Post-mortem genetic testing for Cardiac Ion Channelopathies in stillbirths

Cardiac Ion Channelopathies in stillbirths

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Abstract

**Background** – Although stillbirth is a significant health problem worldwide, the definitive cause of death remains elusive in many cases, despite detailed autopsy. In this study of partly explained and unexplained stillbirths we used next generation sequencing to examine an extended panel of 35 candidate genes known to be associated with ion channel disorders and sudden cardiac death (SCD).

**Methods and Results** – We examined tissue from 242 stillbirths (≥22 weeks), including those where no definite cause of death could be confirmed after a full autopsy. We obtained high quality DNA from 70 cases, which were then sequenced for a custom panel of 35 genes; 12 for inherited long and short QT syndrome genes (LQT1-LQT12 and SQT1-3), and 23 additional candidate genes derived from genome wide association studies. We examined the functional significance of a selected variant by patch clamp electrophysiological recording. No predicted damaging variants were identified in KCNQ1 (LQT1) or KCNH2 (LQT2). A rare putative pathogenic variant was found in KCNJ2 (LQT7) in one case, and several novel variants of uncertain significance were observed. The KCNJ2 variant (p. R40Q), when assessed by whole-cell patch clamp, affected the function of the channel. There was no significant evidence of enrichment of rare predicted damaging variants within any of the candidate genes.

**Conclusions** – Although a causative link is unclear, one putative pathogenic and variants of uncertain significance variant resulting in cardiac channelopathies was identified in some cases of otherwise unexplained still birth and these variants may have a role in fetal demise.
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Introduction

Stillbirth is a major issue worldwide with an estimated 3·2 million occurring each year. In the UK there are around 4,000 stillbirths every year and 1 in every 200 births ends in a stillbirth. In two thirds of cases, the cause is attributed to placental complications, with genetic abnormalities estimated to be between 6-13% on karyotyping. The remaining cases despite a detailed autopsy remain ‘unexplained’ and this is now the most common single contributor to perinatal mortality. It is enormously distressing to the families involved and there is significant unmet clinical need in terms of primary and secondary prevention.

Sequence variants in the genes encoding cardiac ion channels or associated regulatory proteins underlie varying inherited arrhythmic syndromes that may lead to arrhythmias and sudden cardiac death (SCD). In patients under 35 years of age, population studies suggest an incidence of cardiac arrest secondary to primary cardiac arrhythmia of 0·4/100,000 person-years. Long QT syndrome, characterized by prolongation of the QT interval and T-wave morphological changes on the electrocardiogram, has an estimated population prevalence of 1:2000, the molecular basis of which was initially attributed to mutations in three predominant cardiac ion channel genes, although subsequently numerous other genes have been implicated. Given the well-recognized and severe phenotype of infantile long QT syndrome, the condition has long been implicated in the aetiology of sudden infant death syndrome (SIDS), and since the initial description, sequence variants in the three major long QT-associated genes have been identified in 10% of such cohorts. This finding suggests that such genetic variants may also be associated with...
unexplained fetal demise, a hypothesis confirmed by the recent finding that 4% of such cases were associated with mutations in \textit{KCNQ1}, \textit{KCNH2} and \textit{SCN5A}.\textsuperscript{7}

With advances in technology, high quality DNA can be extracted from post mortem tissue, allowing targeted sequencing for mutations that may be associated with a risk of stillbirth.\textsuperscript{8} In this study we used next generation sequencing to investigate an enlarged panel of 35 candidate genes associated with ion channel disorders and SCD in unexplained stillbirths.

\textbf{Methods}

\textbf{Participants}

We prospectively collected fresh tissue (heart, muscle, kidney) from unexplained stillbirths (≥22 weeks) where no definite cause of death could be identified after a full autopsy, from four National Health Service (NHS) hospitals (University College London Hospital, Great Ormond Street Hospital, Southampton University Hospital and Sheffield Children’s Hospital) between 2007 and 2013, after informed parental consent. In addition, we also included previously archived freshly frozen tissue from a similar cohort from Sheffield Children’s Hospital. The Central London Research Ethics Committee 2 approved the study (10/H0713/26).

An experienced perinatal pathologist (NJS) re-classified the unexplained stillbirth cases into three groups using a summary of the autopsy report: (i) fully unexplained intrauterine death (IUD), (ii) unexplained IUD with placental lesions that have been associated with an increased risk of stillbirth, and (iii) partly explained IUD, (Supplementary Table 1).
DNA extraction and sequencing

We extracted DNA from fresh frozen tissue, primarily muscle (Wizard Genomic DNA Purification Kit, Promega Corporation, Madison, USA), and sequenced a custom panel of 35 genes, including 12 inherited long and short QT syndrome genes (LQT1-LQT12), and 23 additional candidate genes derived from genome wide association studies (GWAS) of QT interval \(^4,9-11\), and genetic studies of catecholaminergic polymorphic ventricular tachycardia (CPVT), \(^12\) SCD, \(^13\) and developmental disorders (Supplementary Table 2). \(^14\)

The custom panel was designed using SureDesign (Agilent Technologies, UK), and included all exons and known transcripts for each gene. An additional 5kb upstream of exon 1 and downstream of the last exon was also sequenced for the LQT1-LQT9 genes. The MiSeq Multiplexed Sequencing Platform (Illumina Inc, USA) was used for sequencing (Supplementary Table 2). All samples followed the same pipeline for sequencing, variant calling and annotation, and further details are provided in the Supplementary Material.

Variant filtering and annotation

To identify variants most likely to be functionally damaging we used the algorithm indicated in Figure 1. Briefly, all synonymous (not protein altering) variants were removed and a list of non-synonymous variants was assembled. Conservation scores were used to refine the list of variants (GERP++ scores \(\geq 2\)), and we annotated the variants using ANNOVAR software, which incorporates SIFT, PolyPhen 2, and Mutation Taster prediction algorithms. \(^15\) We brought forward all non-synonymous
variants which were conserved (GERP ++ score >2). Next, the frequency of the variants were checked in the Exome Aggregation Consortium (ExAC) database, and Exome Sequencing Project (ESP) and 1000G databases, and variants with frequencies > 1% across all ethnicities were removed. Finally, a literature search was undertaken to establish if genetic variants were previously associated with long QT or a related phenotype, and if there was any prior ‘in vitro’ testing to assess pathogenicity of the variant. We did not have access to parental DNA to assess inheritance of variants, so variants were subsequently categorised into two broad categories: 1) putative pathogenic or 2) variant of uncertain significance. A variant was considered as putative pathogenic if it was novel or rare (seen in < 0.1% among unrelated and ethnically similar matched controls), and if there was an abnormal electrophysiological phenotype by ‘in vitro’ functional testing. A variant of uncertain significance was a novel or rare variant with no functional data. Variants in the TTN gene were excluded from this analysis due to the extensive number of variants observed in this gene.

**Control samples**

Five hundred and sixty-three samples from the 1000 Genomes project were selected as a “control” sample for burden testing (further information is provided in the Supplementary File). The samples were randomly selected to reflect the same ethnic super code profile as the stillbirth samples; 69.6% European, 3.3% East Asian, and 1.4% African with the remaining 25.7% being randomly selected from the remaining samples in the 1000 Genomes project. As a sensitivity analysis we also selected a sample from 1000 genomes which comprised only European individuals (N = 385).

**Burden testing**
Burden tests were carried out, for each gene, using Fisher’s exact test, to compare the proportion of individuals carrying at least one rare (allele frequency ≤ 0.1%) non-synonymous variant with potential functional consequence, amongst cases versus controls. Two analyses were done: all ancestry and European only. A Bonferroni corrected p value (p < 0.004; i.e. 0.05/13) threshold was used to indicate a significant association for enrichment of rare predicted damaging variants in a gene amongst stillbirth cases. Only 13/35 genes had rare predicted damaging variants in either cases or controls, thus 13 genes were considered in burden tests.

**Power calculation for gene-based tests**

Power calculations for the gene-based Fisher tests were performed using SEQPower software \(^{19}\) applied to the data for the 13 genes tested at MAF < 0.1% available from the Exome Variant Server reference site frequency spectrum data. The LOGIT model using Fisher gene-based test method was used according to the all ancestry sample size of 71 cases and 563 controls. The following assumptions were used: deleterious variants defined as a SIFT score of ≤ 0.05, disease prevalence set as 1/600 and OR range set as 1.2 to 3.

**Functional testing of novel variants**

We selected a variant in *KCNJ2* (p. R40Q) for functional analyses by patch clamp electrophysiological recording to assess its pathogenicity, after reconfirmation of the variant by Sanger sequencing. The KCNJ2 channel (kindly supplied courtesy of Professor Yoshihiro Kubo, National Institute for Physiological Sciences, Japan) was subcloned into pcDNA3.1(+) and site directed mutagenesis was used to introduce the variant.\(^{20}\) The wild-type channel, Thr353Ala variant, Arg40Gln variant or empty vector
pcDNA3.1(+) were transiently transfected into CHO-K1 cells 48 hours before patch clamp analysis. Whole-cell patch clamp was used as previously described to establish if the variant affected the functional properties of the current produced by KCNJ2.\textsuperscript{21} Data are presented as mean and standard error (SEM) and comparisons were made using a t-test and p values <0.05 were considered significant.

**Results**

We extracted the DNA from a total of 242 cases, which were of high quality in 70, and these were successfully sequenced. The demographic characteristics of the stillbirth cases are provided in Table 1. The mean gestational age was 33.95 weeks at death (SD ±6.18), and there was no significant difference between male and female age, 33.6 ± 6.617 versus 34.5 ± 5.734 (p = 0.55). A total of 57% of the cases were male, and 67% were of European ancestry, 4.3% were Asian ancestry, 2.6% were African, and ancestry was unknown in 25.7%. Additional details of the clinical characteristics and categorisation of the 70 stillbirth cases are provided in Supplementary Table 1.

The 70 cases included in this study were sequenced on average to a mean coverage depth of 132x per sample. Ninety-seven percent of the target was covered at least 15 times to ensure accurate calling of heterozygous variants. Following QC, our sequencing analysis identified 1,964 genetic variants across the 70 stillbirth cases. Of these, 683 were exonic and 327 were non-synonymous variants. There were no deletions, insertions, nonsense variants observed in any candidate gene. Splice-site variants were observed in *TTN* and *TRPM7*. Forty-three conserved and predicted potentially damaging non-synonymous variants were found in 41 of 70 cases (58.7%).
Eighteen of these variants were in established LQT genes: one novel in CACNA1C and 17 rare (AKAP9, ANK2, CACNA1C, KCNE1, KCNE2, KCNJ2, and SCN5A). We did not identify any predicted damaging variants in KCNQ1 (LQT1) or KCNH2 (LQT2). Twenty-five predicted damaging variants in non-LQT genes were observed, six of these were novel and 19 were rare (Supplementary Table 3).

One case had a putative pathogenic variant in a LQT gene; KCNJ2 (LQT7; Table 2). Case 27 was an IUD with placental fetal thrombotic vasculopathy (FTV), it was heterozygous for a very rare (0.0015%) variant in KCNJ2 (p. Arg40Gln; Figure 2a-c). We performed ‘in vitro’ electrophysiological studies using whole-cell patch clamping to determine if this variant affected channel function. Another novel non-synonymous variant (p. Thr353Ala) found in an explained fetal demise sample was also tested (Figure 2). CHO-K1 cells transiently transfected with empty vector control (pcDNA3.1) did not possess a large inward current whereas those transfected with KCNJ2-WT or KCNJ2-T353A produced large inward currents with similar levels of current density (Figure 2d). In contrast, the homozygous expression of KCNJ2-R40Q produced currents with significantly reduced current density (p <0.05) in comparison to KCNJ2-WT (Figure 2e). In detail, the R40Q mutation, when expressed in homozygous fashion leads to an approximate 70% reduction in the level of inward current (measured at -150 mV) (Figure 2f). The reduction in current density for the Arg40Gln variant indicated that this variant is likely to result in compromised channel function.

Low frequency variants were observed in three LQT genes: SCN5A (LQT3), KCNE1 (LQT5) and KCNE2 (LQT6) with reported functional data (Table 2). Case 61, an unexplained IUD who had a normal autopsy and placental histology, was
heterozygous for two LQT genetic variants: (p. Asp85Ala: KCNE1) and (p. Thr8Ala: KCNE2). The p. Thr8Ala variant in KCNE2 is low frequency (allelic frequency 0.4%), and is located at the N-terminus, and has been reported in association with antibiotic induced arrhythmia and SIDS.\textsuperscript{22,23} Functional studies have shown that this variant when expressed with the hERG channel has a 15% reduction in current densities compared to wild type, and has an increased sensitivity to treatment with sulfamethoxazole and trimethoprim (agents associated with QT prolongation).\textsuperscript{22} The Asp85Asn variant in KCNE1 has a minor allele frequency of 0.038. This variant has been reported to be associated with drug-induced LQTS, an odds ratio of 9.0.\textsuperscript{24} Functional studies indicate that this variant, when co-expressed with either KCNQ1 or KCNH2, is associated with decreased current density (compared to wild type KCNE1).\textsuperscript{25}

Case 67 was heterozygous for a relatively rare variant, p. Gly615Glu (0.039%) in SCN5A. This variant is located in the DI/DII linker region of the channel protein, and is associated with shorter recovery times from inactivation in a Xenopus oocyte expression system. A similar change in channel function has been observed for other SCN5A variants which have been reported in association with SIDS.\textsuperscript{26}

Case 5, an unexplained IUD with placental lesions was heterozygous for the variant p. Arg481Trp in SCN5A. This variant has previously been reported as increasing the risk of LQT, SCD, torsades de pointes or acquired LQT.\textsuperscript{26-28} It has previously been reported in an African case, and has been shown to affect activation, inactivation or recovery time of the channel depending on the SCN5A splice variant tested.\textsuperscript{28}
Novel variants of uncertain significance

Seven novel variants of uncertain significance were observed in the stillbirth cases (Supplementary Table 4). Case 6 was heterozygous for p. Pro1845Leu in CACNA1C (LQT8); a variant located within the C terminal topological domain (amino acid residues 1525-2221). Five novel variants were observed in five QT-GWAS candidate genes: TRPM7, SCN10A, CREBBP, SRL and BAZ2B. Case 43 was heterozygous for a p. Trp860Met variant in TRPM7, this variant is located in the 2nd transmembrane domain of the ion channel. Case 52 was heterozygous for p. Trp1570Ala variant in SCN10A. We observed five cases that were heterozygous for p. Gln2251Lys in CREBBP. The variant substitutes a glutamine residue from an identified poly-Q region (2199-2216). Case 69 was heterozygous for a BAZ2B variant at p. Asp636Asn. Case 36 was heterozygous for p. Glu22Asp in the SRL gene. We also observed one case (case 46) that was heterozygous for p. His513Tyr in RYR2, a candidate gene for CPVT, however this lies outside the known functional domains where the vast majority of disease-associated mutations cluster.12

Rare variants of uncertain significance

There were several cases with rare variants of uncertain significance that had been previously reported as associated with SUD, arrhythmias or Torsades de pointes. These included a rare RYR2 variant (p. Val2113Met) found from sequencing of a sudden unexplained death (SUD) population.29 The p. Asn687Ser variant in ANK2; has previously been reported in an individual with TdP.30 Finally, a p. R1268Q variant in SCN10A, this has previously been reported in an individual with Brugada syndrome, and functional testing has indicated an 84% reduction in sodium current when expressed with wild type SCN5A compared to wild type SCN10A.31
Testing for enrichment of rare functional variants

A significant number of rare functional variants were observed in public sequence databases, therefore we performed a burden test to detect genes with a significant enrichment in rare predicted damaging variants (minor allele frequency, MAF < 0.1%) in stillbirth cases verses 563 randomly selected controls (see Methods). We did not observe significant evidence of enrichment of rare predicted damaging variants in stillbirth cases within any of the 13 tested genes (Table 3). Similarly, there was no evidence of enrichment in samples of European ancestry only (Supplementary Table 5). A power calculation set at the 5% alpha significance level for a 1-sided test indicated the average power as 51% across the 13 genes.

Discussion

Here we report the data from an extensive molecular autopsy in unexplained stillbirth involving 35 genes associated with ventricular arrhythmia and SCD, using next generation sequencing. We found putative pathogenic variants that might be associated with fetal demise in four (5.7%) cases. Three of these variants had reported functional data – KCNE1 and KCNE2 (1 case); SCN5A (2 cases), and one was a very rare variant in KCNJ2 (p. Arg40Gln) that affected the function of the channel. Predicted damaging variants were found in 37 (53.0%) of the remaining 66 cases, these were of uncertain significance in this setting.

In contrast to a previous study that investigated the role of LQT variants in intra-uterine fetal death,7 we found no predicted damaging variants in LQT1 and LQT2 genes KCNQ1 or KCNH2. We observed three SCN5A (MAF < 1%) variants in our population
with functional data available. This observation is in agreement with an earlier hypothesis of an increased prevalence of sodium channel variants in SIDS compared to potassium channel variants in stillbirth.\textsuperscript{32}

Interestingly, mutations in \textit{KCNJ2} that do not result in Andersen-Tawil syndrome, but affect cardiac function have recently been identified.\textsuperscript{33} These mutations tend to not result in a complete loss of channel function, in a similar manner to the Arg40Gln variant we have identified, which may explain why these patients have an isolated cardiac phenotype and lack extra cardiac dysmorphic features.\textsuperscript{33}

How the predicted damaging variants identified in this study may contribute to the mechanism of intrauterine fetal demise is difficult to determine. We do not have proof of mechanism of death, or recording of fetal arrhythmias, nor do we have evidence from other studies of other patients with the same variants demonstrating convincing pathogenicity. In future studies, pathogenicity could be examined in cardiomyocytes derived from induced pluripotent stem cells that carry these variants. Large scale population based GWAS would also be required to examine potential causal relationships.

Nevertheless, it is possible that a multiple ‘hit’ hypothesis similar to that proposed in SIDS (combination of pathogenic variants and/or of functional polymorphisms triggering lethal cardiac events when combined with specific environmental factors during a critical developmental phase) may also be applicable to unexplained stillbirths. For example, environmental factors may include non-lethal placental pathologies, or milder degrees of intrauterine infection, that might trigger a lethal fetal
cardiac arrhythmia. This hypothesis is supported by recent observations in \textit{kcnj8} knockout mice models in which induced vasospasm by ergometrine, lipopolysaccharide (LPS) induced sepsis, or cytomegalovirus triggered sudden death.\textsuperscript{34} Furthermore, fetal arrhythmias related to ion channelopathies have been reported in settings where there is routine fetal screening.\textsuperscript{35}

Of potential interest is the observation of novel variants in putative candidate genes derived from GWAS of the QT interval. TRPM7 is a ubiquitously expressed non-selective ion channel-kinase which plays a key role in embryonic cardiogenesis and establishing cardiac automaticity. Alongside its role in heart development the channel regulates magnesium homeostasis and a Thr1482Ile variant has been found to be associated with Guamanian neurodegenerative disorder.\textsuperscript{36} Sequencing of \textit{TRPM7} in LQT patients indicated variants in this gene as good candidates for inherited LQT. We observed one stillbirth case that was heterozygous for a novel variant in \textit{TRPM7} (p. Thr860Met) and several other cases were heterozygous for low frequency variants in this gene. Of note \textit{TRPM7} was the only gene which had suggestive evidence of enrichment (prior to Bonferroni correction) in the burden testing. We also observed a splice site variant in this gene in one stillbirth case (Supplementary Table 3). Functional studies of \textit{TRPM7} and its relationship to stillbirth may warrant more detailed analyses.

A second candidate gene of interest is \textit{SCN10A}. SCN10A encodes a voltage gated sodium channel alpha subunit Nav1.8 which consists of four homologous domains each with six transmembrane spanning regions of which one is a voltage sensor. Nav1.8 can also associate with one or more beta units. Mutations in \textit{SCN10A} have
been associated with Brugada syndrome and can modulate \textit{SCN5A} expression (LQT3).\textsuperscript{1} Patients with BrS who had \textit{SCN10A} mutations were more symptomatic and displayed significantly longer PR and QRS intervals compared with \textit{SCN10A}-negative BrS probands.\textsuperscript{2} However, more recent data has put these findings into doubt. Burden testing of \textit{SCN10A} showed a lack of significant enrichment for rare coding variation in cases of BrS in a population of European ancestry.\textsuperscript{37} Likewise our Burden test indicated no significant enrichment of rare functional variants within \textit{SCN10A} amongst stillbirth cases.

The \textit{CREBBP} gene encodes a histone acetyl transferase and coactivator. Interestingly in our study we identified five cases with a novel p. Gln2251Lys nonsynonymous variant. Variants in \textit{CREBBP} have been associated with acute lymphoblastic leukaemia and Rubinstein–Taybi syndrome (RTS), RTS patients have a history of cardiac abnormalities with previous work revealing a link between the syndrome, congenital heart defects and conductive abnormalities. The novel variant we identified lies 100 residues upstream of the nuclear-receptor coactivator-binding domain. Five separate cases contained variants in \textit{BAZ2B} which encodes bromodomain adjacent to zinc finger domain 2B, an epigenetic regulator which has been associated with SCD susceptibility.\textsuperscript{38} Case 36 was heterozygous for a novel \textit{SRL} variant, a gene physiologically involved in calcium storage in muscle tissue.\textsuperscript{39} One case possessed a rare \textit{SMARCAD1} variant, this DNA helicase is required for DNA repair and heterochromatin organization. Another case was heterozygous for a rare variant in \textit{MKL2}, a gene previously noted as required for myogenic differentiation.\textsuperscript{40}
This study is not without limitations. Firstly, our sample size is small owing to the difficulties in post-mortem research consenting, and the phenotype is relatively broad. Some of the stillbirth cases in this cohort could be considered partially ‘explained’, for example the case with presence of placental fetal thrombotic vasculopathy. We did not exclude such cases, since the clinical significance of such focal placental changes remains uncertain and it is possible that cardiac ion channelopathies might increase the risk of death in a compromised fetus. Secondly, we did not have any data of fetal cardiac arrhythmias in the recruited cases. Thirdly, we used data from the 1000 genomes study as “control” data for burden testing. As variants across cases and controls were not called together, it is possible variants in cases are novel due to this issue. Fourthly, the gene-based testing was underpowered (using the best-case scenario of causality assumptions) with only ~51% power achievable from our sample sizes. Finally, parental DNA was not available to assess if the variants were ‘de novo’ mutations or were inherited, nor did we have details on family history of cardiac ion channelopathies. Declining autopsy rates, and challenges associated with consenting of bereaved parents, are major hurdles to research in this area.

Given that cardiac ion channelopathies may be a preventable cause of sudden death (for example the mortality from LQT can be reduced significantly with appropriate therapy), the novel and rare variants we report may be of clinical interest, if future studies are able to establish clearer mechanistic and inheritance pathways.

In summary, these preliminary data indicate that putative pathogenic variants resulting in cardiac channelopathies may exist in some cases of otherwise unexplained stillbirths. It is possible that these variants may have had a role in the fetal demise.
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The sponsor or the funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Disclosures
None

Contributions
ST (Chief Investigator) conceived the idea and designed the study jointly with PBM, CM, AMT, NJS, DA, and DP. PBM, SA, JC and MS undertook the genetic analysis.
ATi, SCH and QA undertook the functional testing. ATe and ID performed the bioinformatics analysis. HRW performed the statistical analysis. PL, MC, BV, DF assisted recruitment. PBM and ST are the guarantors of the study data. All authors contributed to interpretation of the data, manuscript development and approved the final manuscript for submission.
References


Table 1: Demographic characteristics of stillbirth cases

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unexplained Stillbirths (N = 70)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of Gestation – weeks ± SD</td>
<td>33.9 ± 6.2</td>
</tr>
<tr>
<td>Male – no. (%)</td>
<td>40 (57)</td>
</tr>
<tr>
<td>*Ethnicity – no. (%)</td>
<td></td>
</tr>
<tr>
<td>White British</td>
<td>45 (64)</td>
</tr>
<tr>
<td>African</td>
<td>2 (2.8)</td>
</tr>
<tr>
<td>Asian</td>
<td>2 (2.8)</td>
</tr>
<tr>
<td>Eastern European</td>
<td>2 (2.8)</td>
</tr>
<tr>
<td>Indian</td>
<td>1 (1.4)</td>
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<tr>
<td>Unknown</td>
<td>18 (26)</td>
</tr>
</tbody>
</table>

*Self-reported ethnicity
Table 2: Variants in LQT genes observed in stillbirth cases with functional data

<table>
<thead>
<tr>
<th>Case</th>
<th>Clinical presentation</th>
<th>LQT Gene</th>
<th>Amino Acid Change</th>
<th>ExAC Allele Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>Fetal thrombotic vasculopathy</td>
<td><em>KCNJ2</em></td>
<td>Arg40Gln</td>
<td>2</td>
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<tr>
<td>67</td>
<td>Normal autopsy</td>
<td><em>SCN5A</em></td>
<td>Gly615Glu</td>
<td>25</td>
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<tr>
<td>5</td>
<td>Non-specified placental lesion</td>
<td><em>SCN5A</em></td>
<td>Arg481Trp</td>
<td>135</td>
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<tr>
<td>61</td>
<td>Normal autopsy</td>
<td><em>KCNE2</em></td>
<td>Thr8Ala</td>
<td>461</td>
</tr>
</tbody>
</table>

|       |                       | *KCNE1* | Asp85Asn | 1110 |

Long QT: inherited long QT gene; ExAC count = Allele count in Exome Consortium Aggregate Data
Table 3: Burden test results for channelopathy genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Cases (N = 70)</th>
<th>Controls* (N = 563)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKAP9</td>
<td>4.29 (3)</td>
<td>3.20 (18)</td>
<td>0.720</td>
</tr>
<tr>
<td>ANK2</td>
<td>2.86 (2)</td>
<td>3.55 (20)</td>
<td>1.000</td>
</tr>
<tr>
<td>BAZ2B</td>
<td>2.86 (2)</td>
<td>2.31 (13)</td>
<td>0.680</td>
</tr>
<tr>
<td>CACNA1C</td>
<td>2.86 (2)</td>
<td>1.60 (9)</td>
<td>0.354</td>
</tr>
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<td>CREBBP</td>
<td>7.14 (5)</td>
<td>2.84 (16)</td>
<td>0.074</td>
</tr>
<tr>
<td>KCNJ2</td>
<td>1.43 (1)</td>
<td>-</td>
<td>0.112</td>
</tr>
<tr>
<td>NDRG4</td>
<td>1.43 (1)</td>
<td>0.71 (4)</td>
<td>0.449</td>
</tr>
<tr>
<td>RYR2</td>
<td>4.29 (3)</td>
<td>4.62 (26)</td>
<td>1.000</td>
</tr>
<tr>
<td>SCN10A</td>
<td>4.29 (3)</td>
<td>2.31 (13)</td>
<td>0.408</td>
</tr>
<tr>
<td>SCN5A</td>
<td>2.86 (2)</td>
<td>0.89 (5)</td>
<td>0.179</td>
</tr>
<tr>
<td>SMARCA1D1</td>
<td>1.43 (1)</td>
<td>0.18 (1)</td>
<td>0.212</td>
</tr>
<tr>
<td>SRL</td>
<td>1.43 (1)</td>
<td>1.07 (6)</td>
<td>0.566</td>
</tr>
<tr>
<td>TRPM7</td>
<td>5.71 (4)</td>
<td>1.60 (9)</td>
<td>0.047</td>
</tr>
</tbody>
</table>

*The controls were randomly selected from the 1000G dataset to match the self-reported ethnicity of the stillbirth cases (methods); % cases or controls is provided in parentheses; the TTN gene was not included in this analysis due to the extensive number of variants.
Figures

**Figure 1.** Algorithm for prioritising variants in channelopathy genes for reporting and functional testing.

*Legend:* All variants were annotated using ANNOVAR prediction software and the non-synonymous variants were taken forward for further consideration if they were conserved (GERP score ≥2) and predicted to be damaging by at least one algorithm [Sift score >0.95, PolyPhen2 score of >0.85 or Mutation Taster (A or D)]. Rare variants in this study are defined as variants with frequency of 1% or less. Variants with association and/or functional data to support a causative role in channelopathy are reported as putative pathogenic variants. All novel and rare variants with no previous association to channelopathies are reported as variants of uncertain significance.

* 75 TTN variants are not reported or were not included in the burden testing analysis due to extensive number of variants in this gene.

**Figure 2.** The location of the R40Q variant in *KCNJ2* that encodes the Kir2.1 protein and its effect on ion channel function.

*Legend:* A. Genomic layout of *KCNJ2* exons. B. Primary structure of Kir2.1 depicting transmembrane domains flanked with N and C termini. The full-length protein contains two transmembrane domains M1 & M2 joined by a linker pore domain. C. Structural composition of Kir2.1 depicting the R40Q mutation near the N-terminus. D. Voltage protocol used to elicit currents and scale for the representative traces.
shown. E. Representative whole-cell patch clamp recordings for individual cells transiently expressing \textit{KCNJ2-WT}, T353A, R40Q or an empty vector control (pcDNA3.1). F. Current-voltage relationships obtained from cells transiently transfected with \textit{KCNJ2-WT}, R40Q T353A, or an empty vector control. Values are mean ± S.E (n=8-12). * indicates (p<0.05) when compared to \textit{KCNJ2-WT} transfected cells.