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CLINICAL RESEARCH
Channelopathies and cardiomyopathies

Infanticide vs. inherited cardiac arrhythmias

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Aims	In 2003, an Australian woman was convicted by a jury of smothering and killing her four children over a 10-year period. Each child died suddenly and unexpectedly during a sleep period, at ages ranging from 19 days to 18 months. In 2019 we were asked to investigate if a genetic cause could explain the children's deaths as part of an inquiry into the mother's convictions.
Methods and results	Whole genomes or exomes of the mother and her four children were sequenced. Functional analysis of a novel *CALM2* variant was performed by measuring Ca^{2+}-binding affinity, interaction with calcium channels and channel function. We found two children had a novel calmodulin variant (*CALM2* G114R) that was inherited maternally. Three genes (*CALM1-3*) encode identical calmodulin proteins. A variant in the corresponding residue of *CALM3* (G114W) was recently reported in a child who died suddenly at age 4 and a sibling who suffered a cardiac arrest at age 5. We show that *CALM2* G114R impairs calmodulin's ability to bind calcium and regulate two pivotal calcium channels ($Ca_V1.2$ and RyR2) involved in cardiac excitation contraction coupling. The deleterious effects of G114R are similar to those produced by G114W and N98S, which are considered arrhythmogenic and cause sudden cardiac death in children.
Conclusion	A novel functional calmodulin variant (G114R) predicted to cause idiopathic ventricular fibrillation, catecholaminergic polymorphic ventricular tachycardia, or mild long QT syndrome was present in two children. A fatal arrhythmic event may have been triggered by their intercurrent infections. Thus, calmodulinopathy emerges as a reasonable explanation for a natural cause of their deaths.

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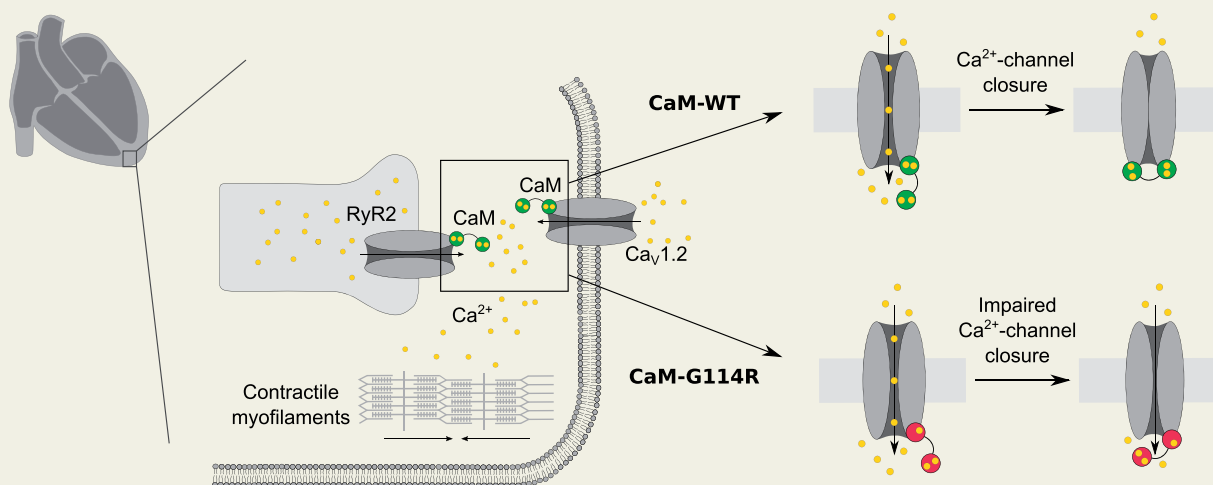
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Graphical Abstract

Keywords Sudden unexpected death • *CALM2* • *BSN* • Calmodulinopathy • Infanticide

What's new

- This study identifies a novel and functional calmodulin variant (*CALM2* G114R) present in a mother convicted of infanticide and her two female children.
- Biochemical and electrophysiological studies of the G114R variant show that it has deleterious effects on calcium binding and regulation of the two pivotal calcium channels involved in cardiac excitation contraction coupling, $Ca_V1.2$ and RyR2, in a similar manner to that of the pathogenic G114W and N98S variants.
- Given the biophysical and functional impact of the *CALM2* G114R variant, we consider the variant likely precipitated the natural deaths of the two female children (Child 3 and 4).
- The two male children (Child 1 and 2) carried biallelic rare missense variants in *BSN*, a gene shown to cause early onset lethal epilepsy in mice when deleted.

Introduction

Few diagnostic odysseys involve such high stakes as the quest to determine whether recurring sudden unexpected deaths (SUD) of children in a single family are the result of natural causes or infanticide. In the late 1990s and early 2000s, a series of high profile convictions of British mothers involved the statistical testimony of eminent paediatrician, Sir Roy Meadow, in support of his general rule that 'one sudden infant death is a tragedy, two is suspicious and three is murder, until proved otherwise'.[1] While the maxim has since been discredited and several mothers, including Sally Clark and Angela Cannings have had their convictions overturned,[2,3] an Australian woman has remained imprisoned.

In 2001, a mother from Newcastle in New South Wales was arrested and charged with smothering and killing her four young children. Each child died suddenly during periods of rest over the course

of 10 years (1989–99) (Supplementary material online, p. 16, *Table S1*) Child 1 (male) died at 19 days having been diagnosed with 'laryngomalacia'. Child 2 (male) died at 8 months from asphyxia due to airway obstruction in turn due to epileptic fits from an encephalopathic disorder of unknown cause associated with blindness. Child 3 (female) died at 10 months old, four days after seeing her general practitioner for a croupy cough and being started on antibiotics (flucloxacillin); autopsy findings included a congested and haemorrhagic uvula and profuse α-haemolytic *Streptococcus* in lung cultures. Child 4 (female) died at 18 months old, two days after being treated with paracetamol and pseudoephedrine for a respiratory infection. At autopsy she was found to have florid myocarditis (*Figure 1*) and spleen cultures grew profuse α-haemolytic *Streptococcus* of two colonial types and moderate *Staphylococcus aureus* (Supplementary material online, p. 16, *Table S1*).[4]

Circumstantial evidence against the mother consisted primarily of tendency and coincidence evidence echoing Meadow's Law, and seemingly inculpatory diary entries.[5] This evidence was thought to outweigh that in favour of death by natural causes.[5] In 2003, a jury found the mother guilty of the manslaughter of Child 1 and the murders of the remaining three children, notwithstanding that there was no history of abuse, no physical signs of smothering, no obvious motive, no eye witnesses, and no admission of guilt.[5] Psychiatrists that assessed the mother in the early 2000s considered her not to be suffering from any psychiatric illness that could explain infanticide, such as personality disorder, psychopathy, psychosis, schizophrenia, bipolar disorder, or Munchausen's syndrome by proxy. A psychiatrist that provided evidence to the inquiry and assessed the mother in March 2019 agreed that she did not suffer from a psychiatric illness that could explain infanticide but considered her to be suffering from complex post-traumatic stress disorder dating to her childhood.[6,7] To date the mother has maintained her innocence.
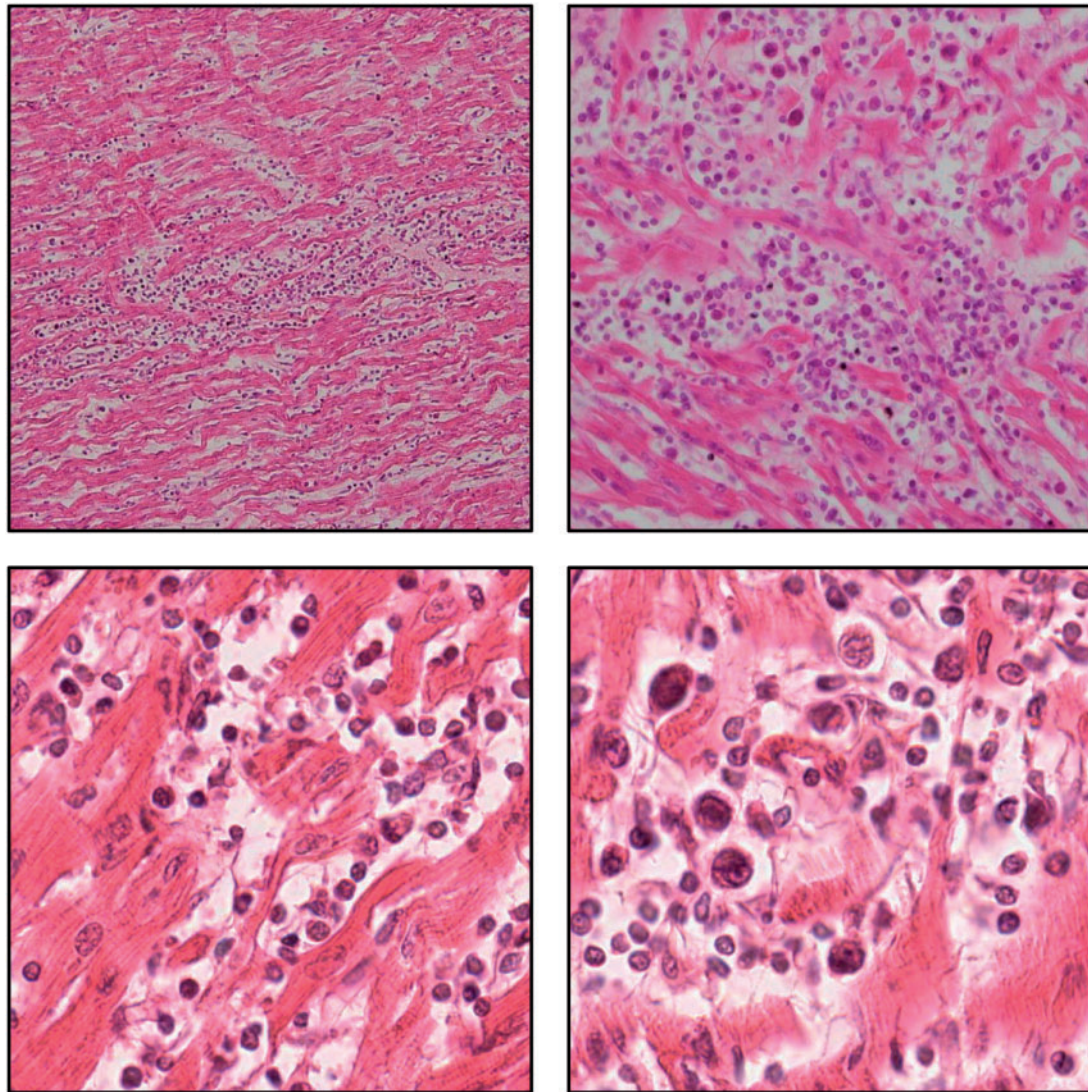
Figure 1 H&E staining of cardiac sections from Child 4's autopsy. Inflammatory infiltrates are seen in the myocardium associated in places with myocyte necrosis (magnification: 100–400×).

In March 2019, a judicial inquiry into the mother's convictions commenced due to concerns raised by several forensic pathologists regarding the medical evidence provided at the trial. The results presented here follow on from genomic investigations conducted at the request of the inquiry. The inquiry concluded before we completed our functional analysis and the mother remains in prison. All the clinical, pathology, and legal case information, including the mother's prison health records, and the children's medical and autopsy records are publicly available in the exhibits to the inquiry and can be accessed via: https://www.folbigginquiry.justice.nsw.gov.au/Pages/documents.aspx.

The hypothesis that a genetic variant causing respiratory failure or arrhythmia could explain the SUD of the children provided the rationale for genomic studies. The four children suffered from conditions of varying clinical significance (Supplementary material online, p. 16, *Table S1*). Child 1 (male) had difficulty breathing from birth attributed to laryngomalacia, Child 2 (male) suffered epileptic seizures from age 4.5 months until his death, Child 3 (female) likely had a respiratory infection at the time of her death, and Child 4 (female) had myocarditis visible on autopsy (*Figure 1*). The children's various conditions suggested that if there was a single underlying natural cause it was likely to have a spectrum of clinical manifestations and lethal triggers. Inherited arrhythmogenic disorders capable of causing sudden cardiac death (SCD) fit this profile and may first manifest as SUD.[8]

Unfortunately, the cardiological assessments of the children were limited. There were no ECG recordings of Child 1. ECG recordings of Child 2 from the early neonatal period and at 4 months of age showed normal sinus rhythm and normal intervals including a QTc of 380 ms. There were no ECG recordings of Child 3. ECG recordings of Child 4 at age 11 days showed normal sinus rhythm and normal intervals including QTc of 380 ms. Aside

from Child 4's myocarditis, examination of each child's heart did not reveal cardiac abnormalities.

Calmodulinopathies are rare life-threatening arrhythmia syndromes caused by pathogenic variants in one of the three genes (*CALM1*, *CALM2*, and *CALM3*) that encode identical calmodulin proteins.[9] Calmodulin (CaM) is a calcium-binding protein that regulates an array of enzymes and ion channels in a calcium-dependent manner, including those essential for regulating cardiac contractility. Specifically, CaM is key to the calcium-dependent inactivation (CDI) of cell membrane calcium channel $Ca_V1.2$, and for timely closure of the cardiac sarcoplasmic reticulum (SR) calcium release channel, RyR2. The first human CaM missense mutation was identified in 2012 in a large Swedish family segregating with catecholaminergic polymorphic ventricular tachycardia (CPVT) and multiple cases of SCD.[10] The first CaM mutations associated with long QT syndrome (LQTS) and cardiac arrest in infants were identified in 2013.[11] The International Calmodulinopathy Registry (ICalmR) ($n = 74$; 35 different pathogenic genotypes) reveals variability in clinical manifestations. Long QT syndrome (49%) and CPVT (28%) present at a median age of 1 year and 6 months and 6 years, respectively. Other presentations include idiopathic ventricular fibrillation (IVF), SUD, overlapping features of CPVT/LQTS and predominant neurological phenotype, while 13.5% are asymptomatic.[9]

Methods

Human subjects and DNA sequencing

The mother provided informed written consent to conduct genomic research and testing of herself and her children. The mother's genomic study complies with ethical regulations of the Australian National University and Australian Capital Territory Health Human Ethics Committees. Whole genome sequencing (WGS) of Child 1 and whole exome sequencing (WES) of Child 4 was performed from neonatal screening cards and WGS of the mother, Child 2, and Child 3 from buccal swab, frozen liver tissue, and a fibroblast cell line respectively. The average coding exon coverage was $77\times$ for the mother, $45\times$ for Child 1, $59\times$ for Child 2, $47\times$ for Child 3, and $172\times$ for Child 4. The coverage was calculated after removal of ambiguously mapped and orphaned reads. Bioinformatics analysis is described in Supplementary material online, pp 4-5. The mother agrees to share the genetic and clinical data of her children upon request for research purposes. Upon request, L.C. and P.J.S. will share the ICalmR data not publically available.

Calmodulin protein production and purification

Calmodulin variants were expressed in an *Escherichia coli* Rosetta (DE3) strain from a modified pET vector with an N-terminal maltose-binding protein and tobacco etch virus cleavage site. Calmodulin purification is described in Supplementary material online, p. 5.

Secondary structure determination by circular dichroism

Circular dichroism spectra were recorded at 37°C using a Chirascan-Plus circular dichroism spectrometer as described previously and summarized in Supplementary material, p. 5.[12]

Calcium binding

Calcium titration experiments were performed as described previously and summarized in Supplementary material, p. 5.[13,14]

Calmodulin binding to the $Ca_V1.2$ IQ-domain and RyR2 CaMBD2

Peptides representing the $Ca_V1.2$ IQ-domain and RyR2 CaM-binding domain 2 (CaMBD2) (Supplementary material, pp. 5–6) with an N-terminal 5-carboxytetramethylrhodamine (5-TAMRA) label were obtained from Proteogenix (>95% purity). Calmodulin binding to the IQ-domain and CaMBD2 peptides was monitored by fluorescence anisotropy of the 5-TAMRA-labelled peptide (Supplementary material, p. 6). Binding was measured at a range of Ca^{2+} concentrations in the presence of 1 mM free Mg^{2+}, controlled by the Ca^{2+}/Mg^{2+}-buffer system described previously and in Supplementary material, p. 5.

Cellular electrophysiology of $Ca_V1.2$

CaM mutations were generated on the human *CALM1* gene in the pIRES2-EGFP vector (Clontech Laboratories, Inc.).[15] For whole-cell patch clamp experiments, HEK293 cells (ATCC) were transiently transfected with human cardiac α_{1C} cDNA,[16] rat brain β_{2a} (M80545), rat brain $\alpha_2\delta$ (NM012919.2) subunits, CaM variants, and simian virus 40T antigen using a standard calcium phosphate method. The details of voltage-clamp recordings, solutions, and quantification of currents can be found in Supplementary material, p. 6.

Store overload induced calcium release assay

Calcium levels in the endoplasmic reticulum of HEK293 cells expressing RyR2 and CaM variants were monitored using single-cell Ca^{2+} imaging and the Ca^{2+}-sensitive fluorescence resonance energy transfer-based cameleon protein D1ER, as previously described and detailed in Supplementary material, p. 6.[17]

Results

The mother reported recurrent episodes consistent with transient loss of consciousness that started in childhood. These episodes have been associated with intercurrent illnesses, physical exertion including swimming and running, emotional stress, and pregnancy (Supplementary material, p. 16, *Table S1*). Her ECGs, cardiac exercise stress test, and 24 h Holter were normal.

We performed WGS of the mother and the first three children and WES of the fourth child. The children's father declined requests to provide a DNA sample, and was not ordered to provide one by the Inquiry. Twenty-nine ultrarare [minor allele frequency (MAF) < 0.0005] variants occurred in 4/4 children (Supplementary material, p. 17, *Table S2*) in genes not known to cause SUD. Next, we searched specifically for ultrarare variants in 424 genes extracted from a literature review of causes of SUD, SCD, SIDS, cardiac or arrhythmogenic conditions, and epileptic encephalopathy (Supplementary material, p. 18, *Table S3*) and identified variants in 12 genes: *CACNA1E, CACNA1G, CALM2, CTNNA3, DMD, DMPK, DOCK7, JUP, KCNAB2, MYH6, MYLK, SCNN1A*, and *BSN* (Supplementary material, p. 19, *Table S4*). In addition, analysis of immune variants identified two missense

variants associated in the literature with susceptibility to bacterial lung infections (*PTPN13*, 4/4 children)[18] or myocarditis (*NLRP1*, novel, 3/4 children)[19] (Supplementary material, p. 19, *Table S5*). Child 2's genome was found to carry a novel *IDS* variant located in the same codon as a known Hunter's Sd mutation (Supplementary material, p. 19, *Table S6*).

Of the 12 cardiac/SCD/SUD/epilepsy variants, only *CALM2* and *KCNAB2* caused human genetic disorders associated with SUD according to the OMIM catalogue. The *KCNAB2* variant (p.Ala11Thr, A11T; c31G>A, MAF = 0.000004) was present in 3/4 children. Although we have shown previously that gain-of-function missense mutations in adjacent residues (R12Q and L13F) are associated with Brugada syndrome,[20] functional analysis of *KCNAB2* A11T was normal (Supplementary material, pp. 3, 9, 20, *Figure S2, and Table S7*). The two male children (Child 1 and Child 2) had biallelic rare missense variants in *BSN* (*Bassoon*): p. L1898M (MAF = 0.000008) and p. A355T (MAF = 0.0022), both predicted to be damaging (Supplementary material, p. 19, *Table S4*). BSN is important for presynaptic function and for formation and function of photoreceptor ribbon synapses of the mammalian retina.[21] In mice, BSN deficiency causes early onset severe epilepsy that is 50% lethal in the first 6 months of life.[22] To date, heterozygous *BSN* variants have been associated with an adult-onset progressive-supranuclear palsy-like syndrome[23] but an autosomal recessive form of this disease has not yet been described.

The *CALM2* variant (p.Gly114Arg, G114R; c.340G>A) was present in the mother and the two female children (Child 3 and Child 4) (*Figure 2A and C* and Supplementary material, pp. 8, 19, *Figure S1, and Table S4*). DNA from the mother's parents was not available. Neither Sanger sequencing nor WGS/WES of the mother's saliva and buccal swab samples was suggestive of mosaicism for *CALM2* G114R [saliva: 76 reference (C): 84 variant (T) reads; buccal swab: 106 reference (C): 104 variant (T) reads] and a blood sample could not be obtained. Sanger sequencing could not be performed on Child 3 and Child 4 due to lack of access to additional DNA. The *CALM2* G114R variant is not found in databases of human genetic variation (ExAC, gnomAD, 1000 genomes, ClinVar). We have not observed the variant in 2580 in-house genomes sequenced at the Australian National University or in genomes from 2485 elderly Australians (MGRB).[24] Overall, *CALM2* is highly intolerant to missense substitution.[25] G114R is predicted as damaging by multiple *in silico* tools; and the phenotype is specific for disease (SUD in infancy and childhood).[26] Indeed, five different CaM mutations causing sudden death or cardiac arrest in children while asleep have been reported (three of them in children <2 years old), all of which are physically close to the G114 residue in the three-dimensional structure of CaM (*Figure 3A*).

Residue G114 in CaM is highly conserved and located in the linker domain between EF-hand III-IV (*Figure 3A*). The *CALM2* G114R variant results in a unique amino acid substitution, but a different substitution in the corresponding codon of *CALM3* (G114W) is known to
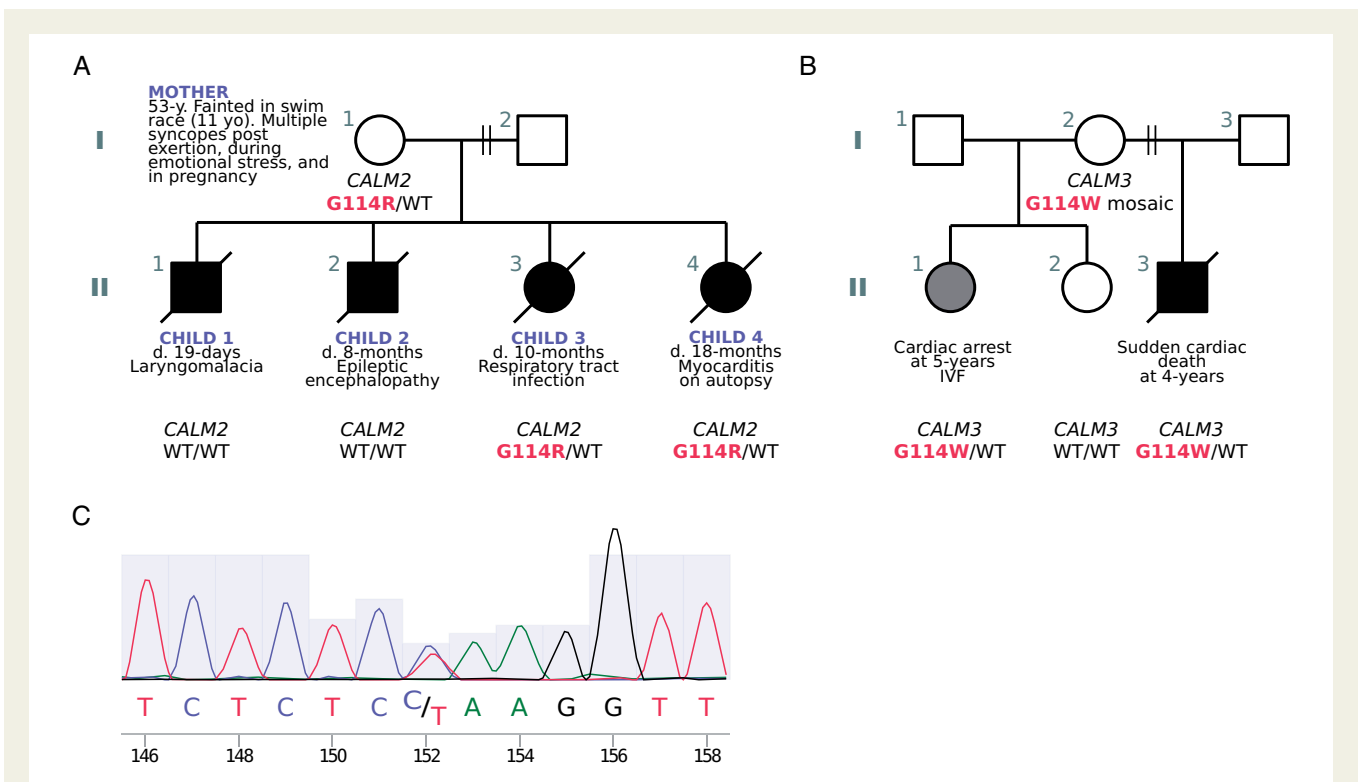
Figure 2 Pedigrees of kindreds with G114R and G114W *CALM* mutations and Sanger sequencing. (A) Family pedigree (kindred A) showing inheritance of the *CALM2* gene and the protein variation glycine (G) at position 114 to arginine (R). (B) Pedigree showing inheritance of the *CALM3* gene and protein variant G114W (tryptophan; W) in kindred B. Shaded symbols represent affected individuals with SUD/SCD/CA. WT=wildtype. (C) Sanger sequencing from the mother's buccal swab highlighting the C→T variant.

Figure 3 G114R and G114W mutations impair the calcium binding strength of CaM. (*A*) Three-dimensional structure of CaM (PDB: 1cll). Ca^{2+}-ions are displayed as black spheres. Mutations at green residues have been associated with SUD/CA during sleep in other patients. (*B*) Ca^{2+}-binding curves (left) of the C-terminal domain of CaM variants N98S, G114R, and G114W, showing reduced Ca^{2+} affinity (right), a likely proarrhythmic substrate. Data displayed as mean ± SD. *$*P \le 0.05$, $**P \le 0.01$, and $***P \le 0.001$ using one-way ANOVA with Dunnett's *post hoc* test against CaM-WT. CA, cardiac arrest; CaM, calmodulin; FI_{norm}, normalized fluorescence intensity; IVF, idiopathic ventricular fibrillation; K_A, association constant; LQTS, long QT syndrome; SCD, sudden cardiac death; SUD, sudden unexplained death.

be pathogenic (*Figure 2B*).[9] *CALM3* G114W was identified in two siblings, a girl who died suddenly at age 5 and a brother who suffered a cardiac arrest at age 4 (*Figure 2B*).[9] Their mother was healthy and found to be a mosaic, with no apparent cardiac phenotype.

Ca^{2+}-sensing and Ca^{2+}-channel regulation by CaM is crucial for maintaining a stable and healthy heart rhythm (Supplementary material, pp. 3–4). Further investigations revealed that CaM-G114R

decreased the Ca^{2+}-binding affinity of the C-domain of CaM, with a similar magnitude of effect to that observed with known arrhythmia-causing mutations, G114W and N98S (*Figure 3B* and Supplementary material, p. 20, *Table S8*). CaM-N98S was included for comparison, as this is one of the best characterized CaM mutations and it has been found in 10 unrelated individuals (5 in *CALM1* and 5 in *CALM2*), causing either SUD (including in a 2-year-old while asleep),

IVF, LQTS, CPVT, or a mixed LQTS/CPVT phenotype.[9] We postulated that the reduced Ca^{2+}-binding affinity results from a structural change conferred by the G114R mutation and observed that in the presence and absence of Ca^{2+}, CaM-G114R contains more alpha-helices and fewer turns and disordered regions (Supplementary material, pp. 10–11, Figures S3 and S4). The structural effect of CaM-G114R was comparable to that of the arrhythmogenic G114W mutation. Thus, both mutations increase the structural stability of the CaM C-domain.

We next investigated the effect of CaM-G114R on calcium flux by investigating CaM binding to the IQ-domain and CaM-binding domain 2 (CaMBD2) of the $Ca_V1.2$ and RyR2 cardiac Ca^{2+}-channels, respectively. We found that the G114R mutation reduces the affinity of CaM for both the IQ-domain (Figure 4A–C) and CaMBD2 (Figure 4E–G) at low and medium Ca^{2+}-concentrations at least as much as the known arrhythmogenic N98S and G114W variants. At high Ca^{2+}-concentrations, there was no significant difference between the CaM variants (Figure 4D and H). Thus, the G114R variant mutes the Ca^{2+}-sensing action of CaM when complexed with the canonical binding domains from $Ca_V1.2$ and RyR2 with an effect similar to or larger than pathogenic variants G114W and N98S (Supplementary material, pp. 12–13, Figures S5 and S6).

We also investigated the action of CaM-G114R on intact $Ca_V1.2$ and RyR2 channel function. Calmodulin controls the CDI of $Ca_V1.2$ (Supplementary material, pp. 3–4, 14, Figure S7). We observed that CaM-G114R decreased CDI of $Ca_V1.2$ to the same extent as CaM-

G114W but less than CaM-N98S (Figure 5A, B and Supplementary material, pp. 3–4, 14, Figure S7), which has been linked to a mixed CPVT/LQTS phenotype. In contrast, the most damaging CaM variants that cause severe LQTS, such as D96V, F142L, or D130G, fully abolish CDI.[15] Finally, we investigated the action of CaM on RyR2 closure in a negative-feedback mechanism (Supplementary material, pp. 4, 15, Figure S8).[17] Normally, CaM exerts its CDI effect on RyR2 by increasing the SR luminal Ca^{2+}-termination threshold for store-overload induced Ca^{2+} release (SOICR). We observed that CaM-G114R decreased the termination threshold for SOICR, equivalent to the effect observed for CaM-G114W and CaM-N98S[14] (Figure 5D). Curiously, CaM-G114W, but not CaM-G114R, also significantly reduced the SOICR activation threshold compared to CaM-WT (Figure 5C). All three mutations resulted in a significant increase in fractional Ca^{2+} release during SOICR[14] (Figure 5E). In summary, these functional studies reveal that CaM-G114R fails to close the $Ca_V1.2$ and RyR2 channels normally, which leads to excess transport of Ca^{2+} through both channels. This dysregulation mechanism is common to arrhythmogenic CaM variants.[27]

Discussion

Our main findings are that the mother and her two female children (Child 3 and Child 4), were carriers of a novel and functional variant in the CALM2 gene associated with sudden death in infancy and
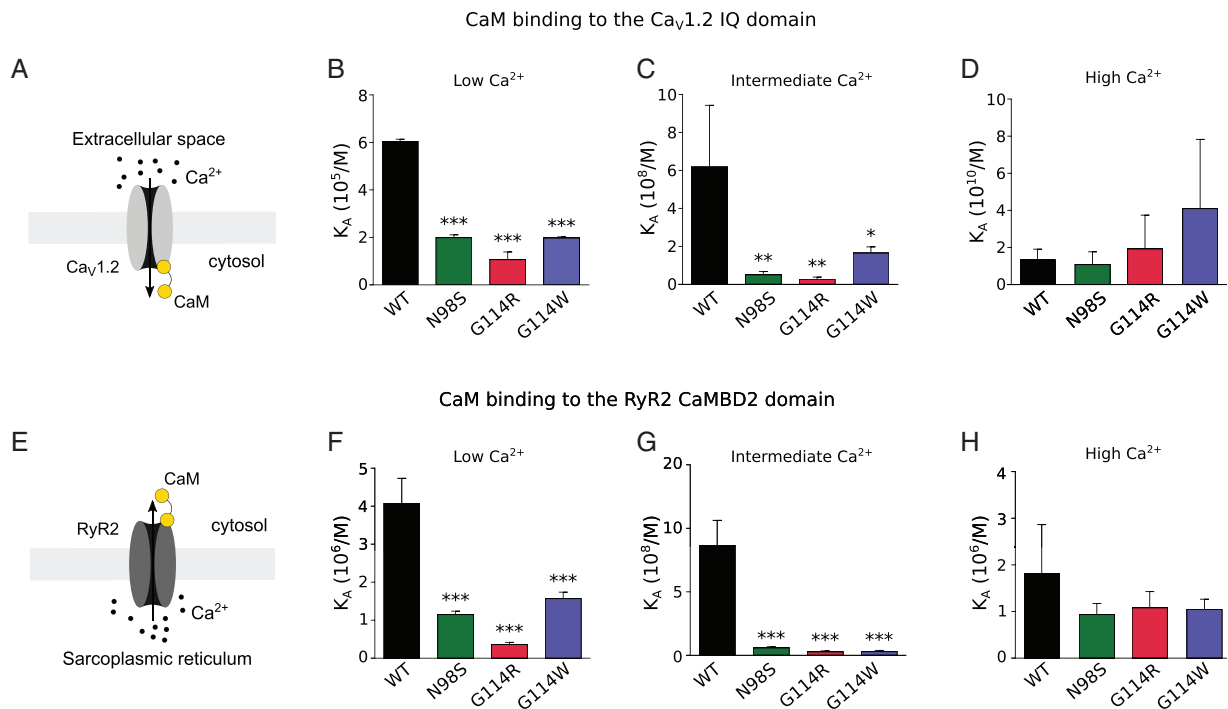
Figure 4 G114R and G114W mutations impair the binding of CaM to calcium channels. (A–D) Ca^{2+}-dependent binding of CaM to the IQ-domain of $Ca_V1.2$. Reduced binding of CaM variants N98S, G114R, and G114W unbalances the fine regulation of $Ca_V1.2$ by CaM at low and intermediate Ca^{2+} concentrations, predisposing to an altered Ca^{2+} inflow through $Ca_V1.2$. (E–H) Similarly, the binding to the RyR2 CaMBD2 domain was decreased at low and intermediate Ca^{2+} concentrations, suggesting an altered Ca^{2+} outflow from the sarcoplasmic reticulum. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ using one-way ANOVA with Dunnett's *post hoc* test against CaM-WT values. CaM, calmodulin.

Figure 5 G114R and G114W mutations cause delayed closure of calcium channels $Ca_V1.2$ and RyR2. (*A*) Exemplar traces of electrophysiological recordings of $Ca_V1.2$ opening (downward deflection) and subsequent CaM-mediated calcium-dependent inactivation (CDI) (return towards baseline) during a 30 mV voltage clamp in the presence of Ca^{2+} (red) or Ba^{2+} (black). (*B*) CDI as quantified by the metric f_{300}, measured at 30 mV. All three CaM mutations significantly reduce CDI, thus increasing the Ca^{2+} inflow through $Ca_V1.2$ and potentially leading to a pathogenic QT prolongation. ***$P < 0.001$ using Student's *t*-test. Quantified RyR2 channel opening (*C*) and closure (*D*) propensity as well as the amount of Ca^{2+} released (*E*) during an open event in store-overload induced calcium release (SOICR) assay. The CaM variant G114W significantly reduced the activation threshold, and the variants G114R and G114W significantly altered the termination threshold and fractional calcium release, potentially leading to an arrhythmogenic enhanced intracellular Ca^{2+} level. Data are displayed as mean ± SEM. *$P \leq 0.05$ and **$P \leq 0.01$ using one-way ANOVA with *post hoc* test. CaM, calmodulin; $Ca_V1.2$, voltage-gated calcium channel isoform 1.2; RyR2, ryanodine receptor type 2; WT, wild type.

childhood, and that the variant is predicted to be arrhythmogenic. The variant found in the female children (G114R) affects the same amino acid as a variant in an otherwise identical protein encoded by *CALM3* (G114W), which was implicated in the SCD of one child and the cardiac arrest of another. The G114R and G114W variants conferred similar structural changes in CaM. Both variants compromise the ability of CaM to sense changes in Ca^{2+} concentration, and impair binding to, and regulation of, the two cardiac Ca^{2+}-channels $Ca_V1.2$ and RyR2. These effects are also similar to those observed with CaM-N98S, which has been found in 10 unrelated patients diagnosed with either LQTS, CPVT, a mixed phenotype, IVF, or suffering from SUD.[9]

It is noteworthy that one N98S carrier experienced SUD while asleep at 2 years of age.[9] Sudden death during sleep is not an exceptional presentation of calmodulinopathies: of the 74 patients reported in the ICalmR, 20 suffered SUD/SCD and 5 of these (25%) died suddenly or suffered cardiac arrests in their sleep. Of those five, three occurred in children below age 2.[9]

Mechanistically and functionally, calmodulinopathies follow a surprisingly consistent scheme.[9] For almost all described cases, *CALM* mutations specifically affect the heart and cause arrhythmia, fibrillation, and/or cardiac arrest.[28] *In vitro* assessment of *CALM* mutations, has revealed an elegant correlation between the clinical manifestation

and their impact on (i) Ca^{2+} sensing and structure of the CaM protein and (ii) how CaM regulates the two Ca^{2+}-channels $Ca_V1.2$ and RyR2.[27] In most cases, the clinical impact is directly reflected in the regulation of these two Ca^{2+}-channel targets. Calmodulin mutations that severely reduce Ca^{2+}-binding to the C-domain and completely abolish CDI are present in patients with severe early onset LQTS.[15] CPVT-associated CaM mutations consistently exert a smaller impact on Ca^{2+}-binding, and minimal or no effect on $Ca_V1.2$ regulation, and have little or no effect on the QT interval.[13,15] Dysregulation of RyR2 Ca^{2+}-release is generally considered the underlying cause of CPVT, and this is also the effect observed for all CPVT-associated CaM mutations.[14,29]

Based on the previous studies and the functional data presented here, we predict that the G114R and G114W variants are pathogenic and that carriers are therefore prone to cardiac arrhythmias of IVF or CPVT-like phenotypes with a potential component of mild LQTS, which could cause cardiac arrest during sleep. This prediction is based on the following observations: (i) Both mutations impair RyR2 binding and regulation to a similar extent as other known CPVT-associated CaM mutations[29]; (ii) Both mutations impair $Ca_V1.2$ CDI to an intermediary extent, consistent with a possible mild LQTS, but likely not severe LQTS[15]; (iii) The mutations are not among the most severe in terms of reduced Ca^{2+} sensing, likely due to the location in a linker region between Ca^{2+}-binding sites; (iv) The effect of the mutations are experimentally similar to the N98S variant, which has been associated with LQTS, CPVT, IVF, or a mixed phenotype; and (v) SUD at a young age has been observed for a number of other calmodulinopathy cases,[9] including a carrier of an N98S mutation (SUD while asleep) which displays a similar functional impact as the G114R and G114W mutations. Of note, the girl with the G114W mutation who suffered a CA was diagnosed with IVF.[9]

In this family, the *CALM2* variant was inherited from their extant mother, who did not appear to be a mosaic. Incomplete penetrance or variable expressivity occur frequently in heritable cardiac arrhythmia syndromes,[8,30] including calmodulinopathies.[31] There are multiple reports of the same CaM mutation giving rise to different clinical manifestations/phenotypes in different individuals, including families, in which the parental carrier has relatively mild symptoms, such as frequent syncope as was the case with the mother, while the children suffer from life-threatening arrhythmias.[9,10,31,32] Familial cases of *CALM*-CPVT and *CALM*-IVF with asymptomatic carriers have been previously reported.[9,32] For example, Marsman *et al.*[32] reported a family with a *CALM1* variant (F90L) that caused IVF in childhood and adolescence. Five children inherited the variant from their mother. Four children suffered cardiac arrests or sudden death, but the fifth child (age 14 years) and the mother (age 60 years) remain asymptomatic after 7 years of follow-up. The ICalmR reports that 13.5% of patients with pathogenic *CALM* variants have experienced no cardiac events.[31]

Phenotypic variation is often the consequence of modifier genes.[33] These are genetic variants capable of either increasing or decreasing the clinical severity produced by a disease-causing mutation. Recently a mechanism of action of a protective modifier gene has been described for LQTS: the family members carrying this protective variant, which compensates the loss of potassium current produced by the disease-causing mutation, had a completely normal ECG and QT interval.[34]

Phenotypic variation also raises the possibility of environmental, iatrogenic, or infectious triggers. Fever has been shown to be a trigger of cardiac arrhythmias and SCD/SUD in childhood.[35] Both Child 3 and Child 4 suffered from respiratory infections immediately prior to their deaths. At present, no research has addressed the influence of infection or fever on the arrhythmogenic risk of calmodulinopathy patients. Serious infections might have caused an elevated catecholamine state,[36,37] which has been associated with trigger activity due to delayed after-depolarizations under conditions of calcium dysregulation.[38] Furthermore, Child 4 was treated with pseudoephedrine, a sympathomimetic that activates α- and β-adrenergic receptors and causes release of endogenous catecholamines, which are the triggers of CPVT. She also suffered from myocarditis, which is thought to be a major cause of SCD in the first two decades of life.[39] Combinations of these factors may have triggered the lethal events.

Child 1 and Child 2 were compound heterozygotes for rare missense variants in *BSN* (*Bassoon*) whose deficiency is known to cause early onset lethal epilepsy in mice.[22] *BSN* is a gene that appears to be highly intolerant to loss-of-function (pLI = 1). These variants require further investigation to determine whether they might account for Child 2's severe epilepsy and blindness.[21]

Conclusions

The data presented here indicate that molecular autopsies including WES/WGS are warranted for SUD infanticide cases where the accused was convicted on purely circumstantial evidence. The growing understanding of inherited cardiac arrhythmias[8] forces a reassessment of the medical foundations, never challenged, on which several mothers were found guilty of infanticide on the basis of assumption instead of evidence. The genomic revolution heralds a new era for the assessment of recurring familial sudden deaths of infants and children, an era that reasserts the presumption of innocence for tragically unlucky families.

Supplementary material

Supplementary material is available at *Europace* online.

Conflict of interest: C.G.V. and T.A. provided unpaid expert testimony in person representing the Canberra Genetics team on April 2019 at the Inquiry into the convictions of the mother. C.G.V., T.A., M.C.C., and M.A. contributed to the written submissions of the Canberra Expert Genetics team from March to July 2019 at the Inquiry (unpaid). D.A.W. provided assistance to the mother's legal representatives and to the scientists and medical practitioners involved in authoring this paper; he received no financial support or other remuneration for doing so.

Data availability

The data underlying this article are available in the article and in its online supplementary material, or will be shared on reasonable request to the corresponding authors.

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