infections.

Chapter 10

DISCUSSION AND CONCLUSION

A critical analysis of the need for molecular autopsies and translatable lessons learned

10.1 Background

The high burden of infant death in South Africa, combined with a lack of routine genetic testing in a forensic pathology setting, prompted the first prospective forensic genetics investigation into SUDI in the country, and in Africa. The aim of this study was to explore the use of molecular autopsies in the medico-legal investigation of SUDI cases in Cape Town, South Africa, with the intention of contributing knowledge towards the development of a locally relevant molecular autopsy.

To this end, it was hypothesised that the molecular autopsy which is informative for the diverse South African context would be different to those already established, due to the majority of previous studies being conducted in developed countries with population groups of mostly European descent. Part of testing this hypothesis necessitated the analysis of known genetic variants commonly screened for in other population groups, as well as sequencing other plausible candidates based on risk factors noted locally. In turn, the testing of these genetic variants required a systematic literature review to identify plausible genetic candidates, the

establishment of a research framework to carry out this research locally (which included the quality assessment of samples), as well as a retrospective review of infant death at Salt River Mortuary for contextualisation and understanding of the target population. Each of these components have been presented as chapters in this thesis, where they have been individually discussed. The purpose of this chapter is therefore not to sequentially repeat those discussions, but rather to examine and integrate key discussion points from the thesis as a whole, and critically discuss these and their implications in a broader context. The synthesis of the study findings, as well as the study limitations, will be critically evaluated to motivate the future direction for this research. In this regard, subsidiary studies which have already been identified through this research will be introduced and the recommendations for the way forward will be presented. An editorial to communicate some key points to a local audience, which has been accepted in the South African Journal of Child Health, is also included as Appendix 11.

10.2 The molecular autopsy

10.2.1 The value of a molecular autopsy

Various forms of molecular autopsies were explored in this study, ranging from the screening of specific mutations, to the massively parallel sequencing of entire clinical exomes. Each approach carried its own advantages and limitations, which together, began the assembly of new and deeper knowledge in the forensic genetic investigation of South African SUDI cases. It is important to critically assess the need for a molecular autopsy from multiple angles, especially considering the legal mandate and resource-limited setting under which FPS operate in South Africa.

Salt River Mortuary services the western metropole of the City of Cape Town (Appendix 5), and investigates approximately 3500 – 4000 total cases per annum. This drainage area includes

the suburb of Nyanga, where the local police station had the highest number of reported murders in the entire country for 2017/2018 ($n = 308$) [409]. Taking into account the current posts for forensic pathologists and registrars (*i.e.* specialist trainees becoming forensic pathologists), the annual caseload results in each professional member of staff conducting over 350 autopsies per year, alongside their teaching, research and administrative duties. This is more than the maximum 250 autopsies endorsed by The National Association of Medical Examiners for pathologists without other responsibilities [410,411]. This recommendation acts as a quality assurance measure to mitigate mistakes, errors in judgment and burn-out [410,411]. Within this context of incredibly high caseloads, combined with staff and resource limitations, the addition of any analysis needs to be strongly justified.

A molecular autopsy can be an expensive investigation, but it has the potential to elucidate a (natural) cause of death. While the costs of high-throughput sequencing are dramatically decreasing, one needs to consider the necessity and value that such an analysis would add to a medico-legal investigation which operates under stringent budgets. From a legal perspective, a molecular autopsy would not be considered a priority for the FPS, as investigation of natural death is not strictly part of their mandate [1,36]. The primary goal in the diagnosis of death in SUDI cases is to determine whether their death was natural or unnatural; and only the latter *requires* further investigations [1]. The legislation does not prevent the investigation of natural deaths to determine cause of death, but this is done at the discretion of the forensic pathologist. At Salt River Mortuary, a SUDI case whose cause of death remains undetermined, and where no foul play was detected, is still considered natural. Although not readily diagnosed at Salt River Mortuary, SIDS is also considered a natural cause of death [16,46]. As such, a molecular analysis to pinpoint an underlying genetic disease, which is also natural, is not strongly motivated from a legal standpoint.

While the legal mandate must be acknowledged, the value of a molecular autopsy must also be critically assessed from medical angles. The FPS falls within the Department of Health and ought to recognise their role within the broader healthcare system. The purpose of determining cause of death is multifaceted, which includes elements of exposing criminal and illegal acts; but establishing cause of death also provides closure to the next of kin. The concept of closure is particularly important in cases of undetermined cause of death or SIDS, where a molecular diagnosis may be the only means to provide families with answers. Moreover, by investigating the deceased, risk factors underlying mortality can be understood and translated into society for the benefit of the living and the local community [412].

In a setting where SIDS is not readily diagnosed due to the present inability to perform the full prerequisite of post-mortem examinations to fulfil its definition and establish said diagnosis, (*e.g.* full autopsies, death scene investigation), it begs the question as to what resources would support molecular tests on an ongoing basis. This is particularly important given the results from the systematic review which showed that a median of only 4 % of SIDS cases enrolled in molecular autopsies were resolved [34]. One might argue against the allocation of resources for the deceased, where the identification of genetic mutations is no longer directly useful to them, and resolution occurs only in the minority of cases.

However, the supplying of resources for a molecular autopsy should be seen as an investment into healthcare in a broader sense, as the primary goal of a molecular autopsy is not just to diagnose the cause of death, but to feed back this information to families who may also be at risk. This objective carries extremely important implications and positive connotations for public health, whereby a molecular autopsy may act as the primary tool to identify highly pathogenic (and novel) variants in the extreme phenotype of sudden and unexpected death.

These molecular investigations may even reveal non-lethal inherited disease which would be of significant value to family members. The results of these analyses can then be used for targeted genetic screening in blood relatives who may also be at risk for sudden death or other inherited diseases. The family screening would be targeted and thus substantially cheaper than the molecular autopsy; moreover, it would hold the potential to save lives. This process must be done cautiously however, as rare variants with an initial ‘unknown significance’ may turn out to be benign [412,413]. It is therefore encouraged that the detection and research of variants by molecular autopsies are centrally reported to aid different laboratories with interpretation of seemingly rare variants [414]. Overall, in areas where the burden of death is so high, it is of utmost importance to recognise innovative areas which have the ability to reduce mortality. This possibility was demonstrated in this study (Chapter 7) in the case of the infant with the homozygous mutation in *GALT* (known to be pathogenic), whose identical twin subsequently demised, likely from the same genetic cause.

10.2.2 The importance of family engagement

In this study, parents of three infants were willing to provide biological samples and engage in further research themselves. In follow up interactions with these families, they have expressed their appreciation for the continued exploration of the death of their infant. The use of their samples has added value to this research, particularly in the investigation of variants involved in pathways not regularly incorporated in SUDI investigations. In Chapter 9, the usefulness of parental DNA samples was demonstrated in the ability to assess inheritance mechanisms and co-segregation, and has resulted in the enlightenment of one potential cause of death and one new avenue to explore in the future. Co-segregation analysis can also aid the interpretation of rare variants which are present in probands and asymptomatic family members, thereby potentially aiding the exclusion of these variants as being causative of the infant’s death [415].

Engagement with families where genetic results were available in the infant however, was not always as expected; for example the mother whose twins demised (Chapter 7) did not wish to learn about the genetic findings in *GALT* and the implications that it may have on her family. However, given her age, personal circumstances, and trauma of losing both her twins, it is understandable that she made this decision. This non-engagement with genetic results was also observed in the infant who was homozygous for the *SCN10A* variant identified via in next generation sequencing (Chapter 8). Despite numerous attempts to contact the family, their response has not been forth-coming. This lack of response and engagement unfortunately weakens the direct value of this concept for families, which is ultimately to benefit them. It is unclear if this attitude is representative of the cohort at large, as only the minority of infants had reportable genetic variants which were also clinically actionable.

The loss to follow up experienced in this study however, is not unique. Glengarry *et al.* (2014) also observed many parents in SIDS studies were also lost to follow up [26]. The authors made the extremely important point that since genetic variants in such cohorts are often novel, the need for family samples and engagement is imperative to understand more about the variant and its potential role in disease [26]. This is in agreement with our experiences in this study as demonstrated by the case reports in Chapter 9. Therefore, the lack of recruitment of family members into this study from the start was a major limitation. Despite extensive efforts to contact family members of infants who were prioritised for next generation sequencing, only three out of nineteen families were reached. This loss of follow up also poses complications for the eventual feedback of results of known pathogenic mutations, which was not anticipated at the start of this study.

Thus, in an attempt to better understand parents' expectations, experiences and perceptions of participating in this research project, a collaborative project was undertaken by a genetic counselling student who qualitatively explored this topic through a semi-structured interview (unpublished). Loss to follow up was also noted in this sub-study, where many families from this study cohort were not contactable due to incorrect or revised phone numbers. What emerged from this study, was that the interviewed participants (n = 11) had a positive experience during the consent process, and they participated to help other families and to find answers for themselves. This was in accordance with a previous study conducted in England, where next of kin of SUDI cases also felt participation was a positive experience as it enabled them to better understand why their baby had died [416]. However, the participants from Salt River Mortuary did not seem to fully recall the genetic details of the study, nor what the implications of receiving genetic results were.

This potential lack of understanding was explored during the interviews, which identified that while participants seemed to understand the project at the time of consent, their immense grief clouded the details and implications of the research project itself. During the consent process it was explained that the family would probably not hear from the researcher again, however, it emerged that parents still had an expectation of being contacted again. Consequently, a letter providing an update on the research project has been drafted and will be posted to participants upon approval from the Institutional Review Board. These findings also motivate for the need for a genetic counsellor to be involved from the start of the process, even during consent taking, to better manage expectations and stress the possible implications of the research.

10.2.3 Quality assurance and validation

While molecular autopsies on SUDI cases may be performed routinely in other countries for cause of death determination, this is not yet the case in South Africa. This research study has provided some of the first insights into genetic variants informative in indigenous Black African and Mixed Ancestry SUDI cases. From a genetics perspective, only the minority of all known genetic variants have a clear clinical significance, and therefore, detecting a plausible genetic variant in a SUDI cases may not be translatable to cause of death. This is a major issue with regards to genomic research on previously understudied populations, such as in (South) Africa, including mostly indigenous Africans, where issues of allele frequencies and penetrance of previously reported disease-causing variants may be different to studies conducted elsewhere.

The interpretation of such genetic variants is complex and needs to be considered in the individual case context of whether the variant could have contributed to death. Some genetic variants initially thought to cause sudden cardiac death have subsequently been found to be present in high frequencies in the background population and are unlikely to be causative of sudden death [417]. Uncovering rare variants which are predicted to be pathogenic *in silico* is also more common than previously thought, with large case-control studies of SIDS cohorts showing no significant difference between the numbers of rare variants in cases versus controls [418,419].

Therefore, interpretation of genetic variants from next generation sequencing data must be done conservatively and caution must be exercised in the construal of results in the case [24,30,417]. Further, before these types of assays are carried out for service-delivery, the assays must be rigorously tested and validated according to ISO 17025 to ensure that findings are consistent

and novel findings are dealt with appropriately [420]. ISO 17025 forms the standards for the regulation of forensic laboratories and all workflows from sample collection to results interpretation must be compliant with these specifications [421].

In this study, an entire chapter was dedicated to the quality assessment of biological samples obtained from the deceased (Chapter 5), and part of this analysis included the assessment of contamination. Such quality control analysis should always be performed prior the molecular autopsy studies, to ensure that the DNA sample obtained is single source and of sufficient quality for downstream processing [422]. The quality of three sample types was assessed using a range of molecular methods which showed that blood was the sample of choice.

Obtaining a sufficient volume of blood from SUDI cases is challenging when only an external autopsy is performed. External autopsies are often conducted on sudden unexpected death of infant cases in South Africa, where clinical history, death scene investigation and a full body X-ray are sufficient to conclude that the death was not unnatural (Chapter 2) [1]. Hence the opening of an infant for the purpose of obtaining a blood sample for a molecular autopsy needs to be strongly justified.

While buccal cells were suitable for targeted testing, the yield of DNA was low, which limited the number of tests performed on the same sample before it was depleted. In a research setting such as this one, assays needed to be designed and optimised for the purposes of this research project; hence DNA yield and quality could not be a limitation. Depleting DNA samples would mean that more participants would need to be recruited to attain a statistically significant cohort. As such, the need to collect blood samples in this study, as opposed to buccal cells, was reasonable and justified. However, this finding does not negate the investigation and use

of other sample types in the future, particularly when streamline testing is performed, and if new technologies are developed to further improve yield of DNA from compromised sources.

The standardisation of a molecular autopsy is anticipated to be a challenging task, as the assay will probably vary in different countries, depending on population group and available resources. For example, founder mutations in *GALT* are likely to be prioritised for SUDI cases in South Africa as opposed to variants common in European cohorts (*e.g.* the *ACADM* variant was not observed in this study cohort, Chapter 7). These sorts of findings need to be considered in a resource-limited setting, where the testing for all possible candidate or even genomes is not (yet) possible.

Forensic scientists typically steer towards standardisation of forensic testing across the globe, to facilitate global comparison of data for human identification purposes. Standardisation of tests, coupled with the validation thereof, also ensures reproducibility, accuracy, precision and thus reliability of results [420]. However, even if molecular autopsies are different from each other, it is essential to ensure that the implemented workflow is validated and commensurate quality requirements that apply to forensic genetic testing. To this end, the Scientific Working Group for DNA Analysis and Methods (SWGDM) may need to consider proposing guidelines for the creation and validation of such tests, should they become more routinely practiced. Guidance is already available pertaining to the application of ISO 17025 to deal with the expressions of opinions and interpretations in forensic science [423]. In a molecular autopsy context, these guidelines could be adapted for the conservativeness with which interpretation must be approached. This is especially true for putatively pathogenic variants which lack functional studies, and how this information should be relayed to families.

An example of where standards were not adhered to, was in the Sally Clark case in the UK [424]. Sally Clark was wrongfully convicted of murdering two of her infant sons, each of whom died suddenly and unexpectedly within the first three months of their lives [424]. An expert witness for the prosecution, Sir Roy Meadow, incorrectly applied the product rule to determine the chances of two SUDI cases occurring in one family. Numerous letters and articles were subsequently published indicating the inaccuracies of the statistics presented [425,426]. In contrast, Professor Ray Hill calculated that there was actually an increased probability of a second sibling also experiencing SUDI, by a factor of 5 to 10 [414].

Upon appeal, it was revealed that the autopsy report had not mentioned that the *S. aureus* bacterium was present in the second infant [427,428]. This finding of infection (natural cause of death) had actually been deliberately changed to smothering (homicide) [429]. This pivotal case prompted a review of other similar cases, and Sally Clark and three other women were released from prison. Sir Roy Meadow had testified in each of these cases. This case example demonstrates the importance of validation in all processes, including interpretation, no matter how complicated the task. This further emphasises the need to be conservative in the interpretation of genetic data.

10.3 Immunological factors

An important consideration for molecular autopsies in a local context is that they must be fit for purpose. A finding that differed between SUDI cases at Salt River Mortuary and those reported in other international studies, was that the peak age of death occurred earlier: 0 - 3 months of age compared to 2 - 4 months of age [16,126]. This was seen in both the retrospective study (Chapter 2) and prospective SUDI cohort (Chapter 6). The rise in SUDI after the first month of life has previously been explained by the gradual decrease of maternal IgG in an

infant, rendering the infant vulnerable to infections. The earlier shift in at-risk age might be due to less maternal IgG transferred to the infant, which has been shown previously in other African population groups in other contexts [430,431]. As Palmeira *et al.* (2012) reviewed, IgG transfer depends on several factors, including total IgG and specific antibodies available, IgG sub-class, integrity of the placenta, and the gestational age of infants [432]. In this study, 40.55 % of SUDI cases were born prematurely (Figure 2.9), which highlights this as an important risk factor in the local context.

Another contributory factor may be the severe burden of disease in overcrowded living spaces as seen in the informal settlements in Cape Town, and the inability of the maternal IgG to appropriately protect the neonate against infection [128,129]. In this 5-year retrospective study, a remarkable 66 % of all SUDI admissions had an infectious cause of death (Figure 2.7). Unfortunately, the pathogens underlying these deaths were only investigated in the minority of cases due to resource limitations and the mandate of the FPS (Figure 2.6). A similar observation was made in the prospective study, where 68 % of included SUDI cases had infectious causes of death, yet microbiology and/or virology was only requested in 44 (37.9 %) of these cases. Of the six positive virology tests, the most common was adenovirus ($n = 4$) and in two of these cases, human bocavirus was also detected. For microbiology testing, *S. aureus* was the most common pathogen, which was observed in three of the fifteen positive cultures.

The binding of staphylococci bacteria to buccal cells have been shown to increase when cells were exposed to cigarette smoke [433]. This finding is supportive of second-hand smoke exposure being a risk factor for SUDI, particularly when the infant has also been exposed to *S. aureus*. Studies have also shown that cotinine, a metabolite of nicotine, is detectable in the urine of infants who were exposed to second hand smoke [434]. A systematic review of

infection in sudden unexpected death of all ages by our research group also showed that tobacco smoke exposure and co-sleeping were the two most common risk factors reported in infant deaths [106].

Further, the risk for SUDI has shown to increase significantly when infants were simultaneously exposed to tobacco smoke and co-slept [112]. This combination of factors may indeed be significant; however, it has also been proposed that families in which combinations of risk factors are present, are also of lower socio-economic status and less educated [90]. It is therefore unclear if these risk factors are merely associated due to social circumstances, or if they are directly contributory and modifiable [100,103]. Nevertheless, in this retrospective study, a total of 53.11 % of SUDI cases were exposed to tobacco smoke (most of who also co-slept), which highlights the need to educate families about the risks and dangers of smoking cigarettes around pregnant women and infants.

Due to the large role of infection in SUDI cases in South Africa [4,119,215], this study went on to investigate the possible association of three genetic variants in a prospective SUDI cohort from Salt River Mortuary. These variants were selected based on their previous associations with respiratory infections and sepsis [245,255,262], one of which had also been associated with SUDI in a British population [244]. A total of 59 SUDI cases had at least one of the genetic variants, and two infants were heterozygous for all three variants tested. One of these latter infants had a cause of death of meningitis based on histology findings, although microbiology results were negative; the second infant's death is still under investigation.

Comparison of allele frequencies to those from the background population suggested that *IL-6* (c.-174G>C, rs1800795) and *TNF- α* (c.-488G>A, rs1800629) were significantly associated

with infection-related SUDI in South Africa. A significant difference was also found for each of the three variants (*i.e.* for *TLR4* c.896A>G, rs4986790 as well) when allele frequencies from the Black African population group were stratified and compared to background data from African individuals on gnomAD (publicly accessible: <http://gnomad.broadinstitute.org>).

While associations were found in this study, this finding needs to be verified in a larger cohort, where matched controls are also available. Even then, the association does not indicate that the genetic variants were causative of the death, but rather is suggestive that the genetic variant may have increased the infant's vulnerability to infection. The role of infection needs to be carefully assessed by the pathologist, who ideally would have access to comprehensive data pertaining to clinical history, autopsy, microbiology, virology and histology, in order to formulate the cause of death.

The role of infection emerged again during the analysis of the clinical exome of a two month old female from the Mixed Ancestry population. An external autopsy showed signs of respiratory tract infection on the Lodox[®] Xmplar-dr, which is a natural cause and thus does not require further medico-legal investigation in South Africa [36]. Genetic screening of the three candidate variants described in Chapter 6 was negative, as was the analysis of four mutations causative of metabolic disorders, and 43 genes associated with inherited cardiac arrhythmias. However, the analysis of 4 811 genes in the clinical exome revealed a homozygous missense variant in *MASP1*, which is currently of unknown clinical significance. Each parent was heterozygous for the variant, and it was predicted to be pathogenic *in silico*. Based on previous literature, it is hypothesised that the variant may play a role in MBL deficiency [399] [401] [402], which is associated with respiratory tract infections [403,404].

These genetic findings, combined with the rates of infection-related causes of death in SUDI cases and the relatively low yield of positive virology and microbiology results, advocate for microbiome research in SUDI cases, as well as the investigation of immunological testing in post-mortem ancillary investigations of SUDI cases. This should be paralleled with genetic testing for primary immunodeficiency diseases (PIDs), which are a group of non-communicable diseases which affects immunological function [435]. The type and severity of infection that manifests varies depending on the underlying cause of PID [436]. There is a strong genetic basis for PIDs, making the diseases heritable. There are more than 10 000 genes associated with the immune system, with at least 300 monogenic causes of PIDs established to date [437].

Based on the reported prevalence of PIDs in other countries, the number of South African individuals estimated to have a PID is 2 850 – 45 723; however, in 2014, less than 500 cases of PID had been reported in South Africa [438]. The mean age of diagnosis of individuals has also decreased from 51 months (during 1983 - 2000) to 35 months (during 2001 - 2009). Out of the 168 individuals seen at Red Cross Hospital, Cape Town, South Africa between 1983 and 2009, recurrent infections accounted for 74 % of patients' symptoms, and 25 % of all patients were known to have passed away [439].

The under-diagnoses in South African populations may be due to a range of reasons, including: an unawareness of symptoms by the patient; a lack of seeking medical advice; an underestimation of the significance of recurring infections, especially if patients are seen by multiple healthcare practitioners (often the case in the primary healthcare and district healthcare settings); an absence of routine testing in clinics to confirm diagnoses of PIDs; a lack of diagnosis as many symptoms are similar to those of human immunodeficiency virus; a

lack of awareness of healthcare practitioners and others. Another hypothesis for the under-diagnoses could be that infants are demising within their first year of life, before multiple infections occur. Since infants are particularly vulnerable within their first few months of life, an infection, in the presence of a predisposing genetic variant, could perhaps result in death; thus contributing towards the lower PID incidence that expected. Further, because these diseases are not actively being screened for at birth or at autopsy, it is not clear what the rate of incidence is, or their possible role in SUDI.

10.4 Newborn screening

Many countries have recognised the importance of diagnosing inherited metabolic diseases at an early age, and introduced routine screening for newborn babies. Although not widely used, such tests are available in South Africa, but mostly within the private medical scheme (medical insurance) sector, which the majority of the population cannot afford. Public healthcare facilities do not offer routine metabolic screening at birth, resulting in many patients going undiagnosed. Important newborn screening research studies were conducted at Red Cross Children's Hospital, Cape Town (2002 and 2010), which investigated two founder mutations in large cohorts, and estimated carrier frequencies [289,290]. Yet, the expected numbers of affected individuals are not reporting to the public healthcare system, which is worrisome.

The carrier frequency for *GCDH* c.877G>A/T was a remarkable 1 in 36 unrelated South African Black newborn babies [290], translating to an estimated affected status of 1 in 5 184. For *GALT* c.404C>G/T, the 1 in 60 carrier frequency equates to 1 in 14 400 Black African newborns being affected [289]. In this study, 1 infant out of 102 of Black African SUDI cases was affected, which hints at its detrimental role in vulnerable infants. Due to the relatively small sample size, this frequency could be an overestimation of affected galactosaemia

individuals in SUDI cases, and thus requires testing in a larger number of cases. This would now be possible in post-mortem investigations, since this molecular assay has been established in-house, and it is also offered by the NHLS.

It is also noteworthy that metabolic screening is a requirement for the full diagnosis of SIDS. However, current resources and available ancillary investigations at Salt River Mortuary do not cater for metabolic screening, nor are results available from newborn screening. Hence, the absence of this information prevents the diagnosis of SIDS from being made. More importantly though, if it is known that undiagnosed metabolic disorders can be fatal, it begs the question as to why more efforts are not being made to include metabolic screening as a routine test in newborn babies. The wealth of international literature, combined with locally relevant mutations in South Africa, strongly motivates for newborn metabolic screening to be rolled out nationally, as a preventative measure for mortality. While the inclusion of metabolic screening could be part of the post-mortem tests in the interim, we ought to rather focus resources on preventing these deaths in the first instance. In this regard, the policy for ‘Genetic and Congenital Disorders’ is currently being updated by the National Department of Health (personal communication: Professor Raj Ramesar, 2019).

The motivation for a temporary inclusion of metabolic screening in the forensic post-mortem setting is persuaded by the findings of the present study and that newborn screening will take years to be implemented routinely due to the lack of national healthcare resources. In the interim, metabolic screening should be incorporated into forensic post-mortem investigations in order to identify at-risk relatives and inform parents about future babies. This was demonstrated in the example, where one SUDI case was found to be homozygous for the *GALT* c.404C>G/T variant, and her identical twin also subsequently demised. Given that the genetic

assays for two variants important to the South African population are already optimised in this setting, the validation of these processes would enable the tests to be offered as a service to FPS. Alternatively, facilities are set up at the NHLS Chemical Pathology unit, where these and other metabolic disorders can be tested. The results from metabolic testing, whether in a forensic post-mortem context or in the form of newborn screening in the future, would unleash the possibilities of saving lives.

10.5 Awareness campaigns

Due to the paucity of research on SUDI cases in South Africa, and particularly Salt River Mortuary, one of the first objectives of this study was to retrospectively review infant deaths admitted to Salt River Mortuary to elucidate the burden of SUDI and associated risk factors. The purpose of this part of the study was primarily to obtain a baseline as to why babies in Cape Town were dying. It was hoped that this information would aid the estimated number of cases where a molecular autopsy may be worthwhile. Previous studies have shown improved results and better use of resources when selecting specific cases for molecular autopsies [26,320].

The 5-year retrospective review undertaken as part of the present study identified 1 199 SUDI admissions to Salt River Mortuary between 2013 and 2017, which comprised 6.67 % of the total caseload. This was substantially more than the collated data from five other forensic mortuaries in the country, which documented a total of 2 704 SUDI over a 5-year period, accounting for 3.36 % of their combined caseload [33]. While that study focused on the years 2005 – 2009, the multicentre review forms the most comprehensive and recent data available for SUDI in the country. Within the collated data, Tygerberg Mortuary (Cape Town) had the most SUDI cases ($n = 1\,271$), which were not too dissimilar to those at Salt River Mortuary.

These figures therefore highlight both the burden of SUDI in South Africa, particularly in Cape Town, as well as the lack of research in this important field.

The overwhelming majority of infants in this study co-slept in an adult bed on their side (Figure 2.9). Side-sleeping has previously been identified as a risk factor for SIDS, but this finding has not been reproducible in all studies [440,441]. It has been suggested that side sleeping may be a risk factor as it is easier for the infant to turn into the prone position [85,442]. Another study found that the combination of side sleeping in a premature infant with low birth weight at the age of 13 to 24 weeks old was a particularly risky combination [443]. Co-sleeping is a well-documented risk factor for SIDS, where there are higher chances of (accidental) asphyxia, hyperthermia, and hypoxia of the infant, especially if the co-sleepers were smokers [73,440,444].

In this study, it was not possible to assess the risk ratio or association of side sleeping position and/or co-sleeping with SUDI cases due to the absence of sleeping data from healthy controls. It may be that the majority of infants in the background population co-sleep and/or sleep on their side, with many babies thriving and leading healthy lives. The unavailability of this data was a limitation in this study, as while extensive data is accessible for deceased infants (from the forensic post-mortem investigation), similar questions to this extent are not readily asked of living babies in clinics, schools or other contexts. A prospective case-control study, including healthy living babies, is sorely lacking in South Africa and is needed to gather data from matched controls to facilitate statistically-powered analysis. This ought to be done prior to an awareness campaign to educate mothers about locally relevant risk factors.

Awareness campaigns pertaining to safe sleeping environments have been emphasised in developed countries, but are understated or non-existent in many low and middle income countries [5,20]. Hauck and Tanabe have reviewed international trends following the launching of awareness campaigns which showed a definite drop in SIDS rates in all countries [5]. They also observed that the stabilisation of such decline calls for further action as well as the need for better standardisation in cause of death diagnosis [5,20]. Similar efforts need to be made in South Africa, given the large number of SUDI cases with modifiable risk factors. These results strongly advocate for resources to be allocated for this purpose.

A host of modifiable risk factors, specifically pertaining to maternal behaviour, were also noted from the retrospective review, which included smoking and substance use; the missing of antenatal care visits and vaccination appointments; and not seeking medical attention when infants showed signs of illness. Reasons for this behaviour has previously been attributed to lower socio-economic status of families [445], which was indeed the case in this study. A Rwandan study found that older maternal age, a single marital status and a lack of social support were associated with the underutilisation of antenatal care services [446].

The reasons why women of low socio-economic missed antenatal care have qualitatively been explored in Arizona, and reasons identified included finances, transport, missing work, system difficulties, lack of knowledge, fear and low motivation [447]. In the UK, similar barriers have been noted in themes of denial, concealment and disadvantage [448]. Exploring these themes further revealed reasons relating to psychology, empowerment and socio-cultural dynamics [449]. A systematic literature review which aimed to identify reasons for missing vaccine appointments, also identified family characteristics, attitudes and knowledge as some of the reasons why mothers missed the vaccine appointments for their children [450]. The

understanding of why mothers behave in certain ways is of utmost importance when considering an intervention, such as an educational campaign.

10.6 Limitations and future work

Some of the limitations of this study have been discussed throughout the body of the thesis and this chapter, including the small number of controls, the limited number of parental samples, as well as missing data in medico-legal cases files. This section will therefore focus on the limitations of the overall study, as well as the way forward.

A larger limitation of the overall study was that the infants recruited into this study did not all have full post-mortem investigations. The absence of empirical results for full autopsies, histology, toxicology, microbiology and virology limited the baseline information available for cases with which to interpret genetic results. This lack of information also hindered the broader understanding of the cohort and underlying mechanisms for death which may have been missed.

Due to the investigation of most SUDI cases at Salt River Mortuary not fully meeting the international requirements to fully diagnose SIDS (*i.e.* as a direct consequence of the lack of full autopsies), a range of other terms were used to classify cause of death, such as ‘unascertained’, ‘undetermined’, ‘under investigation’ or ‘consistent with natural causes’. This nonconformity with terminology is a limitation in a broader sense, as it makes local data difficult to situate in a global context, which restricts assessments of trends and comparisons with other studies.

The absence of standardised investigations for SUDI and use of terminology was also a limitation to this study. For example, the noting of patchy lungs on the Lodox[®] Xmplar-dr scan led to the use of phrases such as ‘signs of respiratory tract infection’, which guided death being diagnosed as infection-related; often with different degrees of evidence being woven into the diagnosis phrasing (*e.g.* ‘associated with infection’ or ‘consistent with hypoxic events’ or ‘likely pneumonia’). The phrases used suggest a lack of evidence for the diagnosis, and this is most likely due to the external autopsies which were most frequently performed, the reasons for which have already been discussed extensively. To diagnose that a death was due to infection is a complex process, requiring results from not only the autopsy, but also histology, microbiology and/or virology tests. It is suggested that causes of death are guided and standardised according to the International Classification of Diseases (ICD) published by the Centre for Disease Control and Prevention (publicly accessible: <https://www.who.int/classifications/icd/en/>, 31/01/2019).

The definition of SIDS makes provision for cases which fall short of the full definition, and which have signs of mild infection [2]. It is therefore unclear if the lack of SIDS cases in this study is precise and if there were cases which were indeed subclasses of the full SIDS definition. It is likely that the number of SIDS (and subcategories of SIDS) cases are under-represented at Salt River Mortuary, and standardised investigations should be implemented to elucidate more specific diagnoses [33]. Of course, this requires resources, as well as a change in the mandate and/or mind-set of the FPS, that all SUDI cases will be investigated fully, even if they are natural cases. Indeed, it is these sorts of cases which should be prioritised for molecular autopsies, and in this study, it was not always clear as to which cases would be most relevant. Consequently, all SUDI cases were recruited as per normal operations, and the placing of the molecular autopsy within this context was challenging. The absence of full autopsies

thus may have been a confounding factor in the interpretation of the results obtained in this study.

Another aspect of the SUDI investigation process revealed in this study, is that the death scene investigation component requires attention. Analysis of scene records showed that these were mostly incomplete and often indicated contradictory information to that provided by the South African Police Service (SAPS) and/or the family during their interview. Death scene investigation has not always been prioritised in SUDI cases in South Africa, as previously noted by Dempers *et al.* (2016) [81]. However, the death scene is where crucial clues must be collected, particularly those which might indicate (accidental) asphyxia, hyperthermia, and hypoxia of the infant during co-sleeping [3,451]. To this end, an ancillary project has been initiated at Salt River Mortuary to investigate the accuracy of current death scene investigation practices and to suggest feasible ways to improve this process commensurate with international guidelines.

The role of molecular investigations in a SUDI cohort in Cape Town is two-fold. The first is that molecular investigations need to be carried out in a research context to better understand the disproportionately high burden of infection-related death in infants. This might prompt collaborative research with experts in immunology to assess the hypothesis of the immunocompromised infant. Gozal (1996) also hypothesised that a failure of an infant to express heat shock proteins in response to stressors experienced in early life may contribute towards SUD [452]. Such response mechanisms are imperative in the protection of cells from damage. Heat shock proteins are transcribed and translated in response to stress from temperature, hypoxia and infections. The author points out that these three stressors are also major stressors associated with SUDI [452]. Perhaps genetic variants exist in the genes or

promotor regions of heat shock proteins which prevent their optimal functioning, and which may become transparent in the early stages of post-partum life.

On another level, those deaths which remain undetermined must be prioritised for a validated molecular autopsy within a service-delivery context. This referral will be easier once SUDI investigation is standardised, such that ‘true’ SIDS cases are specifically diagnosed, and thus easily identified and prioritised. It is envisaged that this latter autopsy will test for metabolic disorders in the short term, but these may be phased out once newborn screening for metabolic disorders is implemented in South Africa. By harnessing lessons from the deceased, newborn screening for metabolic disorders is strongly argued for in this thesis.

The scope of the latter molecular autopsy requires future work such that it is optimal within a resource limited setting and informative for a currently under-studied population. A limitation of the genetic component of this study was that not all candidate variants were tested, due to available funding and the scope of this PhD. There are many other candidate variants that are plausible to test in this cohort, including the South African founder mutation in *KCNQ1* (rs12720459, c.1022C>T, p.Ala341Val) [453–455], as well as those identified in *SCN5A* in a recent retrospective study of SIDS cases in Pretoria [167]. This project forms a solid foundation for a long-term prospective forensic genetic study into SUDI cases in South Africa, which has the advantage of being linked with Salt River Mortuary FPS within an appropriate ethical framework [32].

Further, variations in the mitochondrial DNA (mtDNA) may be worthwhile exploring, due to their emerging association with SUDI [385]. Since mtDNA is largely involved with energy production which is vital for survival, many variants in the mtDNA are not compatible with

life. Consistent with this, only a small number of infants who have died suddenly and unexpectedly have been reported to have a variant in the mtDNA [27,384]. Further, most variants that are compatible with life seem to occur sporadically, as mothers and offspring of affected individuals do not typically carry the same variant. However, a case report in 2002 described an infant, who died at one year of age, who was found to have the same mtDNA variant as his mother who had ocular myopathy, demonstrating that mtDNA variants can be germline and can contribute to SUDI [456]. Opdal *et al.* (1998) also observed that SUDI cases exhibited more substitutions in the D-loop of mtDNA compared to controls [29].

10.7 Conclusion

The high IMR in South Africa is indicative of the overall poor health of the population. Despite efforts to reduce infant mortality on a national and global scale, infant death remains high in South Africa. At Salt River Mortuary, more than 250 SUDI cases are investigated annually. A 5-year retrospective study ascertained that SUDI cases form 6.67 % of the total caseload at Salt River Mortuary. The large number of SUDI cases thought to be associated with infection may also be indicative of other underlying diseases, such as inherited metabolic diseases which are triggered by fevers and infections. Yet, these are not screened for at birth or at forensic autopsy in South Africa.

Molecular autopsies have shown value in SIDS cases, in that they were able to elucidate genetic variants which could sufficiently explain death. A systematic review was performed, and showed that no molecular autopsy studies had yet been performed in Africa on indigenous African SUDI cohorts. Hence, a prospective research was launched by the University of Cape Town, in collaboration with Salt River Mortuary FPS, to investigate genetic variants which may be of relevance to the local population of SUDI cases.

A complex research framework, aligned with service operations and ethical standards was established at Salt River Mortuary. A pilot cohort of infants was recruited to optimise sample collection procedures and molecular assays, which justified that blood was the sample of choice for this research study. More work is still required to fully optimise DNA extraction from retrospective FFPE tissue samples, including increasing the number of tissue sections used.

A statistically significant cohort of SUDI cases was then recruited (n = 201). DNA was isolated from biological samples and candidate genetic variants were analysed. Allele frequencies in three variants previously associated with infections (*IL-6* rs1800795 G>C; *TNF- α* rs1800629 G>A; *TLR4* rs4986790 A>G) were significantly different between the Black African SUDI cases with infection-related death and the African individuals reported on the global online repository, gnomAD (publicly accessible: <http://gnomad.broadinstitute.org/>). This is suggestive of a possible association between these variants and infection-related cause of death in SUDI, but this requires confirmation in a larger cohort comprising more controls.

Four infants were found to be heterozygous G/A for the rs121434366 variant, meaning that they were carriers of glutaric aciduria type I (inability to metabolise certain proteins). Another infant was found to be homozygous T/T for the rs111033690 variant *GALT* gene, indicating that this infant had galactosaemia (inability to metabolise galactose). Both of these founder variants have previously been observed at Red Cross Children's Hospital in patients of African ancestry with respective metabolic disorders [289,290]. The infant affected by galactosaemia was an identical twin, who unfortunately subsequently demised in a different region of South Africa. Interestingly, the mother did not wish to learn about the genetic finding. She had found closure and did not want to revisit old wounds.

Even so, this event highlights the potential value of molecular autopsies and the ability it holds in saving lives, but emphasises that genetic testing must be done within appropriate turnaround times. Considering the resource-limited setting in South Africa, the introduction of a molecular autopsy needs to be carefully rationalised, and perhaps prioritising the service for SUDI cases who have a living identical twin is the start. Until such time that newborn screening becomes routine and accessible in South Africa, molecular autopsies should include testing for inherited errors of metabolism. This is also motivated by the well-characterised known mutations and their functional role in disease.

In this study, a subset of selected SUDI cases (n = 19) underwent also next generation sequencing alongside parental samples from three families. Four infants had potentially pertinent genetic variants in cardiac arrhythmia-related genes, one of which was homozygous T/T for rs750771811 in the *SCN10A* gene. Functional analysis of this amino acid residue has previously indicated that this variant is likely to cause BrS, but this is the first time it has been observed in a SUDI victim.

Finally, DNA from two infants and their parents underwent clinical exome sequencing and plausible genetic variants were identified in each of the infants. The genetic findings in one case were based on a polygenic hypothesis, which illustrates the value of parental samples to assess the segregation of these variants. The molecular analysis in the second case suggested an immune-compromised infant, which acts as an important reminder about these less studied mechanisms of death, which may have particular importance in a South African context. The importance of a genetic counsellor must be reiterated in their role in both the consent process as well as during the feedback of results.

This study was the first prospective forensic genetic investigation of SUDI cases in South Africa, and possibly in Africa. The successful fulfilment of the study's objectives has added new knowledge which is important for the progression of molecular autopsies in a local context. In the first instance the traversing of the highly sensitive issues of recruiting families (who are usually under great emotional duress for the access to their next of kin for this kind of investigation, and the attendant logistical and ethical issues) was of immense value, and serves to preface future and very necessary work in this regard. This body of work has also served to raise awareness of the need for a deeper investigation in a research-naïve environment around SUDI and SIDS in South Africa. While the study setting was situated in Cape Town, it is thought that the lessons learnt in this study will be informative to other resource-constrained countries and that the genetic findings may also be applicable to SUDI cohorts in other parts of (South) Africa. However, what bears emphasis is the value of this research in raising awareness, and perhaps plotting previously unthought-of stakeholders in a multidisciplinary approach to identifying the true burden of SIDS, and its investigation. These results are just the beginning of ongoing research in this area, where we hope to collaborate with other researchers to gain further insight into mechanisms of death and ultimately reduce infant mortality.

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This reference list is formatted according to the guidelines provided by Forensic Science International.

Appendix 1: Power calculation

A power calculator was used to estimate the number of SUDI cases required to yield a statistically large enough cohort for this study.

To calculate the number of individuals, n:

$$n = \frac{p(1-p)z^2}{d^2}$$

Equation A: Power calculation used to determine a significantly large cohort

$p = 0.087$, the frequency of SIDS cases among SUDI cases reported in five out of seven major medico-legal centres South Africa during 2005 – 2009 [33].

$z = 1.96$, indicating a 95 % confidence interval

$d = 0.0435$, constant

Substituting the above numbers into the equation, $n = 161.3$

Therefore the minimum number of infants required in this study is 162.



UNIVERSITY OF CAPE TOWN
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Human Research Ethics Committee



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12 October 2015

HREC REF: 445/2015

Prof R Ramesar
Human Genetics
FHS

Dear Prof Ramesar

PROJECT TITLE: SUDDEN UNEXPECTED DEATH IN INFANTS: POTENTIAL GENETIC CONTRIBUTIONS IN A SOUTH AFRICAN COHORT-(PhD-candidate-L Heathfield)

Thank you for your response letter addressing the issues raised by the Human Research Ethics Committee (HREC).

It is a pleasure to inform you that the HREC has formally approved the above-mentioned study.

Approval is granted for one year until the 30th September 2016.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the following student: - Laura Heathfield is also involved in this project.

Please quote the HREC reference no in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.

Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research

Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

IMPORTANT: Always bring this booklet when you visit any health clinic, doctor or hospital

ROAD TO HEALTH GIRLS

Child's first name and surname:

Date of Birth:

DD/MM/YYYY

This booklet must be issued at birth by the health services concerned.

If birth takes place at home, the first opportunity after delivery should be used to issue the booklet.

The booklet must be issued **FREE OF CHARGE**, irrespective of delivery taking place at a public or private health facility.



health

Department:
Health
REPUBLIC OF SOUTH AFRICA

WELL CHILD VISITS – RECORDING SHEET FOR CHILDREN										
Record the following information for each visit on the spaces that are not shaded. Refer to the page numbers given in this booklet and complete the relevant section.							Remember to check the following. Tick if done, and record details on the relevant page			Date of next visit
Age	Date	Growth (IMCI) (page 14)	PMTCT/ HIV status (IMCI) (page 7&8)	TB status (IMCI)	Feeding (EBF/EBF/ mixed feeding for first 6 months)	Immunisations (page 6)	Vitamin A (page 9)	Deworming (page 9)	Development (page 13)	Oral Health (page 20)
3-6 days										
6 wks										
10 wks										
14 wks										
4 mths										
5 mths										
6 mths										
7 mths										
8 mths										
9 mths										
10 mths										

Age	Date	Growth (IMCI) (page 14)	PMTCT/ HIV status (IMCI) (page 7&8)	TB status (IMCI)	Feeding (EBF/IEFF/ mixed feeding for first 6 months)	Immunisations (page 6)	Vitamin A (page 9)	Deworming (page 9)	Development (page 13)	Oral Health (page 20)	Date of next visit
11mths											
12 mths											
14 mths											
16 mths											
18 mths											
20 mths											
22 mths											
24 mths											
30 mths											
36 mths											
42 mths											
48 mths											
54 mths											
60 mths											
72 mths											
12 yrs											

DETAILS OF CHILD AND FAMILY (To be completed at birth)	
Child's first name and surname: _____	
Child's ID number:	
Mother's ID number:	
Date of birth dd / mm / yyyy	Name of facility where child was born:
Child's residential address:	
Mother's name:	Mother's birth date:
Father's name:	Who does the child live with?
How many children has the mother had (including this child?)	
Number born (including stillbirths)	Reason(s) for death(s):
Number alive now	Date information given: / / / dd mm yyyy
Child in need of special care (mark with X) (Complete at delivery or at first contact with health services)	
Is the baby a twin, triplet, etc? Yes No	Does the mother need additional support to care for the child? (Specify) Yes No
Any disability present (including birth defects?) Yes No (Specify)	Other: (Specify)
Stamp of facility and name and signature of official who issued booklet	

IMMUNISATIONS					
Name and surname:			ID number:		
Age group	Batch no.	Vaccine	Site	Date given dd/mm/yy	Signature
Birth		BCG	Right arm		
		OPV0	Oral		
6 weeks		OPV1	Oral		
		RV1	Oral		
		DTaP-IPV-Hib1	Left thigh		
		Hep B1	Right thigh		
		PCV 1	Right thigh		
10 weeks		DTaP-IPV-Hib2	Left thigh		
		Hep B2	Right thigh		
14 weeks		DTaP-IPV-Hib3	Left thigh		
		Hep B3	Right thigh		
		PCV2	Right thigh		
		RV2	Oral		
9 months		Measles1	Left thigh		
		PCV3	Right thigh		
18 months		DTaP-IPV-Hib4	Left arm		
		Measles2	Right arm		
6 years		Td	Left arm		
12 years		Td	Left arm		

HEAD CIRCUMFERENCE AT 14 WEEKS AND AT 12 MONTHS

14 Weeks: _____ (Range: 37 - 42 cm) **12 Months:** _____ (Range: 42 - 47.5)

REFER if head circumference is outside range

NEONATAL INFORMATION			
Birth weight:		Birth length:	
Head circumference at birth:			
Gestational age (weeks)		Rh factor	
Mother's RPR			
Antenatal (Maternal history):		Intrapartum (including mode of delivery)	
APGAR		1 min	5 min
Neonatal problems: (identify high risk problems):			
Neonatal Feeding: ☐ Exclusive breast ☐ Exclusive formula			
Special care plan / input required (e.g. Kangaroo Mother Care)			
Specify:			
Post-discharge plan (if baby was admitted in a neonatal ward/premature):			

PMTCT/HIV INFORMATION

Child's first name and surname:

Child's ID Number:

Signature of consent: _____

Date:

Fill in this section on discharge from Midwife Obstetric Unit (MOU) or obstetric ward or at first subsequent visit if not yet done

Mother's latest HIV test result ☐ Positive ☐ Negative ☐ To be done

When did mother have the test? ☐ Before pregnancy ☐ During pregnancy ☐ At delivery

Is the mother on life-long ART? ☐ Yes ☐ No

If yes, duration of life-long ART at time of delivery ☐ < 4 weeks ☐ > 4 weeks ☐ Before pregnancy

Document ARVs the mother received:

Did the mother receive infant feeding counseling? ☐ Yes ☐ No

Decision about infant feeding ☐ Exclusive breast ☐ Exclusive formula

Document Nevirapine given:

All HIV exposed infants should receive Nevirapine for a minimum of 6 weeks

Has the mother disclosed to anyone in the household? ☐ Yes ☐ No

Has the mother's partner been tested? ☐ Yes ☐ No

Remember to offer testing for all the mother's other children if not yet done

Offer a mother with unknown HIV status a rapid HIV test.

If mother's HIV rapid test is positive, perform an HIV DNA PCR test on infant if $\geq 6/52$

Fill in this section if infant is HIV exposed			
6 week visit			
What feeds has the infant received? ☐ Exclusive breast ☐ Exclusive formula ☐ Mixed feeding			
HIV PCR test done? Date:	☐ Yes ☐ No	Affix NHLS tracking barcoded sticker here	
Cotrimoxazole started?	☐ Yes ☐ No		
Infant feeding discussed?	☐ Yes ☐ No		
Has the child received Nevirapine? ☐ Yes ☐ No		If yes: ☐ Stop now ☐ Continue	
Stop Nevirapine if the mother is on life-long ART or the child has stopped breast-feeding. If not, continue until breastfeeding stops			
10 week visit, or earlier if ill			
PCR result		☐ Positive ☐ Negative	
Post test counseling done?		☐ Yes	☐ No
Referred for ART?	☐ Yes ☐ No	Stop Nevirapine if PCR is positive	
Cotrimoxazole given?		☐ Yes ☐ No	
Has child received Nevirapine? ☐ Yes ☐ No		If yes: ☐ Stop now ☐ Continue	
Encourage a mother whose baby is HIV positive to continue breastfeeding			
Retest HIV negative children 6 weeks after cessation of breastfeeding, or if clinical suspicion.			
An HIV exposed child should be retested with a rapid HIV Antibody test at 18 months			
Repeat PCR test ☐ Positive ☐ Negative Date:		HIV antibody test ☐ Positive ☐ Negative Date:	
Post test counseling done?		☐ Yes	☐ No
Referred for ART	☐ Yes ☐ No	Stop Nevirapine if PCR is positive	
Cotrimoxazole given?		☐ Yes ☐ No	
Has child received Nevirapine? ☐ Yes ☐ No		If yes: ☐ Stop now ☐ Continue	
Tick if there is additional information on HIV status in clinical notes ☐			

VITAMIN A SUPPLEMENTATION						
	At age	Date given dd/mm/yy	Signature	At age	Date given dd/mm/yy	Signature
100 000 IU	6 mths	/ /				
200 000 IU every 6 months	12 mths	/ /		42 mths	/ /	
	18 mths	/ /		48 mths	/ /	
	24 mths	/ /		54 mths	/ /	
	30 mths	/ /		60 mths	/ /	
	36 mths	/ /				
ADDITIONAL DOSES:						
For conditions such as measles, severe malnutrition, xerophthalmia and persistent diarrhoea. Omit if dose has been given in last month. Measles and xerophthalmia: Give one dose daily for two consecutive days. Record the reason and dose given below.						
Date	Dose given	Reason	Signature	Date	Dose given	Reason
DEWORMING TREATMENT (Mebendazole or Albendazole)						
Dose	At age	Date given dd/mm/yy	Signature	At age	Date given dd/mm/yy	Signature
	12 mths	/ /		18 mths	/ /	
	24 mths	/ /		48 mths	/ /	
	30 mths	/ /		54 mths	/ /	
	36 mths	/ /		60 mths	/ /	
	42 mths	/ /				

HEALTH PROMOTION MESSAGES

Up to 6 months

Feeding:

- Breastfeed exclusively (give infant only breast milk and no other liquids or solids, not even water, with exception of drops or syrup consisting of vitamins, mineral supplements or medication);
- Breastfeed as often as the child wants, day and night;
- Feed at least 8 to 12 times in 24 hours;
- When away from the child leave expressed breast milk to feed with a cup;
- Avoid using bottles or artificial teats (dummies) as this may interfere with suckling, be difficult to clean and may carry germs that can make your baby sick.



Why is exclusive breastfeeding important?

- Other foods or fluids may damage a young baby's gut and make it easy for infections (including HIV) to get into the baby's body;
- Decreases the risk of diarrhoea;
- It decreases risk of respiratory infections;
- It decreases risk of allergies;

If you have chosen to formula feed your baby, discuss safe preparation and use of formula with the health care worker

Play: Provide ways for your child to see, hear, feel, and move.
Have colorful things to see and reach

Communicate: Look into your child's eyes and smile at him or her
Talk to your child and get a conversation going with sounds or gestures.



HEALTH PROMOTION MESSAGES

6 - 12 months

Feeding:

For all children start complementary foods at 6 months

- ◆ Continue breastfeeding;
- ◆ Always breastfeed first before giving complementary foods;
- ◆ Start giving 2—3 teaspoons of mashed dried beans and/or locally available animal foods daily to supplement the iron in the breastmilk. Examples include egg (yolk), minced meat, fish, chicken/chicken livers, mopani worms. Give soft porridge, vegetables and then fruit;
- ◆ Gradually increase the amount and frequency of feeds.
- ◆ Children between 6—8 months should have two meals a day. By 12 months this should have increased to 5 small meals per day, whilst frequent breastfeeding continues;
- ◆ Offer your baby safe, clean water regularly;
- ◆ If the baby is not breastfed, give formula or at least 2 cups of full cream cow's milk (cow's milk can be given from 9 months of age)



Play: Give your child clean household things to handle, bang and drop.



Communicate:

Respond to your child's sounds and interests. Tell your child the names of things and people.

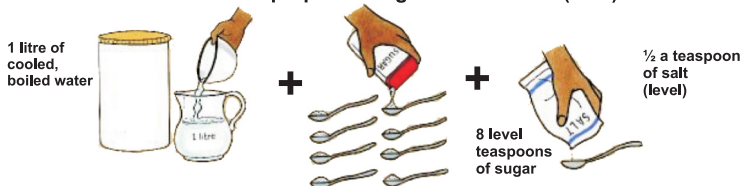
Encourage feeding during illness

Suggest an extra meal a day for a week after getting better

Feeding recommendation for DIARRHOEA

- ◆ Follow feeding recommendations for the child's age, but give small frequent meals (at least 6 times a day);
- ◆ Give a sugar-salt solution (SSS) in addition to feeds. Give SSS after each loose stool, using frequent small sips from a cup (half cup for children under 2 years and 1 cup for children 2—5 years). If the child vomits, wait for 10 minutes then continue, but more slowly

How to prepare a sugar-salt solution (SSS) at home



HEALTH PROMOTION MESSAGES

Feeding: 12 months up to 5 years

- If the child is breastfed, continue breastfeeding as often as the child wants until the child is 2 years and beyond;
- If not breastfeeding, give at least 2 cups of full cream milk, which could be maas, every day;
- Encourage children to eat a variety of foods;
- Feed your children five small meals a day;
- Make starchy foods the basis of a child's main meals;
- Children need plenty of vegetables and fruit every day;
- Children can eat chicken, fish, eggs, beans, soya or peanut butter every day;
- Give foods rich in iron and vitamins A and C;

Iron-rich foods: Liver, kidney, dark green leafy vegetables, egg yolk, dry beans, fortified cereal;

Remember that tea interferes with the absorption of iron. Iron is best absorbed in the presence of vitamin C;

Vitamin A-rich foods: Liver, dark green leafy vegetables, mango, paw paw, yellow sweet potato, full cream milk;

Vitamin C-rich foods: Citrus fruit (oranges, naartjies), guavas, tomatoes;

- If children have sweets, treats or drinks, offer small amounts with meals;
- Offer clean, safe water regularly;
- Encourage children to be active every day.



Play and communicate: 12 months to 2 years

Play: Give your child things to stack up, and to put into containers and take out.



Communicate: Ask your child simple questions. Respond to your child's attempts to talk. Play games like "bye".

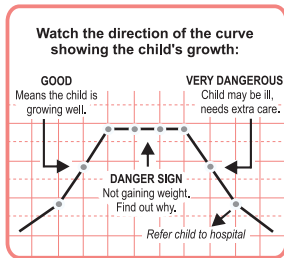
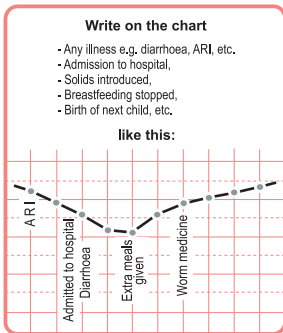
Play and communicate: Above 2 years

Play: Help your child count, name, and compare things. Make simple toys for your child.

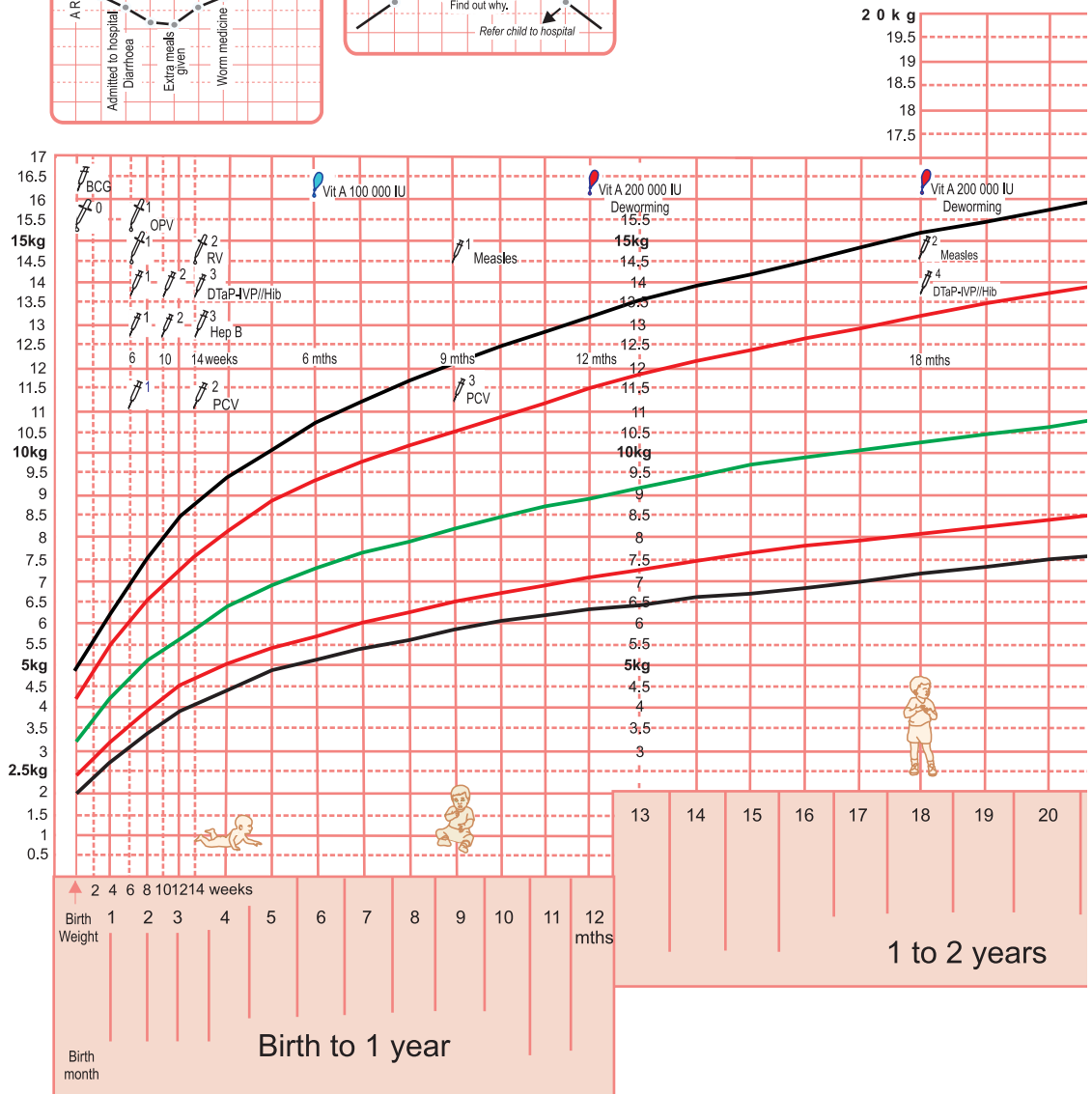


Communicate: Encourage your child to talk and answer your child's questions. Teach your child stories, songs and games.

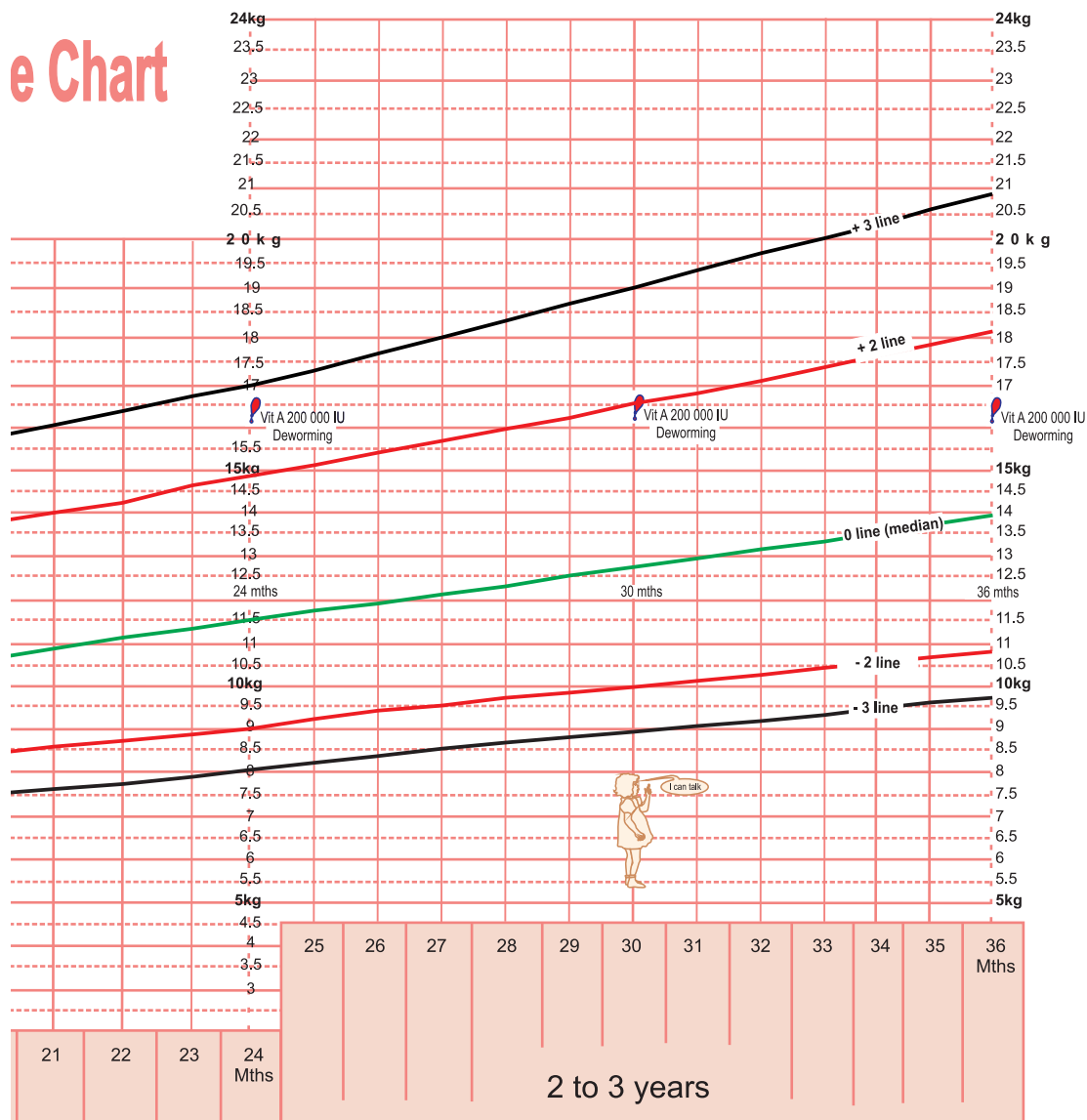
DEVELOPMENTAL SCREENING			
	VISION AND ADAPTIVE	HEARING AND COMMUNICATION	MOTOR DEVELOPMENT
ALWAYS ASK	Can your child see?	Can your child hear and communicate as other children?	Does your child do the same things as other children of the same age?
14 weeks	Baby follows close objects with eyes	Baby responds to sound by stopping sucking, blinking or turning	Child lifts head when held against shoulder 
6 months	Baby recognises familiar faces	Child turns head to look for sound	Child holds a toy in each hand 
9 months	Child's eyes focus on far objects Eyes move well together (No squint)	Child turns when called	Child sits and plays without support 
18 months	Child looks at small things and pictures	Child points to 3 simple objects Child uses at least 3 words other than names Child understands simple commands	Child walks well  Child uses fingers to feed
3 years	Sees small shapes clearly at 6 metres	Child speaks in simple 3 word sentences	Child runs well and climbs on things
5-6 years: School readiness	No problem with vision, use a Snellen E chart to check	Speaks in full sentences and interact with children and adults	Hops on one foot  Able to draw a stick person
REFER	Refer the child to the next level of care if child has not achieved the developmental milestone. Refer motor problem to Occupational Therapist/Physiotherapist and hearing and speech problem to Speech therapist/Audiologist if you have the services at your facilities.		



Girl's Weight-for-Age



e Chart



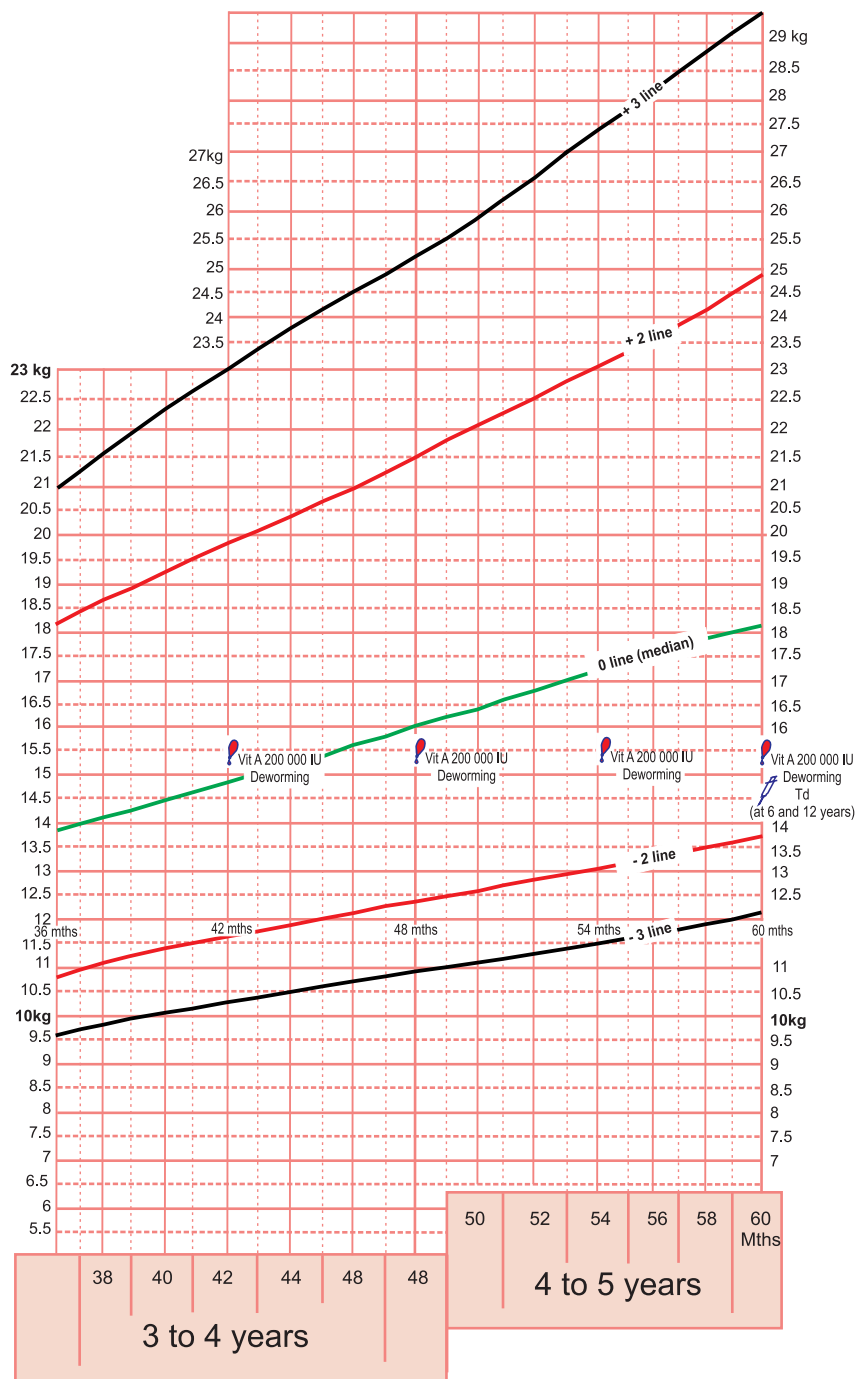
Interpretation of lines:

This Weight-for-Age Chart shows body-weight relative to age in comparison to the Median (0-line).

A girl whose weight-for-age is below the -2 line, is underweight. A girl whose weight-for-age is below the -3 line, is severely underweight. Clinical signs of Marasmus or Kwashiorkor may be observed.

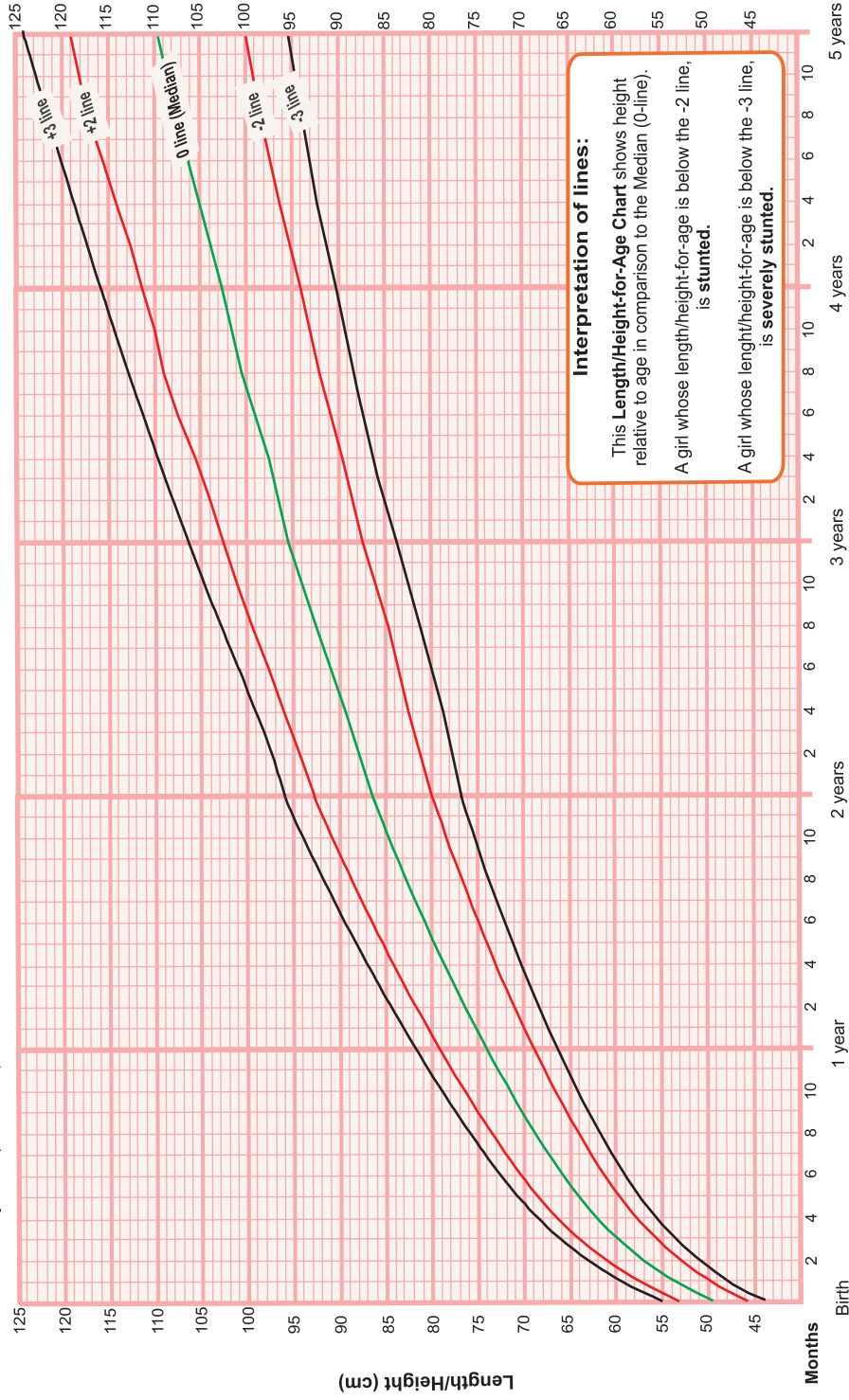
If her line crosses a z-score line and the shift is away from the median, this may indicate a problem or risk of a problem.

If her line stays close to the median, occasionally crossing above or below it, this is fine.



Length/height -for-age GIRLS

Birth to 5 years (z-scores)



FOR PERIODIC USE

(every 6 months) Indicate under "Growth" (page 2 & 3) if child is stunted

Interpretation of lines:

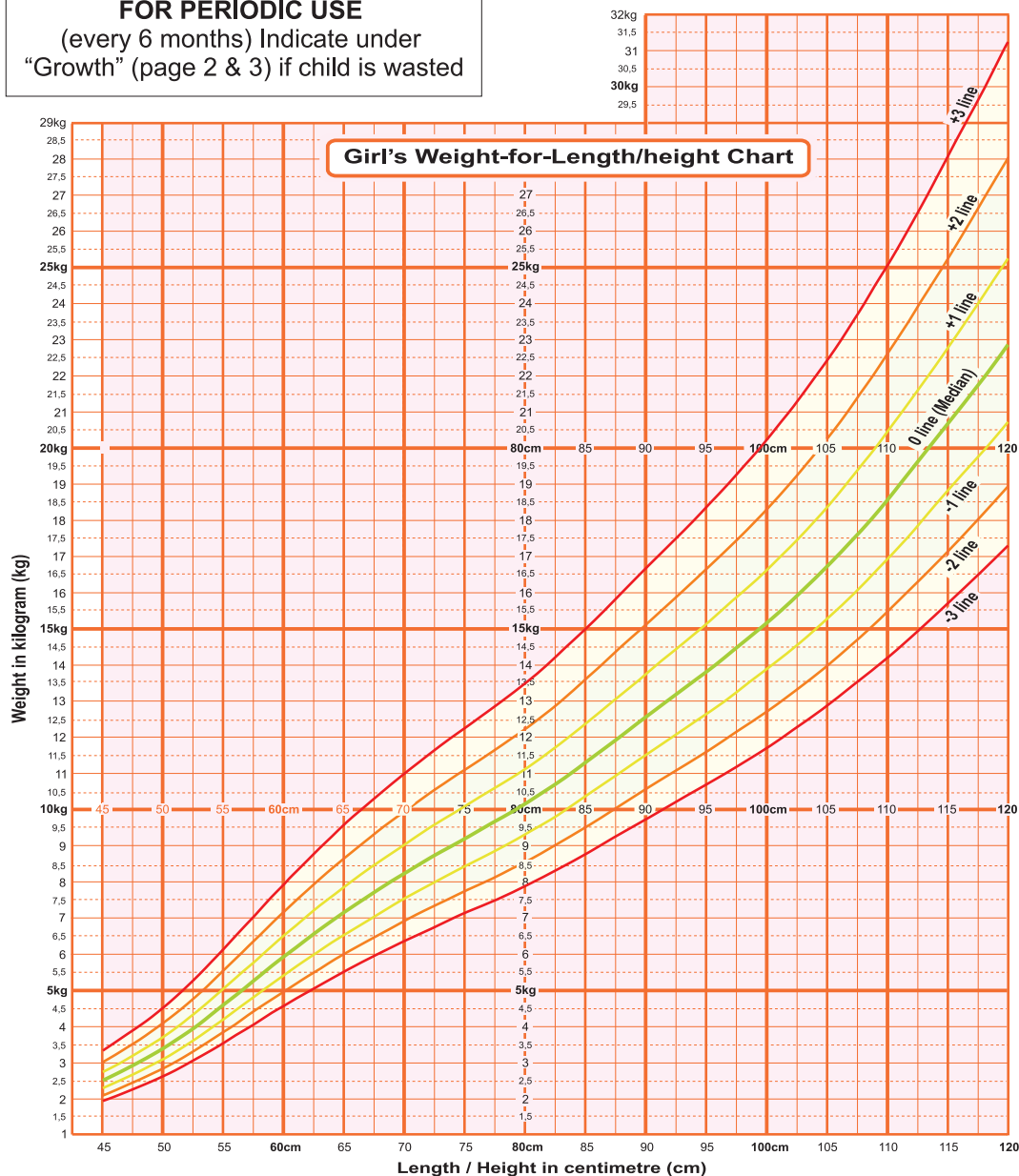
This Length/Height-for-Age Chart shows height relative to age in comparison to the Median (0-line).

A girl whose length/height-for-age is below the -2 line, is **stunted**.

A girl whose length/height-for-age is below the -3 line, is **severely stunted**.

Age (completed months and Years)

FOR PERIODIC USE
(every 6 months) Indicate under
“Growth” (page 2 & 3) if child is wasted



This **Weight-for-Length/height Chart** shows body-weight relative to length/height in comparison to the Median (the 0 Z-score line).

A girl whose weight-for-length/height is above the +3 line, is **obese**.

A girl whose weight-for-length/height is above the +2 line, is **overweight**.

A girl whose weight-for-length/height is above the +1 line, shows possible risk of **overweight**.

A girl whose weight-for-length/height is below the -2 line, is **wasted**.

A girl whose weight-for-length/height is below the -3 line, is **severely wasted. Refer for urgent specialised care.**

MID-UPPER ARM CIRCUMFERENCE (MUAC) (Every 3 months)							
Date of visit	MUAC	Date of visit	MUAC	Date of visit	MUAC	Date of visit	MUAC
< 11.5 cm indicates severe acute malnutrition (REFER urgently) ≥11.5 < 12.5 cm indicates moderate acute malnutrition (Manage as in IMCI guide-lines)							
HOSPITAL ADMISSIONS							
Hospital name	Admission number	Date of admission dd/mm/yyyy	Date of discharge dd/mm/yyyy	Discharge diagnosis			
		/ /	/ /				
		/ /	/ /				
		/ /	/ /				
		/ /	/ /				
		/ /	/ /				
		/ /	/ /				
		/ /	/ /				
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		/ /	/ /				
		/ /	/ /				
		/ /	/ /				
		/ /	/ /				
NAME OF CLINIC(S) VISITED							
Clinic 1:				Clinic 2:			
Clinic 3:				Clinic 4:			

ORAL HEALTH EXAMINATIONS

**Refer child if scheduled examinations have not been done.
To be completed by Dentist, Dental Therapist or Oral Hygienist.**

Schedule of visits:

1st visit on appearance of first tooth

Examiner: _____ Health facility: _____ Date: _____

At age 12 months, when attending immunizations

Examiner: _____ Health facility: _____ Date: _____

In the 2nd year, with other health checks

Examiner: _____ Health facility: _____ Date: _____

In the 3rd year, with other health checks

Examiner: _____ Health facility: _____ Date: _____

In the 4th year, with other health checks

Examiner: _____ Health facility: _____ Date: _____

In the 5th year, with other health checks

Examiner: _____ Health facility: _____ Date: _____

Use a clean cloth to clean your baby's gums
Use a small soft toothbrush to clean the baby's teeth

☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

CLINICAL NOTES		
Date	Assess and classify	Counsel and treat

CLINICAL NOTES		
Date	Assess and classify	Counsel and treat

Take your child to the nearest clinic when any of the these danger signs occur:

Vomiting everything



Unable to breastfeed



Convulsions



Child lethargic or unconscious



Cough and breathing rate more than 50 breaths per minute



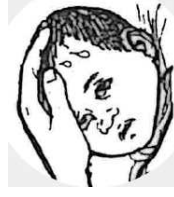
Diarrhoea with sunken eyes or sunken fontanelle

Diarrhoea with blood

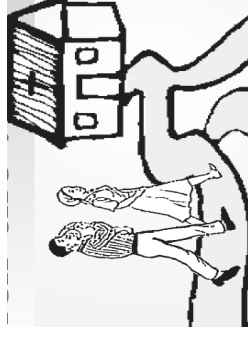


Child under 2 months and:

- is not feeding
- has fever



Chest indrawing





Forensic Pathology Services

SUD Questionnaire

FORENSIC PATHOLOGY SERVICE

To be completed in all individuals 5 years of age and younger who have died suddenly and unexpectedly.

FPS laboratory _____

WC _____

Name of baby _____

Part 1: Scene Questionnaire and Observations

Date: _____

Time: _____

Name of Forensic Pathology officer: _____

**1. A: Who gives the history/ information in this case
e.g. mother / father / granny / grandpa / other relative (give details)**

Name:	Relationship:
Address:	Contact telephone number:
ID Number or Date of Birth:	

1. B: Deceased's Details

Full name:	
Home Address:	
Age:	Date of birth:
Race:	Gender:

1. C: Person(s) at/called to the scene and relationship

Name/relationship	Date	Time
Name/relationship	Date	Time
Name/relationship	Date	Time
Police response/name	Date	Time

Paramedic response/name		Date	Time
When was the death certified/by whom		Date	Time
Was the deceased taken to hospital? If yes, provide name of hospital		Yes	No
Name of hospital:			
Date of arrival:		Time of Arrival:	
Name of doctor seen / declared death: (Comment: Get copies of doctors notes)			
Was the deceased's resuscitated or treated by the paramedics? If Yes, get copies of the ambulance voucher		Yes	No
1. D: Household environment at scene:			
Place (dwelling) where the deceased lived at time of death:	House	Shack	Other (specify)
Number of rooms and bedrooms within household?	Bedrooms		Total rooms:
Number of individuals living in household?	Adults:	Children:	Total:
Is there enough fresh air circulating through the room in which the deceased was found?	Yes	No	
Are there odours present in the room where the deceased slept? If Yes, specify	Yes	No	
Was there peeling paint in the room in which the deceased slept?	Yes	No	
Was the peeling paint anywhere near the deceased's food?	Yes	No	
Was the room damp and/or fungal (mould) present within household?	Yes	No	
Did people smoke cigarettes within the household?	Yes	No	
Are there any pets in the household? If Yes, provide type and number	Yes	No	
Provide a brief history of the events leading up to death:			
Is there any suspicion of foul play? If Yes, please describe below	Yes	No	

List items retained by FPO at scene (e.g. medications, syringes, clothing etc.)
Comments by FPO who attended scene:

Date:	Signature of deponent:

I certify that the above answers to the questionnaire at the scene was taken down by myself and that the deponent has acknowledged that he / she knows and understands the contents hereof.

Date _____ Time: _____ Place: _____

Signature of Forensic Pathology Officer: _____

Part 2: Facility Questionnaire

Date:_____ Time:_____

Name of Forensic Pathology officer:_____

2. A: Who gives the history/ information in this case**Ideally to be provided by mother, legal guardian or primary caregiver**

Name:	Relationship:
Address:	Contact telephone number:
ID Number or Date of Birth:	

2. B: Deceased's Details

Full name:	
Home Address:	
Age:	Date of birth:
Race:	Gender:

2. C: Circumstances of death / details about events before death

1. When was the baby last seen alive?	Date	Time
2. Who last saw the baby alive? Name and relationship		
3. When was the baby found dead?	Date	Time
4. Who found the baby dead at the scene? Name and relationship		
5. Was the deceased's body moved when found dead? If Yes, provide details below	Yes	No
6. Was the deceased ill prior to death?	Yes	No
a) If Yes, Was the deceased taken to doctor, hospital, clinic, pharmacy or traditional healer for treatment? Indicate which option(s) and dates below	Yes	No

b) If deceased was ill and not taken to doctor or clinic, provide reasons why?					
c) Provide names of medication given to deceased (including traditional). FPO to retain all medications for pathologist.					
7. Where was the baby found dead	Bed	Cot	Couch	Floor	Other
Other:					
8. Did the baby sustain any injuries – e.g. by falling or being hit: If yes:			Yes	No	
a) When did it happen?					
b) How did it happen?					
c) Where did it happen?					
d) What did the caretaker do about it?					
9. a) On what surface was the baby placed to sleep?	Bed	Couch	Cot	Floor	Other
b) Specify Other:					
c) If Bed/cot, Indicate mattress type.	Foam Rubber	Inner Spring	Other (specify):		
10.a) Was there a pillow present under the head?			Yes	No	
b) If Yes, was the face pressed against pillow		Yes	No	Don't know	
11.a) In what position was the deceased's body found?	Back	Stomach	Side (R/L)	Other	
b) Specify Other:					
c) In what position was the deceased's face found?	To side (R/L)	Face up	Face down	Other	
d) Specify Other:					
e) Was there anything covering the deceased's head or face?	Yes	No	Don't Know		
f) If Yes, provide details.					

12. Was the head, neck or chest squashed or wedged between any objects?	Yes	No	Don't know
If Yes, provide details			
13. Did the deceased use a dummy (pacifier)?	Yes	No	Don't know
14.a) Did the deceased sleep in the same bed as the mother, father or another person?	Yes	No	Don't know
b) If Yes, describe position	In the arms	Alongside	On Chest
c) Number of people that slept in same bed with deceased?			
d) Was anyone found laying on top of deceased (overlay)?	Yes	No	Don't know
15. Did the mother or anyone in the house smoke while the deceased slept on the night/day of death?	Yes	No	Don't know
16.a) Did the mother/caregiver use alcohol before going to bed with the baby on the night/day the baby was found dead?	Yes	No	Don't Know
b) If Yes, Indicate what and how much?			
17.a) Did the mother/caregiver use drugs before going to bed with the baby on the night/day the baby was found dead?	Yes	No	Don't know
b) If Yes, Indicate what and how much			
18.a) Did the mother/caregiver give the baby medication on the night/day of death?	Yes	No	Don't know
b) If Yes, Indicate what medication and how much.			
17.a) When was the deceased last fed?		Date	Time
b) What was the deceased fed?			
2. D: About the baby			
1.a) Where was the baby born?	Hospital	Clinic	Home
b) Name of clinic/hospital, or specify other			
2.a) How was baby born?	Normal Vaginal delivery	Caesarian Section	Forceps or Ventouse
b) Indicate reason for Caesarian Section or assisted delivery			
3. Birth weight	4. Birth Length		
5. Specify number of weeks gestation and indicate term	Weeks:	Preterm	Full Term
			Post-dates (overdue)
6. Did the deceased receive Kangaroo care (skin to skin contact)?		Yes	No
7.a) Was the baby	Exclusively Breast fed	Exclusively Bottle fed	Mixed Bottle and Breast fed
b) Provide name of Formula Milk			
c) Was boiling water used to make the bottle?		Yes	No
d) Provide names of any other foods used to feed deceased.			
8. Does the mother have the clinic card? If Yes, make copies for pathologist. If No, ask family to bring to facility.		Yes	No
9.a) Are the immunizations up to date for age?		Yes	No

b) If No, provide reasons				
10. Was the deceased sick before it died?	< 24 hours	1 day to 2 weeks	> 2 weeks	Never
Cold or Runny Nose				
Coughing				
Diarrhoea (Runny tummy)				
Unusually restless or irritable				
Crying more than usual				
Change in appetite or feeding				
Vomiting				
Seizures or fits				
Fever or Warm to touch				
Lethargic, floppy, no energy				
Cyanotic (Blue) or suddenly stop breathing				
11.a) Did the deceased come into contact with someone who was sick in the past 2 weeks?			Yes	No
b) if Yes, provide details of sickness and who the person was:				
12.a) Is the deceased known to be allergic to anything?		Yes	No	Unknown
b) If yes, what?				
13.a) Did the deceased visit another province or country prior to the death?			Yes	No
b) If yes, provide details of where and when.				
14. What did the baby wear when it died? (list clothing)				
2. E: About the mother				
1. Is the mother		Single	Married	In relationship
2.a) Is the mother employed?			Yes	No
b) If Yes, what works does she do?				

3. Age of the mother?		
4. What standard of schooling did she achieve?		
5.a) Is the mother the primary caregiver	Yes	No
b) If No, who is caregiver and why?		
6. Was she on contraception before she fell pregnant?	Yes	No
7. Did she take iron and vitamin tablets during her pregnancy?	Yes	No
8. Did she receive antenatal care?	Yes	No
9. Did the mother have diabetes in the pregnancy?	Yes	No
10. Did the mother have high blood pressure in pregnancy	Yes	No
11. Did the mother gain weight adequately in pregnancy?	Yes	No
12.a) Was she diagnosed with any illness during the pregnancy e.g. HIV?	Yes	No
b) If Yes, What?		
13.a) Was the mother on any medication during the pregnancy?	Yes	No
b) If yes, what medication:		
14.a) Were there any difficulties during the delivery?	Yes	No
b) If yes, what?		
15.a) Were there any problems with the baby after the delivery?	Yes	No
b) If yes, what?		
16.a) Was any specific instruction given about specific health care for the baby?	Yes	No
b) If yes, what?		
17.a) Was she depressed after the pregnancy?	Yes	No
b) If Yes, is she on any treatment?		
18.a) How many children does she have?		
b) How old are they?		
c) Do they all have the same father?	Yes	No
d) If No, provide details		
e) Do they all live with her?	Yes	No

f) If No, Provide reasons why?						
g) Are they all healthy?				Yes	No	
h) If No, provide details						
i) Do any of the children have learning disability?				Yes	No	
19.a) Did the mother smoke during the pregnancy?				Yes	No	
b) If yes, how many cigarettes per day?						
c) Does the mother smoke after the pregnancy?				Yes	No	
d) Does the mother know that smoking harms the unborn baby?				Yes	No	
20.a) Did the mother drink alcohol during the pregnancy?				Yes	No	
b) What did she drink?		Beer	Wine	Spirits	Other	
c) Other:						
d) How often did she drink?		Daily	Weekly	Occasionally		
e) How much did she drink?						
f) Does the mother drink after the pregnancy?				Yes	No	
g) Does the mother know that alcohol harms the unborn baby?				Yes	No	
21.a) Did the mother use drugs?				Yes	No	
b) What drugs did she use?	Cannabis	Cocaine	Heroin	Mandrax	Tik	
c) Other:						
d) How often does she use drugs?		Daily	Weekly	Occasionally		
22. Does the husband/partner drinks?				Yes	No	
23. Do the parents of the mother drink?				Yes	No	
24.a) Did the mother have a previous baby that died suddenly?				Yes	No	
b) If yes, how many died?						
c) At what age did they die?						
d) Was a PM done?				Yes	No	
e) If yes, Provide details as to when it was done, where it was done and what the cause of death was.						
25.a) Did the mother have a previous stillbirth?				Yes	No	
b) If Yes, provide details						

2. F: Household environment				
1.a) Was the following in the room where the deceased slept to heat the room?	Electric heater	"Galley"	Fire	Other
b) Describe other:				
2. Number of people in dwelling	Adults:	Children:	Total:	
3. Estimated monthly income?				
4. Number of smokers in the dwelling?				
5. Are there mentally retarded/ challenged people in the dwelling?			Yes	No

Comments from the FPO who conducted the interview:	
List any items retained at the interview	
Date:	Signature of deponent:

Date _____ Time: _____ Place: _____

Department of Health
Forensic Pathology Laboratory

Appendix 5: Drainage area for Salt River Mortuary, Cape Town

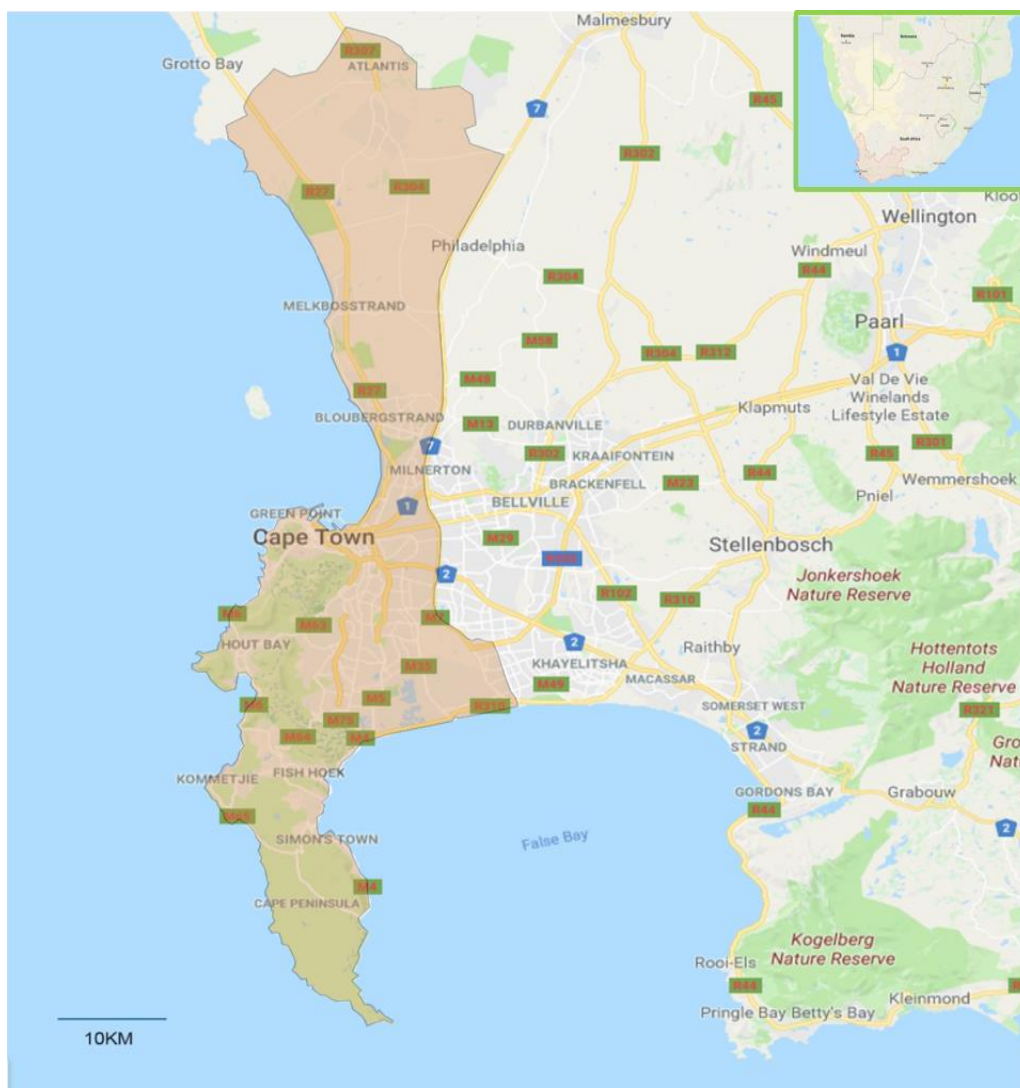


Figure A: Map of the city of Cape Town and surrounding areas, with the brown highlighted region indicating the drainage area for Salt River Mortuary.

Image courtesy of Calvin Mole.

Appendix 6: Informed consent form



INFORMATION FORM AND INFORMED CONSENT FORM

INFORMATION FORM

Study title: Sudden unexpected death in infants: a forensic genetic investigation in a South African cohort

Principal investigator: Laura Heathfield

Co-investigators: Prof Raj Ramesar, Prof Lorna Martin and Dr Sairita Maistry

Introduction to the study

You are invited to participate in research study which is a collaborative project between the Divisions of Human Genetics and Forensic Medicine at the University of Cape Town. It will be conducted by Laura Heathfield who is a researcher in the Division of Forensic Medicine at the University of Cape Town, working towards her PhD degree.

The purpose of the research study is to try and understand why some infants experience sudden and unexpected death. This form explains what you will be asked to do if you decide to participate in this study. Please read it carefully and feel free to ask any questions you like before you make a decision about participating.

Background

Sudden unexpected death of infants is a devastating loss and is often caused by infections, inborn errors of metabolism or environmental factors that surround an infant at a vulnerable

time of their natural development. Sometimes after post-mortem investigation, the cause of death still cannot be determined. This suggests that maybe there are genetic variations that may contribute to the death of the infant or predispose them to contracting an infection.

All humans are made up of many cells. Each cell contains DNA which is made up of genes. It is these genes that contain information on how a person will look or whether they get a certain disease. Sometimes these genes may predispose someone to be more vulnerable to an environmental factor, such as getting an infection or not being able to sleep on their tummy. This study wants to investigate what genetic factors could be responsible for causing a sudden and unexpected death of an infant less than one year old.

To be able to do this; different biological samples such as blood must be collected from infants who experience a sudden and unexpected death and the genetic material extracted and analysed. This will help to find genetic variations that may have contributed to the cause of the death. Once this research has been done and validated, this information will eventually be able to help determine the cause of death of other infants who experience sudden unexpected death and help family members in case they want other children.

Procedure

You will be asked to give permission to allow blood to be collected at autopsy by the Forensic Pathologist from your deceased family member:

- Blood: 5 ml will be collected.

Once the sample is collected, there will be laboratory tests on the samples to extract the DNA and to study the DNA more closely (for *e.g.* DNA sequencing and genotyping). Please note

that technology in the laboratories are always getting better so in time there might be newer laboratory techniques invented that may become accessible to the researcher. You will also be asked to allow the biological samples collected to be used in these newer technologically advanced methods if and when they become available.

The biological material collected from your deceased family member and the extracted DNA will be stored for a period of 20 years at the Division of Human Genetics (authorised institution) at the University of Cape Town. After this time the samples will be appropriately discarded.

The name of your family member will not be made known. Instead the samples, extracted DNA and generated data will be coded which means that any identifiers will be replaced with numbers or symbols. The confidentiality of the samples and data will be maintained in the following ways:

1. Every person involved in the project will be required to fill in a confidentiality agreement.
2. Only the primary investigator will have access to the database.
3. All samples, genetic data and results generated will be stored in locked filing cabinets and password protected program in the Division of Human Genetics at the University of Cape Town.
4. Sharing of experimental data will be done on the basis of alphabetical and numerical codes.
5. The results of the study may be published or presented at meetings but the identity of the deceased and the family will not be revealed.
6. Your participation will be kept confidential.

The biological samples will not be used for any research unless the research study is reviewed and approved by the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee. This committee is responsible for protecting the rights and welfare of individuals who volunteer for participation in research studies.

There are several things you need to know before allowing biological samples to be taken from your deceased family member:

1. When research is carried out it is not the policy of the University of Cape Town to provide genetic information about the deceased to the family members.
2. Participation in this study is voluntary. You are free not to participate or to withdraw at any time, for whatever reason, without negative consequences. In such cases no samples will be collected and your choice will not affect the way you will be treated at the Salt River Mortuary.
3. If you participate in the study, you can change your mind later and decide that you don't want to participate anymore and you do not want the tissue to be used in this study. Please let us know and we will destroy the samples. If the sample has already been analysed at the time you change your mind, your results and other data may have already been shared with other investigators. In that case, we will not be able to destroy this data. The data will be removed from the secured database. That means that no additional researchers can get the data.
4. You will not receive feedback of possible genetic variations that are found in the DNA of the deceased. This study is unlikely to benefit you or your family directly, but it is hoped that it will contribute to knowledge about sudden unexpected death in infants in the future.

5. There will be no cost to you and there will no compensation for your participation.

Making your choice

Please read each sentence below and think about your choice. After reading each sentence; please tick the Yes or No box. No matter what you decide, it will not negatively affect you or your deceased family member in any way.

If you may have any questions or require referral to a grief centre or psychological support please don't hesitate to ask the person taking the consent. If you may have any questions with regards to the rights and welfare of a research subject in the study, please contact the Chairperson of the University Of Cape Town Faculty Of Health Science Human Research Ethics Committee, **Professor Marc Blockman** on (021) 406 6496. If you require any further information about this study please contact Laura Heathfield at (021) 406 6569 or email at laura.heathfield@uct.ac.za

If the spouse/partner/major child/parent/guardian/major sibling agrees then the consent form needs to be read and informed consent will be taken. **Please note that the information and consent forms will be translated into the family member's language of choice.**

- Thank you for your time -

Please find attached the consent form to be signed if you wish to proceed.

CONSENT FORM

I, _____ (full name),
the spouse/partner/major child/parent/guardian/major brother/major sister (circle relationship)
of the deceased; fill in the Western Cape death register number: WC11

I confirm that I have:

	Yes	No
a) Read and understood contents of this form and agree to be a part of the research study.		
b) Been informed about this study's purpose, procedures, possible benefits, and risks.		

I give consent and agree that:

	Yes	No
a) Biological samples in the form of [blood] can be taken from my deceased family member.		
b) The biological samples can be subjected to laboratory tests at the Division of Human Genetics in which DNA will be extracted and analysed to better understand sudden unexpected death of infants.		
c) Agree that the samples and extracted DNA may be stored in the Division of Human Genetics at the University of Cape Town for a period of 20 years after which it will be appropriately discarded.		
d) The stored genetic material may only be used further research studies which have been reviewed and approved by the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee.		
e) I may be contacted in the future by someone from the University of Cape Town who may ask me to take part in research that may develop from the results of the study.		

I further understand that:

	Yes	No
a) The treatment and management of the biological samples of my deceased family member will be in accordance with guidelines of the University Of Cape Town Faculty Of Health Science Human Research Ethics Committee.		
b) The genetic laboratory is under obligation to respect medical confidentiality.		
c) This study is unlikely to benefit me or my family directly.		
d) I can at any time withdraw my consent and that I have to notify the primary investigator of my decision to withdraw.		
e) Research conducted with this DNA may result in publication, but neither the deceased nor the family of the deceased will be identified.		

I, have explained to _____ (full name)
who is the spouse/partner/major child/parent/guardian/major brother/major sister of the
deceased; the purpose, procedures, possible benefits and discomfort of this research study; and
how the samples will be collected and stored in the Division of Human Genetics at the
University of Cape Town for use in the study and in possible further research by other
individuals within the Division of Human Genetics at the University of Cape Town.

Full name of person obtaining consenting

Signature of person obtaining consenting

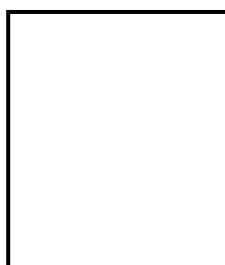
Date

Full name of person authorising consent for
collection of samples at autopsy for use in research study

Signature of person authorising consent for
collection of samples at autopsy for use in research study

Date

Thumb print of the spouse/partner/major child/parent/guardian/major brother/major sister of
the deceased:



Full name of witness

Signature of witness

Date

Appendix 7: Primers and properties

Table A: Primers designed to amplify target regions in this study, alongside their properties.

The underlined sequence represents a tail. Primer properties were assessed using OligoAnalyzer 3.1 (publicly accessible: <https://eu.idtdna.com/site/account/login?returnurl=/calc/analyzer>, date accessed 18/06/2016 and 27/04/2017).

Gene	rs number	Direction	Primer sequence (5' - 3')	Length (bp)	T _m (°C)	GC content (%)
<i>IL6</i>	rs1800795	Forward	CAGCATTACTCTTTGTCAAGAC	22	51.6	40.9
		Reverse	GCAGAATGAGCCTCAGAC	18	53.0	55.6
		Internal	<u>TTGTTGTTGTTGTTGTTG</u> TCCTTTAGCA (Reverse direction)	29	59.5	37.9
<i>TNFα</i>	rs1800629	Forward	AGCATTTCCCTCCAACC	17	52.2	52.9
		Reverse	CTCTGCTGTCCTTGCTG	17	53.1	58.8
		Internal	AGGCAATAGGTTTTGAGGGGCAT	23	59.1	47.8
<i>TLR4</i>	rs4986790	Forward	GTCTGGCTGGTTTAGAAGTC	20	52.9	50.0
		Reverse	TCAATAGTCACACTCACCAGG	21	54.0	47.6
<i>GALT</i>	rs111033690 & rs75391579	Forward	GTAGCACAGCCAAGCCCTA	19	57.2	57.9
		Reverse	CCATTAATCCTGTTCCCATGTCCAC	25	57.7	48.0
<i>ACADM</i>	rs121434278	Forward	ATGTAGCTGGTATAAAGACC	20	48.9	40.0
		Reverse	TCCAGTCCACAAACATACC	20	52.4	45.0
<i>GCDH</i>	rs121434371	Forward	TGGTTCTGCATAGGCCCTC	19	57.1	57.9
		Reverse	CCCATGGTATAGAGAGTGAAACCC	24	56.6	50.0
		Internal	<u>AGAGAGGCTGCCTGAACAAC</u>	20	57.3	55.0

Appendix 8: Sample information regarding positive microbiology results

Table B: Positive microbiology results alongside sample type and days between death and post-mortem

A total of 14 SUDI cases had positive microbiology results, out of a total of 37 requests. Various sample types returned positive results, and the number of days between death and post-mortem varied between two and six days.

SUDI ID	SAMPLE RETAINED	RESULTS	DAYS BETWEEN DEATH AND POST-MORTEM
8	Pleural fluid/aspirate	<i>Staphylococcus aureus</i> (ampicillin resistant)	3
12	Aspirate	<i>Serratia fonticola</i>	5
14	Tracheal Swab	<i>Staphylococcus aureus</i>	4
37	Blood	<i>Staphylococcus aureus</i>	6
38	Intraoperative swab	<i>Streptococcus pneumoniae</i>	4
42	Cerebrospinal fluid	<i>Coagulase negative staphylococcus</i> (doubtful significance)	4
45	Tracheal aspirate	<i>Enterobacter cloacae</i> complex (ampicillin, amoxicillin-clavulanic acid, cefuroxime (parenteral; oral) and cefoxitin resistant)	2
74	Blood	<i>Neisseria meningitidis</i>	5
	Cerebrospinal fluid	<i>Neisseria meningitidis</i>	
139	Tracheal swab	<i>Klebsiella pneumonia</i>	3
147	Blood	<i>Proteus vulgaris</i> (ampicillin, cefuroxime (parental) and cefuroxime (oral) resistant)	4
148	Swab 1 (superficial)	<i>Escherichia coli</i>	5
	Swab 2 (superficial)	<i>Escherichia coli</i>	
149	Swab (superficial)	<i>Haemophilus influenzae</i>	4
157	Swab 1 (superficial)	<i>Streptococcus pyogenes</i>	4
	Swab 2 (superficial)	<i>Streptococcus pyogenes</i>	
	Swab 3 (superficial)	<i>Streptococcus pyogenes</i>	
	Pleural fluid/aspirate	<i>Streptococcus pyogenes</i>	
173	Tissue (lung)	<i>Mycobacterium tuberculosis</i> complex	2

Appendix 9: Variants observed in GALT

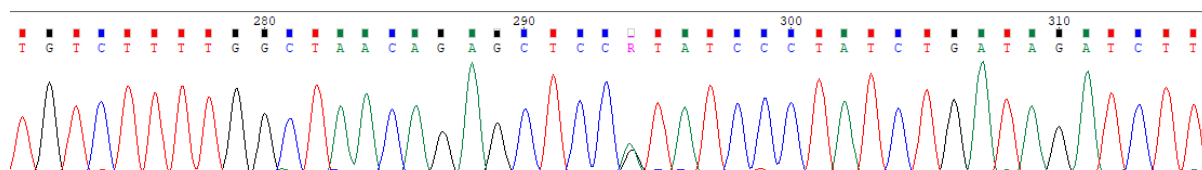
rs12000481

3' UTR variant; heterozygous G/A

Benign on ClinVar (URL: <https://www.ncbi.nlm.nih.gov/clinvar/>; date accessed: 12/11/2018)

Observed 3 SUDI cases:

- 2 SUDI cases with infection-related cause of death
- 1 procedure-related case



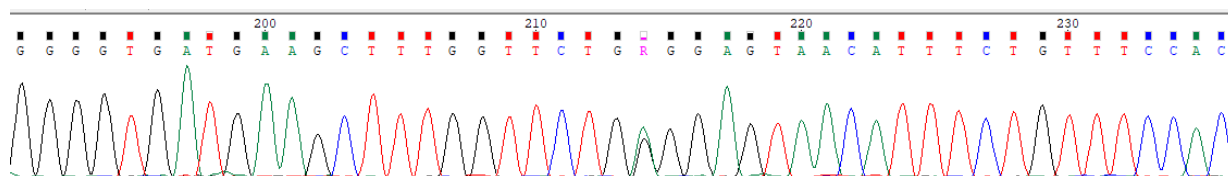
rs2277202

Non-coding exonic variant; heterozygous G/A

Benign on ClinVar (URL: <https://www.ncbi.nlm.nih.gov/clinvar/>; date accessed: 12/11/2018)

Observed 1 SUDI case:

- 1 SUDI cases with undetermined cause of death



rs41274867

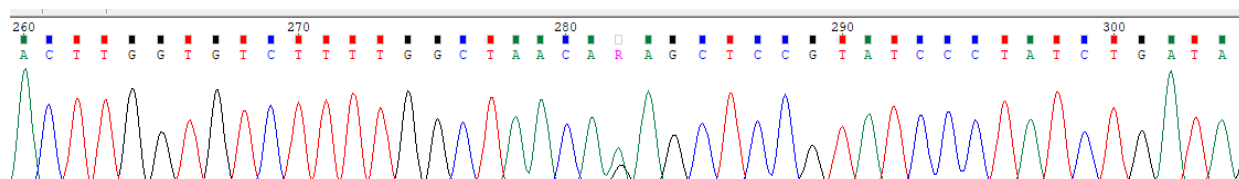
Heterozygous G/A

Splice acceptor variant

Benign on ClinVar (URL: <https://www.ncbi.nlm.nih.gov/clinvar/>; date accessed: 12/11/2018)

Observed 1 SUDI case:

- 1 SUDI cases with undetermined cause of death (same as case with rs2277202 above)



Appendix 10: List of 43 genes included in the molecular autopsy panel

Table C: List of genes and accession numbers included in the molecular autopsy panel

A total of 43 genes were investigated using next generation sequencing. HGNC: HUGO Gene Nomenclature Committee

No.	Gene name (HGNC)	Gene abbreviation (HGNC)	Chromosomal Location
1	<i>A-kinase anchoring protein 9</i>	<i>AKAP9</i>	NC_000007.14
2	<i>ankyrin 2</i>	<i>ANK2</i>	NC_000004.12
3	<i>calcium voltage-gated channel subunit alpha1 C</i>	<i>CACNA1C</i>	NC_000012.12
4	<i>calcium voltage-gated channel auxiliary subunit alpha2delta 1</i>	<i>CACNA2D1</i>	NC_000007.14
5	<i>calcium voltage-gated channel auxiliary subunit beta 2</i>	<i>CACNB2</i>	NC_000010.11
6	<i>calmodulin 1</i>	<i>CALM1</i>	NC_000014.9
7	<i>calmodulin 2</i>	<i>CALM2</i>	NC_000002.12
8	<i>calmodulin 3</i>	<i>CALM3</i>	NC_000019.10
9	<i>calsequestrin 2</i>	<i>CASQ2</i>	NC_000001.11
10	<i>caveolin 3</i>	<i>CAV3</i>	NC_000003.12
11	<i>desmin</i>	<i>DES</i>	NC_000002.12
12	<i>discs large MAGUK scaffold protein 1</i>	<i>DLG1</i>	NC_000003.12
13	<i>desmocollin 2</i>	<i>DSC2</i>	NC_000018.10
14	<i>desmoglein 2</i>	<i>DSG2</i>	NC_000018.10
15	<i>desmoplakin</i>	<i>DSP</i>	NC_000006.12
16	<i>glycerol-3-phosphate dehydrogenase 1 like</i>	<i>GPD1L</i>	NC_000003.12
17	<i>hyperpolarization activated cyclic nucleotide gated potassium channel 4</i>	<i>HCN4</i>	NC_000015.10
18	<i>junction plakoglobin</i>	<i>JUP</i>	NC_000017.11
19	<i>potassium voltage-gated channel subfamily D member 3</i>	<i>KCND3</i>	NC_000001.11
20	<i>potassium voltage-gated channel subfamily E regulatory subunit 1</i>	<i>KCNE1</i>	NC_000021.9
21	<i>potassium voltage-gated channel subfamily E regulatory subunit 5</i>	<i>KCNE5</i>	NC_000023.11
22	<i>potassium voltage-gated channel subfamily E regulatory subunit 2</i>	<i>KCNE2</i>	NC_000021.9
23	<i>potassium voltage-gated channel subfamily E regulatory subunit 3</i>	<i>KCNE3</i>	NC_000011.10
24	<i>potassium voltage-gated channel subfamily H member 2</i>	<i>KCNH2</i>	NC_000007.14
25	<i>potassium voltage-gated channel subfamily J member 2</i>	<i>KCNJ2</i>	NC_000017.11
26	<i>potassium voltage-gated channel subfamily J member 5</i>	<i>KCNJ5</i>	NC_000011.10
27	<i>potassium voltage-gated channel subfamily J member 8</i>	<i>KCNJ8</i>	NC_000012.12
28	<i>potassium voltage-gated channel subfamily Q member 1</i>	<i>KCNQ1</i>	NC_000011.10
29	<i>lamin A/C</i>	<i>LMNA</i>	NC_000001.11
30	<i>plakophilin 2</i>	<i>PKP2</i>	NC_000012.12
31	<i>phospholamban</i>	<i>PLN</i>	NC_000006.12
32	<i>ryanodine receptor 2</i>	<i>RYR2</i>	NC_000001.11
33	<i>sodium voltage-gated channel alpha subunit 10</i>	<i>SCN10A</i>	NC_000003.12
34	<i>sodium voltage-gated channel beta subunit 1</i>	<i>SCN1B</i>	NC_000019.10
35	<i>sodium voltage-gated channel beta subunit 2</i>	<i>SCN2B</i>	NC_000011.10
36	<i>sodium voltage-gated channel beta subunit 3</i>	<i>SCN3B</i>	NC_000011.10
37	<i>sodium voltage-gated channel beta subunit 4</i>	<i>SCN4B</i>	NC_000011.10
38	<i>sodium voltage-gated channel alpha subunit 5</i>	<i>SCN5A</i>	NC_000003.12
39	<i>sarcolemma associated protein</i>	<i>SLMAP</i>	NC_000003.12
40	<i>syntrophin alpha 1</i>	<i>SNTA1</i>	NC_000020.11

41	<i>transmembrane protein 43</i>	<i>TMEM43</i>	NC_000003.12
42	<i>triadin</i>	<i>TRDN</i>	NC_000006.12
43	<i>transient receptor potential cation channel subfamily M member 4</i>	<i>TRPM4</i>	NC_000019.10

Appendix 11: Editorial accepted for SAJCH

Original Publication

Heathfield LJ, Martin L, Ramesar R. Considerations of molecular autopsies in forensic cases of sudden unexpected infant death in South Africa. S Afr J Child Health. (Accepted, 2018).

Nature of the publication: Editorial

Journal: South African Journal of Child Health

Online ISSN: 1999-7671 | Print ISSN: 1994-3032

Publisher: Health & Medical Publishing Group

Candidate contribution:

LJH: Conceptualisation; data curation; formal analysis; investigation; writing of original draft (lead author).

Co-authors contributions:

LJM and RR: Supervision; review and editing of original draft.

Editorial: Considerations of molecular autopsies in forensic cases of sudden unexpected infant death in South Africa

Fatalities which are sudden and unexpected are referred for medico-legal investigation under the National Health Act No 61 of 2003 in South Africa [36]. Post-mortem investigation of these cases may include an autopsy, death scene investigation and clinical history review, which is usually sufficient to determine cause of death. However, in some instances, the cause of death remains undetermined [37].

A retrospective study at Tygerberg Forensic Pathology Service (Cape Town) between 2001 and 2005 reported that sudden unexpected death (SUD) comprised 6.1 % (n = 826) of their

total caseload (n = 13 536), and of these cases, 8.8 % (n = 72) remained undetermined after post-mortem investigation [37]. This was more than double the proportion of undetermined SUD cases reported in England (4.1 %; n = 23) during a similar time period [38]. Further, the number of infants (less than 1 year of age) who demise suddenly and unexpectedly represent an unusually high a burden of mortality in South Africa. A multicentre study in South Africa estimated that 8.7 % of sudden unexpected infant death cases admitted for forensic investigation remained as having an ‘undetermined’ cause of death [33].

The use of genetic analysis is becoming increasingly relevant, particularly in research settings, to investigate cases which have remained undetermined following post-mortem investigation [319,457–459]. The analysis of DNA from a deceased individual with the aim of identifying genetic variants associated or causative of a fatal disease (particularly those with a sudden onset) is referred to as a molecular autopsy. The genetic variants targeted during a molecular autopsy are most often associated with diseases which do not manifest with obvious and specific pathological changes [24]. Examples include some variants in *KCNQ1* and *SCN5A* which are causative of suboptimal electrical functioning of the heart (*e.g.* Brugada syndrome) [320], as well as variants associated with metabolic disorders which are often masked by signs of mild infection at autopsy[22,319]. Hence, it is unlikely that these genetic-based diseases would have been detected during a conventional medico-legal autopsy.

The value of a diagnosis in an otherwise ‘undetermined’ case brings closure to the family which has suffered loss, and this accurate mortality data has positive implications for both the legal and public health sectors [31]. Some studies have also demonstrated the clinical value of a forensic genetic analysis, when reportable genetic mutations were relayed back to blood-relatives [25,30]. These individuals were then linked with appropriate clinical and genetic

screening to better understand and/or manage their own risk of possibly having the same disease which caused the death of their relative. In the case of infant deaths, parents may find this genetic information valuable for future pregnancy management.

Studies have shown varying degrees of resolution of previously undetermined cases following a molecular autopsy; with Anderson *et al.* (2016) demonstrating up to 44 % resolution in a SUD cohort aged 1-19 years old [21]. The successful resolution reported in various studies appears to be dependent on numerous variables; such as the age of the cohort, selection criteria of the cohort, and the genes targeted for genetic analysis [26,34,320]. The potential resolution of cases, combined with the value for the family, have made molecular autopsies an attractive prospect in investigations of SUD.

It is important to note that there is no single ‘sudden death gene’[27] and therefore finding a plausible genetic variant that contributed to sudden death may require repeated genetic testing. This approach therefore requires the availability of a biological sample which yields sufficient quantity and quality of DNA for multiple tests. Retrospectively speaking, tissue may have been collected from sudden death cases at forensic autopsy for histopathology analysis and stored. Tissue is typically formalin fixed and paraffin embedded to preserve cell morphology; however, this poses significant difficulties in the amount and quality of usable DNA that can be subsequently retrieved for a molecular autopsy [173].

If collecting samples prospectively, 20 ml of blood is the recommended sample for post-mortem genetic testing [157]. Realistically, this volume of blood is exceptionally challenging to obtain from deceased infants, as post-mortem changes, including clotting, increase the viscosity of the blood. If an internal autopsy is necessary to determine cause of death, a

sufficient volume of blood can be obtained from infants (*e.g.* heart blood) for a molecular autopsy. In our experience researching molecular autopsies on cases at Salt River Mortuary, Cape Town, even 1 ml of blood has sufficed (yielding a median of 103 µg of DNA).

A ‘massively parallel sequencing’ approach may also be used to analyse many genes simultaneously, which requires less DNA (as, technically, only one test is performed). This method requires complex instrumentation and highly skilled personnel, both of which are not routinely found in forensic pathology settings. As such, collaboration between fields is of utmost importance. While the cost of processing samples using massively parallel sequencing has decreased, and is anticipated to decrease further, the limiting factor is still the time required to analyse and interpret the genetic results, whereby the more genes analysed, the more time required for interpretation.

The value of performing molecular autopsies to resolve forensic cases of sudden unexpected death which would have otherwise remained undetermined is well-documented. Its application has been demonstrated in other countries, yet research into this important area of forensic genetics is currently lacking in South Africa [34]. To this end, the University of Cape Town together with Salt River Mortuary have engaged in collaborative research to investigate sudden unexpected death in Cape Town and the applicability of molecular autopsies in a local setting. We urge other academic and forensic to also engage in the recruitment of statistically-powered cohorts unexplained cases of SUD (particularly of infants and children), with possibly bio-banking blood samples for future genetic analysis. Guidance pertaining to the ethical, legal and logistical challenges which accompany such research is provided by Heathfield *et al.* (2017) [32]. Together we can strive towards better and integrated health care, even after death.

Appendix 12: Data statement

Due to the nature of this data being related to actual forensic casework in South Africa, original, raw data cannot be made open access due to confidentiality reasons.

Appendix 13: Thesis-related scientific outputs

This list of scientific outputs relates to research conducted by the PhD candidate or directly supervised by the PhD candidate, which is associated with this primary study of sudden and unexpected death of infants.

SUMMARY

Journal articles: **6**

Conference papers: **1**

Conference oral presentations: **8**

Conference poster presentations: **3**

JOURNAL ARTICLES

Reid, K.M., Maistry, S., Ramesar, R.S and **Heathfield, L.J. (2017)**. A review of the optimisation of the use of formalin fixed paraffin embedded tissue for molecular analysis in a forensic post-mortem setting. *Forensic Science International*. 280, 181-187. doi:10.1016/j.forsciint.2017.09.020

Heathfield, L.J., Maistry, S., Martin, L.J., Ramesar, R.S., and de Vries, J. (2017). Ethical considerations of forensic genetics research on tissue samples collected post-mortem. *BMC Medical Ethics*. 18(66), 1-8. doi:10.1186/s12910-017-0225-6

Kruger, M.M., Martin, L.J., Maistry, S., and **Heathfield, L.J. (2018)**. A systematic review exploring the relationship between infection and sudden unexpected death between 2000 and 2016: a forensic perspective. *Forensic Science International*. 289, 108-119. doi: 10.1016/j.forsciint.2018.05.023

Heathfield, L.J., Martin, L.J., and Ramesar, R.S. (2018). A systematic review of molecular autopsy studies in sudden infant death cases. *Journal of Pediatric Genetics*. 07(04): 143-149. doi: 10.1055/s-0038-1668079

du Toit, C., Martin, L.J., and **Heathfield, L.J. (2018)**. Investigation into abandoned neonates admitted to Salt River Mortuary, Cape Town. *Forensic Science International*. 292, 232-241. doi: 10.1016/j.forsciint.2018.09.031

Heathfield, L.J., Martin, L.J., and Ramesar, R.S. (2018). Considerations of molecular autopsies in forensic cases of sudden unexpected death in infants and children in South Africa. *South African Journal of Child Health*. Accepted (in press).

PEER-REVIEWED CONFERENCE PAPERS

Reid, K.M., Martin, L.J. and **Heathfield, L.J. (2019)**. Evaluation of DNA profiles obtained from deceased individuals at Salt River Mortuary (South Africa). *Australian Journal of forensic science*. Accepted (in press).

CONFERENCE ORAL PRESENTATIONS

Heathfield, L.J. and Reid, K.M. Success rate of forensic DNA profiling from cotton swabs and blood samples obtained from deceased infants at Salt River Mortuary. The SAPS Forensic Services 4th Annual Conference, 2 March 2017.

Heathfield, L.J. Molecular analysis of deceased infants DNA reveals variant associated with the immune system. The African Society of Forensic Medicine 6th Annual Conference, 6-10 March 2017.

Heathfield, L.J. Sudden unexpected deaths of infants: the value of a molecular autopsy. Paediatrics Research Symposium, Red Cross Hospital Cape Town, November 2017.

Heathfield, L.J. Genetic analysis in sudden unexpected infant deaths with mild infection. 56th International Congress of the Federation of South African Societies of Pathology (FSASP); PathCape 2018. Spier Estate, Cape Town, 16-18 August 2018.

Bennett, T. and **Heathfield, L.J.** International trends in death scene investigation performed for sudden and unexpected death of infant (SUDI) cases. Safety and Violence Initiative Conference, Cape Town, October 2018.

Louw, S. and **Heathfield, L.J.** Forensic genetic research on sudden unexpected death in an infant (SUDI) at Salt River Mortuary: experiences and perceptions of parents. Paediatrics Research Symposium, Red Cross Hospital Cape Town, October 2018.

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Heathfield, L.J. Sudden unexpected death of infants: the value of a molecular autopsy. Child Death Review Research Symposium, Cape Town, November 2018.

CONFERENCE POSTER PRESENTATIONS

Kruger, M.M., Reid, K.M. and **Heathfield, L.J.** The ongoing need for education on risk factors for sudden unexpected death of infants. Child Health Priorities Conference, Groote Schuur Hospital, Cape Town, November 2016.

du Toit, C., Martin, L.J., and **Heathfield, L.J.** Retrospective analysis of abandoned live births, stillbirths and non-viable foetuses admitted to Salt River Mortuary, Cape Town. Paediatrics Research Symposium, Red Cross Hospital Cape Town, November 2017.

Young, A., Dube, F., Martin, L.J., and **Heathfield, L.J.** Insights into infection related death in infancy in Cape Town: a retrospective review. Paediatrics Research Symposium, Red Cross Hospital Cape Town, November 2017.