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Cardiac ion channels associated with unexplained stillbirth – an immunohistochemical study

https://doi.org/10.1515/jpm-2022-0227
Received May 10, 2022; accepted May 30, 2022;
published online June 22, 2022

Abstract

Objectives: Despite the use of post-mortem investigations, approximately 20% of stillbirths remain unexplained. Cardiac ion channelopathies have been identified as a cause of death in Sudden Infant Death Syndrome (SIDS) and could be associated with unexplained stillbirths. This study aimed to understand if the expression or localisation of cardiac ion channels associated with channelopathies were altered in cases of unexplained stillbirths.

Methods: A case control study was conducted using formalin-fixed cardiac tissue from 20 cases of unexplained stillbirth and a control group of 20 cases of stillbirths from intrapartum hypoxia. 4 μm tissue sections were stained using haematoxylin and eosin, Masson's trichrome (MT) and Elastic van Gieson (EVG). Immunohistochemistry (IHC) was performed using antibodies against CACNA1G, KCNJ2, KCNQ1, KCNH2 and KCNE1. The cardiac conduction system in samples stained with MT and EVG could not be identified. Therefore, the levels of immunoperoxidase staining were quantified using QuPath software.

Results: The nuclear-cytoplasmic ratio of sections stained with haematoxylin and eosin was higher for the hypoxia group (hypoxia median 0.13 vs. 0.04 unexplained, $p < 0.001$). CACNA1G (unexplained median 0.26 vs. hypoxia 0.30, p=0.009) and KCNJ2 (unexplained median 0.35 vs. hypoxia 0.41, p=0.001) had lower staining intensity in the unexplained stillbirth group. There were no statistically significant differences in the staining intensity of KCNQ1, KCNH2 and KCNE1.

Conclusions: Two ion channels associated with channelopathies demonstrated lower levels of expression in cases of unexplained stillbirth. Further genetic studies using human tissue should be performed to understand the association between channelopathies and otherwise unexplained stillbirths.

Keywords: cardiac ion channels; channelopathies; immunohistochemistry; unexplained stillbirth.

Introduction

Examinations performed after a stillbirth are important as they can provide parents with an understanding about why their baby or babies died; this information can be used for the management of future pregnancies and for research on stillbirth prevention [1]. The three fields of investigations that yield the most useful information when managing stillbirth include: autopsy, which provides additional information in 24.7% to 84.5% of cases; placental histology which provides additional information in 69.5% to 95.7% of cases and fetal chromosomal analysis which provides additional information in 11.7% to 29.0% of cases [2]. These examinations can provide a cause of death in 40.1% of cases and information for the management of the next pregnancy in 51% of cases [3].

Systems to classify stillbirths are crucial to categorise the different causes that can lead to these deaths in order for measures to be instituted for their prevention in future pregnancies. To date, the Wigglesworth Classification System has been the most commonly used system, but this records high levels of unexplained stillbirth [4]. More modern classification systems such as the relevant condition at death (ReCoDe) and cause of death and associated conditions (CoDAC) systems take into account the key factors that contributed to the cause of death and allow for a more diverse categorisation, which reduces the proportion

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of unexplained stillbirth [5, 6]. However, despite the use of objective hierarchical classification in conjunction with post-mortem examination approximately 20% of stillbirths are classified as unexplained and half of these cases have no clinical, fetal or placental lesions associated, thus being entirely unexplained [7].

The lack of an explanation for fetal death despite thorough investigation for a significant proportion of stillbirths has led to exploration of novel factors. One such avenue of exploration is cardiac channelopathies with the hypothesis that these diseases could be associated with previously unexplained stillbirth cases [8]. One argument for exploring cardiac ion channelopathies in unexplained stillbirth is their presence in cases of Sudden Infant Death Syndrome (SIDS) - the sudden death of a child before 1 year of age that despite clinical and pathological investigations remains unexplained [9]. SIDS is often caused by genetic alterations that can be inherited and lead to arrhythmic syndromes that can manifest with or without structural heart alterations [10, 11]; these diseases linked to genetic alterations of cardiac ion channels are known as channelopathies and have been found in 30% of autopsies of individuals of up to 15 years of age [12]. Channelopathies such as Brugada syndrome (BrS), short QT syndrome (SQTS), long short QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT) are associated with SIDS and caused by pathogenic gene alterations that encode ion channels or associated proteins (see Figure 1) [13]. Similar channelopathies have been detected in stillbirths, though to date their identification is limited to molecular studies, as they do not cause morphological changes to cardiac anatomy [14, 15].

Further work is needed to understand the association between channelopathies and unexplained stillbirth cases.

Brs	LQTS	SQTS	CPVT
	KCNE1		
	KCNE2		
SCN5A	SCN5A		
KCNH2	KCNH2	KCNH2	
CACNA1C	CACNA1C	CACNA1C	
	KCNQ1	KCNQ1	
	KCNJ2	KCNJ2	KCNJ2

Figure 1: Genes associated with Brugada syndrome (BrS), short QT syndrome (SQTS), long QT syndrome (LQTS) and catecholaminergic polymorphic ventricular tachycardia (CPVT).
Adapted from: Fernández-Falgueras et al. 2017 [12].

If a robust association is demonstrated, genetic testing could be offered to families affected by unexplained stillbirth to identify their genetic predisposition and risk of recurrence of stillbirth associated with cardiomyopathy-related genes [8]. This study addressed the hypothesis that defects in the cardiac conduction system resulting from abnormal cardiac ion channel expression could be associated with unexplained stillbirth in humans. The presence of cardiac ion channels was studied in cardiac tissue obtained at post-mortem examination and processed into paraffin blocks from cases of unexplained stillbirths and cardiac tissue of intrapartum stillbirths caused by hypoxia.

Materials and methods

Participants whose tissue samples were used in this study had previously given consent for their use in research studies during the consent process for a post mortem (PM) examination of their stillborn child. Approval for the study was given by the Health Research Authority (East Midlands–Derby Research Ethics Committee, 17/EM/0362).

Fetal cardiac tissue was obtained from two groups of stillborn infants. Firstly, those whose mode of death was intrapartum hypoxia (as a control group whose cause of death was a known hypoxic insult). Samples were included in this group if the fetus was of 24 weeks' gestational age or greater, had a PM report detailing events of intrapartum hypoxia that led to fetal demise and absence of significant findings and a placental histology examination report and maternal clinical history that could provide a cause of death other than intrapartum hypoxia. Secondly, samples were obtained from stillborn infants whose death was unexplained. Inclusion criteria for this group were: 24 weeks' gestational age or greater, PM report concluding an unexplained cause of death and absence of significant findings in the PM report, placental histology examination report and maternal clinical history that could provide a cause of death. In both groups participants were included if their cardiac tissue was sampled and blocks had sufficient tissue to be used for research purposes.

Equally-sized groups of unexplained stillbirth cases (n=20) and cases of stillbirth due to intrapartum hypoxia (n=20) were obtained. A power calculation was not performed as there was insufficient prior data upon which to base the calculation. Formalin-fixed paraffin-embedded (FFPE) tissue blocks containing cardiac tissue from the cases were retrieved and 4 µm sections obtained for histochemical and immunohistochemical staining. Haematoxylin and Eosin (HE) staining was performed for assessment of cardiac tissue morphology using a Leica Autostainer XL (Leica Biosystems, Milton Keynes, United Kingdom). Elastic van Gieson (EVG) (VWR, Lutterworth, United Kingdom) and Masson's Trichrome (MT) (Atom, Hyde, United Kingdom) were performed manually according to standard histochemical protocols to identify the cardiac conduction system.

Immunoperoxidase staining was performed using antibodies directed against CACNA1G (Proteintech Rosemont – IL, USA), KCNJ2 (Abcam, Cambridge, United Kingdom), KCNQ1 (Atlas Antibodies, Stockholm, Sweden), KCNH2 (Invitrogen, Waltham, MA, USA), KCNE1 (Invitrogen, Waltham – MA, USA) and SCN54 (Abcam, Cambridge, United Kingdom) for assessment of expression of proteins linked to

cardiac ion channel abnormalities and channelopathies. The immunohistochemical (IHC) protocols and optimisation of antibodies were performed using the automated Ventana BenchMark ULTRA (Roche, West Sussex, United Kingdom). The final concentration used for each antibody was achieved using Dako Antibody Diluent (Dako Agilent, Santa Clara, CA, USA). The IHC protocols and concentrations of antibodies used for this research are detailed in Table 1. All samples were stained in the same run. Negative controls were achieved via omission of primary antibody and substitution with matched concentrations of non-specific immunoglobulin from the same host species as the primary antibody. All slides were imaged using a Panoramic 250 Flash III Slide Scanner (3D Histech, Budapest, Hungary), which outputted the files as.MRXS files, with a maximum width and height of 9,000 × 130,000 pixels and each pixel corresponding to 0.2428 μm^2.

The HE scans were analysed using QuPath v0.2.3, loaded onto the software with the image type set to Brightfield HE and labelled with their image name to prevent bias selection. Each scan was visualized at 0.2x magnification to prevent selection bias of more cellular areas and 7 random annotation areas of 1.157×10^6 μm^2 marked and merged for analysis in each scan. Using the pixel classification option, two thresholds were created to identify and measure the total nuclear area (Supplementary Figure A) and the total tissue area (Supplementary Figure B) of the selected annotations in each scan. A script containing the 2 thresholders was created and run for the HE slides and the obtained measurements downloaded and saved as a separate.excel file. The cytoplasm area of each scan was calculated by subtracting the total nuclear area by the stromal area. The nuclear/cytoplasm ratio of each scan was calculated by dividing the total nuclear area by the cytoplasm area.

The IHC scans were analysed using QuPath v0.2.3, loaded onto the software with the image type set to Brightfield H-DAB and labelled with their image name to prevent bias selection. Each scan was visualized at 0.2x magnification to prevent selection bias of more cellular areas and 7 random annotation areas of 1.157×10^6 μm^2 marked and merged for analysis in each scan. Using the cell detection option, the software calculated the mean intensity of DAB in the selected annotations (Supplementary Figure C). A script was created to calculate the mean DAB intensity for the annotations of each scan and the obtained measurements were downloaded and saved as separate Microsoft Excel files.

The MT and EVG slides were microscopically examined by a Consultant Histopathologist for identification of the cardiac conduction system on the samples stained for this study. The optimisation of KCNE1 revealed staining of specific cells in the cardiac tissue, which when microscopically evaluated by a Consultant Histopathologist were hypothesised to be fibroblasts. In order to evaluate if the stained cells were fibroblasts, stained slides for SMA (Dako Agilent, Santa Clara, CA, USA) and Vimentin (Dako Agilent, Santa Clara, CA, USA) from control tissue were evaluated by a Consultant Histopathologist alongside the control tissue stained slide with KCNE1 and the positive areas seen on all tissue sections were identified to most likely represent fibroblasts.

Data were collected from the two groups compiled for this study: Intrapartum Hypoxia stillbirth group (n=20) and Unexplained stillbirth group (n=20). The statistical information used for the examination of the HE scans was the mean nuclear/cytoplasm ratio of each sample, with a total of 40 data points. The statistical information used for the examination of the IHC scans was the mean DAB intensity for the antibodies CANA1G, KCNJ2, KCNQ1, KCNH2 and KCNE1 of each sample, resulting in 40 data points per antibody (total IHC data points=200). Positive and negative control sections were microscopically visualized to ensure appropriate quality control procedures were met for each of the IHC runs. Statistical analysis was performed using GraphPad Prism software (Version 7.04, Graphpad, La Jolla, CA, USA). Data distribution was tested using the Shapiro-Wilk test and was found to be non-normally distributed. Therefore, Mann-Whitney U test was used was used to compare groups. A p-value of <0.05 was considered to be statistically significant.

Table 1: Immunohistochemistry protocols utilised in the project.

Antibody, AB	Protocol	AB concentration
CACNA1G	CC1 64' 97 °C AB incubation 32' Haematoxylin 24' Bluing 20'	8 µg/mL
KCNJ2	CC2 44' 97 °C AB incubation 1H 44' Amplification step Haematoxylin 24' Bluing 20'	4 µL/mL
KCNQ1	CC2 80' 100 °C AB incubation 2H Amplification step Haematoxylin 24' Bluing 20'	1.6 µg/mL
KCNH2	CC2 8' 90 °C Protease 4' AB incubation 2H Amplification step Haematoxylin 24' Bluing 20'	20 µg/mL
KCNE1	CC2 68' 100 °C AB incubation 32' Haematoxylin 24' Bluing 20'	5 µg/mL

Results

Demographic characteristics for both sample groups are shown in Table 2. Maternal body mass index (BMI) was not available for 11 cases in the hypoxia stillbirth group and 7 cases of the unexplained stillbirth group. Otherwise, the demographic data collected for the two groups did not vary significantly.

The mean nuclear/cytoplasm ratio of samples from the hypoxia group was higher than for the unexplained group ($p < 0.00001$, Figure 2C). As this could reflect myocardial apoptosis a potential relationship between the period between death and PM examination was explored. There did not appear to be a correlation between nuclear/cytoplasm ratio and the interval between death and post-mortem examination in either group (Figure 2D). The MT and EVG slides stained for the study were microscopically evaluated by a Consultant Histopathologist. The identification of specific cellular features that could represent the cardiac conduction system was not successful, thus ion channel

Table 2: Demographic characteristics of samples from hypoxia stillbirth group and unexplained stillbirth groups.

	Hypoxia group (n=20)	Unexplained group (n=20)
Maternal BMI	30 (24–38)	25 (20–32)
Gravidity	2 (1–8)	2 (1–4)
Parity	1 (0–4)	0 (0–1)
Fetal sex	Female=13 (65%) Male=7 (35%)	Female=6 (30%) Male=14 (70%)
Gestational age, weeks	38 (31–41)	38 (28–41)
Birthweight, kg	3.02 (1.20–4.42)	2.84 (0.87–3.96)
Heart weight, g	18.20 (7.15–29.58)	15.81 (4.13–22.70)
Death to PM, days	7 (1–15)	8 (3–15)

Maternal body mass index (BMI), gravidity, parity, gestational age at delivery, birthweight, heart weight and period from death to post mortem (PM) is presented as mean and range within parenthesis. The fetal sex data are presented as a percentage of each sex for each group.

expression in myocardium, rather than the conduction system was explored.

CACNA1G demonstrated a cytoplasmic pattern of staining in cardiac tissue (Figure 3A–C). Analysis of the intensity of immunoperoxidase staining for CACNA1G showed a statistically significant difference between the two groups (p=0.0088) with lower intensity seen in the unexplained group.

KCNJ2 is evident in cardiac tissue in a cytoplasmic pattern of staining, with some cytoplasmic granular areas (Figure 3D–F). The analysis of the KCNJ2 scans demonstrated a significant statistical difference in the mean DAB intensity between the hypoxia and Unexplained groups (p=0.00142), again with lower stain intensity for the hypoxia group.

KCNQ1 shows a slightly granular cytoplasmic pattern of staining in cardiac tissue (Figure 4A–C). No significant statistical differences were found for the intensity of

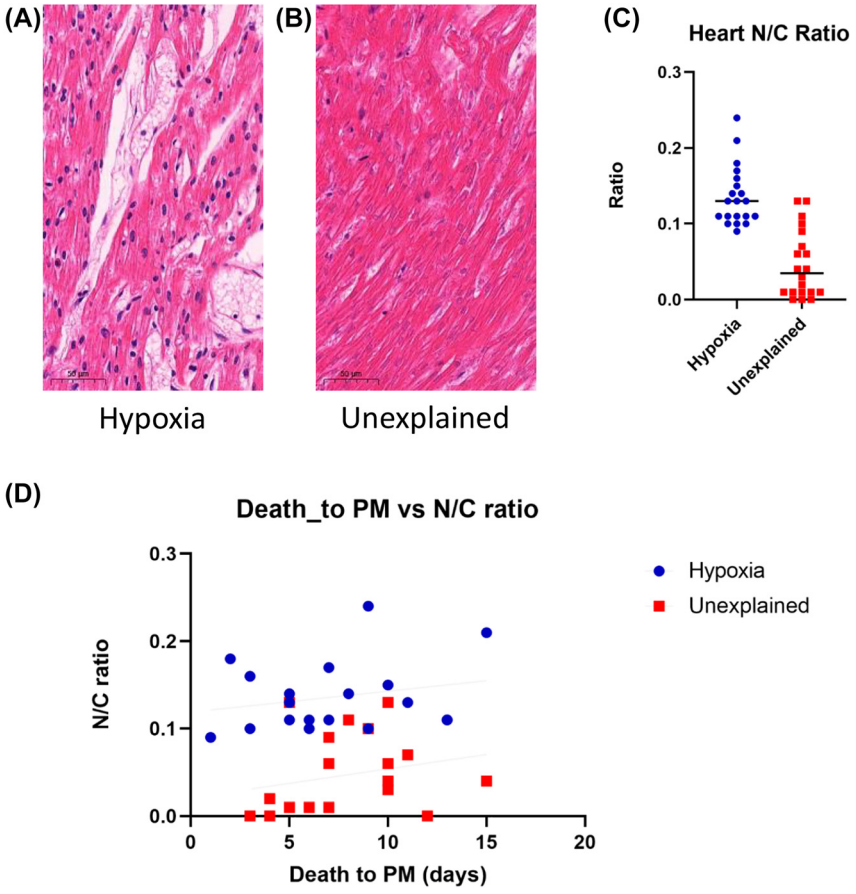

Figure 2: Haematoxylin and eosin staining for hypoxia stillbirth group (A) and unexplained stillbirth group samples (B). (C) The distribution of mean nuclear/cytoplasm ratio in sample groups analysed for the study. The black line represents the median of each group. Scatter plot (D) of period of death to PM and N/C ratio for hypoxia and unexplained groups. The graph demonstrates no correlation between the period of death to PM examination and the nuclear/cytoplasm ratio for both groups.

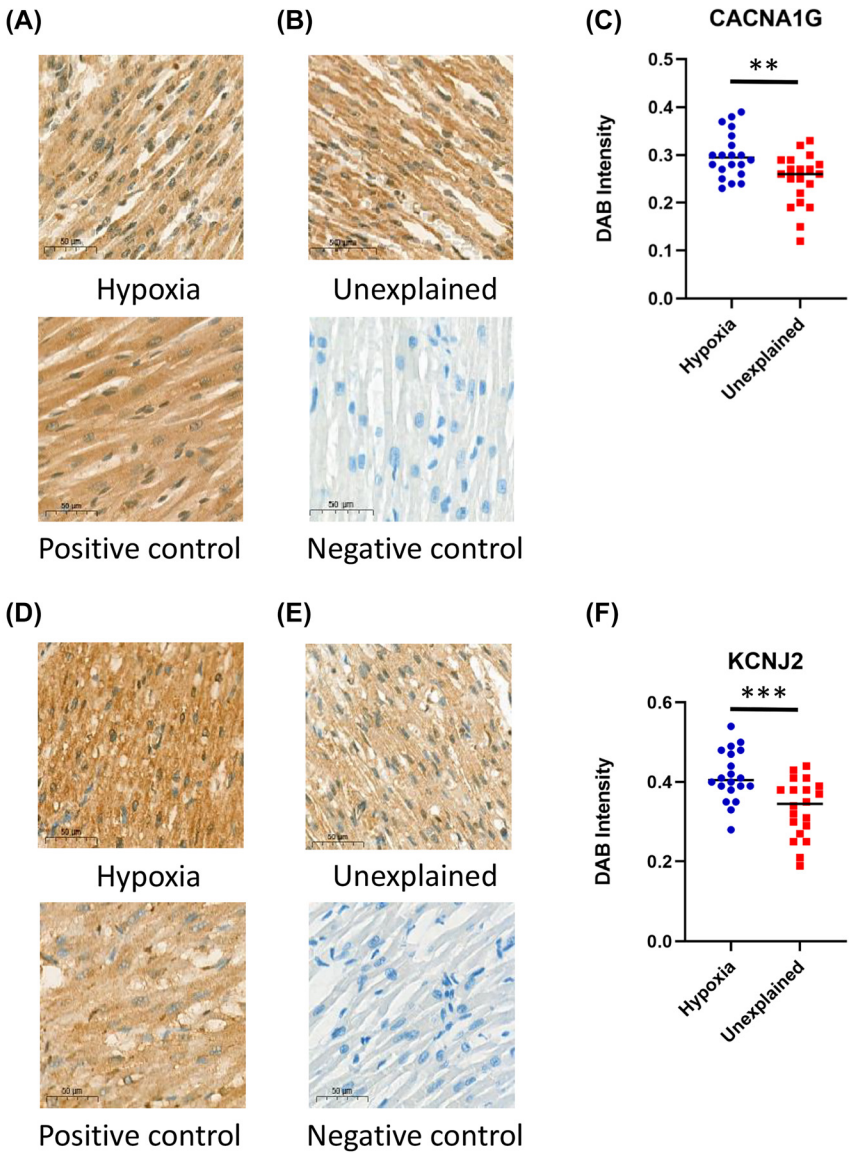

Figure 3: CACNA1G stains for hypoxia stillbirth group (A) and unexplained stillbirth group (B) samples with positive and negative controls. The graph (C) shows the distribution of the samples by group and mean DAB intensity of each scanned image analysed for the study. KCNJ2 scans for hypoxia (D) and unexplained stillbirth (E) groups with positive and negative controls stained with IHC runs for this antibody. The graph (F) demonstrates the distribution of the samples of both groups according to their mean DAB intensity. Black lines represent the median DAB intensity for each group.

staining of KCNQ1 between the study groups (p=0.14). The staining pattern for KCNH2 is cytoplasmic in cardiac tissue (Figure 4D–F). The results for this antibody in both groups showed no significant statistical differences for their staining (p=0.55). The IHC results for the KCNE1 antibody were demonstrated with staining of specific cells of cardiac tissue, most likely fibroblasts (Figure 4G–I). There were no significant statistical differences for the staining intensity of KCNE1 between both study groups (p=0.57).

Discussion

This study demonstrates that the expression of some ion channels in the cardiac conduction system, previously associated with unexplained stillbirths, differ between cases of unexplained stillbirth and stillbirths related to intrapartum hypoxia (controls for this study). There is a paucity of data regarding cardiac morphology and the expression of ion channels in cardiac tissue from

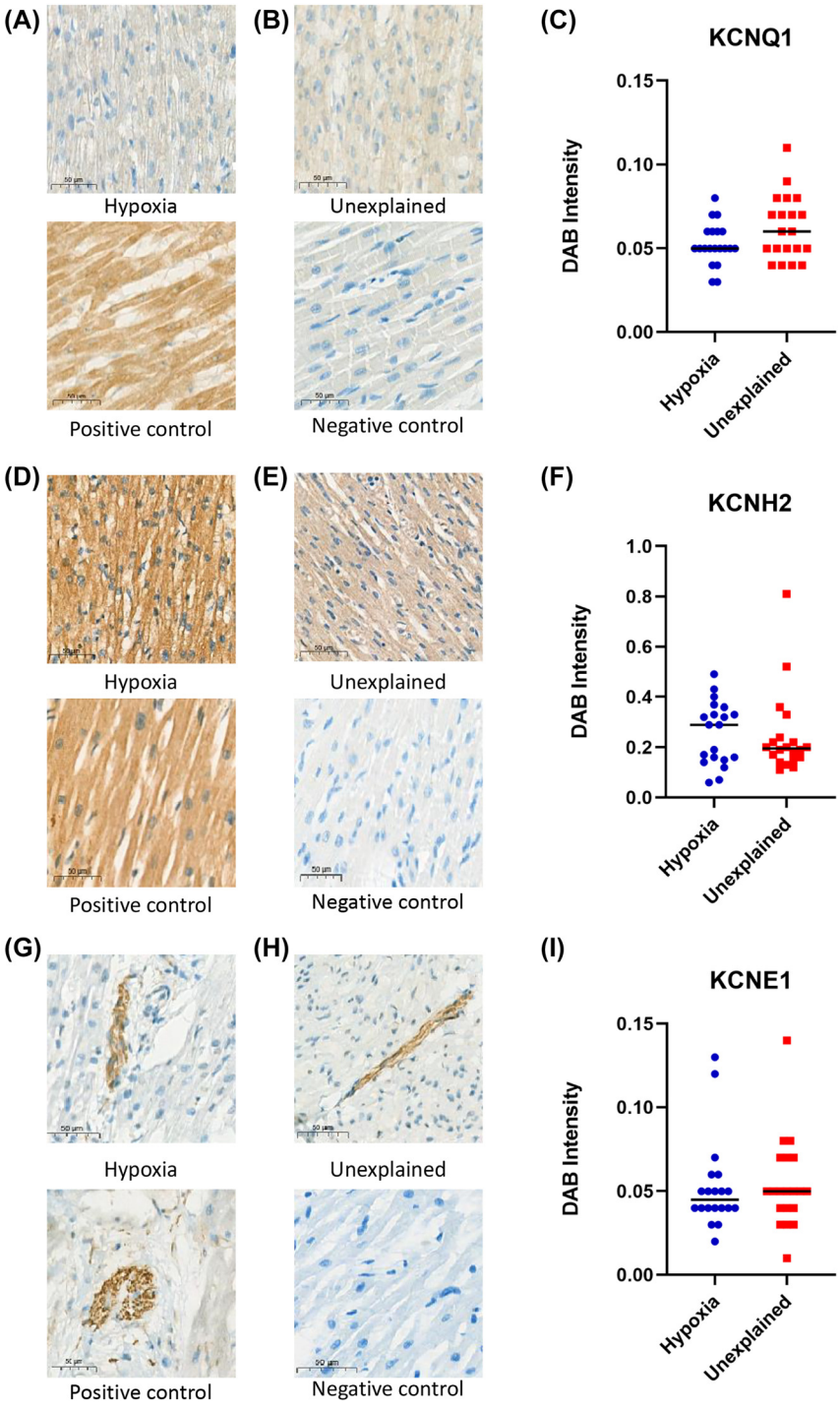

Figure 4: Representative images of KCNQ1 staining from hypoxia (A) and unexplained (B) groups, with positive and negative controls for this antibody. The mean DAB intensity for each sample can be seen in the graph (C). Representative images of KCNH2 staining for hypoxia stillbirth (D) and unexplained stillbirth (E) group, with positive and negative controls used during IHC runs for this antibody. The mean DAB intensity distribution for the stained samples is plotted in the graph (F). Representative images of KCNE1 immunostaining for hypoxia group (G) and unexplained stillbirth (H) with positive and negative controls used during IHC runs for this antibody. The graph exhibits the mean DAB intensity of the stained samples (I). Median values of each group are represented by the black lines.

Table 3: Previous retrospective studies associating channelopathy genes and unexplained stillbirth cases. Adapted from: Wilkins-Haug 2020 [16].

Publication	Retrospective unexplained stillbirth population	Genes assessed	Results
Crotti et al., 2013	91	KCNQ1, KCNBH2, SCN5A	3 pathogenic variants; 5 rare variants with functional effect
Munroe et al., 2018	70	35 channelopathy genes	Probably pathogenic on functional *in vitro* studies
Sahlin et al., 2019	290	70 channelopathy and cardiomyopathy genes	Pathogenic variants for channelopathies

stillbirths. This study is the first to use immunohistochemical methods in a retrospective unexplained stillbirth population to identify ion channels associated with channelopathies and evaluate their expression and localisation in cardiac tissue (Table 3) [16].

The higher nuclear/cytoplasm ratio seen in the hypoxia group is consistent with this cause of death, as hypoxia is thought to promote apoptosis and results in cell shrinkage, thereby increasing the nuclear to cytoplasm ratio of these samples [17]. The lack of correlation between the period of death to PM examination and the nuclear/cytoplasm ratio suggests that the increase of this ratio for the hypoxia group is not an artefact of death and the cause of death for each group does play a part in the alteration of the cell architecture. Intrapartum hypoxia can lead to myocardial necrosis, which has been demonstrated by IHC staining of the ninth component of complement (C9) in necrotic myocardium [18]. The demonstration of C9 using IHC has been performed in stillbirth samples and can be used to further understand histological features of cardiac ischaemia in these cases [19].

The intensity of IHC staining for CACNA1G and KCNJ2 was reduced in samples from unexplained stillbirth, which may indicate that proteins encoded by these genes are less effectively expressed in this group. This finding supports the hypothesis proposed in this study that CACNA1G and KCNJ2 could be implicated in a possible channelopathy related cause of death. These genes have been implicated as causative of channelopathies such as SQTS, LQTS and BrS [20] and have previously been identified through genetic testing in stillbirth cases with no definite cause of death [21].

However, there was no difference in staining intensity for the antibodies KCNQ1, KCNH2 and KCNE1 in unexplained stillbirth. Variants of these genes have also been identified in other genetic studies performed with stillbirth cases [21, 22]. The results for this research concerning these genes could be linked to the antibody chosen for this methodology and the limitations of optimising antibodies using one specific immunohistochemical staining platform available. IHC staining platforms use different reagents and variations of this technique influence the ability to successfully optimise antibodies for research and routine clinical use. As there are no comparable data regarding the expected staining pattern of these antibodies, these results should be interpreted with caution. It is possible that KCNQ1, KCNH2 and KCNE1 could be altered only in the cardiac conduction system, which this study was not able to identify with certainty in the selected samples. Further studies are needed as this study confirms their expression in myocardium and genetic testing studies have associated their genes to channelopathies [23]. Therefore, prospective studies, where the cardiac conduction system is purposively sampled may be required to explore whether there is altered expression of KCNQ1, KCNH2 and KCNE1 in unexplained stillbirth.

This study was limited by the use of pre-sampled tissue from PM examinations. This tissue was sampled following PM dissection guidelines, which do not specifically aim to obtain cardiac tissue with areas of conduction system. As such, this study is unable to state with all certainty if the selected samples had cardiac conduction system present on them. We were also unable to match the samples to DNA to perform linked analysis to determine whether channelopathies were present in these samples.

Following the results obtained for CACNA1G and KCNJ2, genetic studies such as sequencing samples from both groups should allow the demonstration of the differences in genetic expression that lead to the differences in protein expression identified using immunohistochemistry. These would further ascertain whether there could be defects resulting in altered expression of these genes and their associated channelopathies as possible causes of death for the unexplained stillbirth cases. Genetic studies associating the pathogenicity of gene variants to certain channelopathies continue to help our understanding of this disease in the general population [24]. A similar study conducted using 58 samples of FFPE myocardial tissue and 9 control blood samples from sudden unexplained infant death cases identified 31 variants of the SCN5A gene, of which 5 had been previously reported has pathogenic, 12 identified variants were considered novel and 2 of the novel variants were predicted as “probably damaging” [25]. Given the numerous

variants identified, the pathogenicity of each sequencing variation should be established against strict criteria such as the guidelines provided by the American College of Medical Genetics [26].

Establishing an association between specific genes and channelopathies that can be diagnosed with tests such as electrocardiograms would allow the identification of fetuses and future children at risk of SIDS [27]. Thus, there is a need for more genetic, epidemiologic and PM investigations to be performed in order to help explain the association between channelopathies and SIDS or unexplained stillbirth [28]. Given the "Triple Risk Hypothesis" model for SIDS, which has been adapted for late stillbirth (which implicates cardiac arrhythmias as a causative element), a more exhaustive PM examination investigating the cardiac conduction system should be performed for otherwise unexplained stillbirth cases [29]. The information gained from such studies could then be used to direct further research for unexplained stillbirth cases.

Acknowledgments: The authors wish to acknowledge the contribution of parents who at the time of their bereavement gave consent for the use of samples for research purposes.
Research funding: This project received financial support via a donation from Grace Jorgensen and Walter Grattidge. AEPH receives salary support from Tommy's Charity, UK.
Author contributions: AEPH, SQB and GB were responsible for the project design. GB, GP and SQB identified samples for analysis. AK, CB, AH and SB undertook immuohistochemical analysis. SQB and AEPH undertook the statistical analysis. All authors contributed to the writing and review of the manuscript and approved its submission.
Competing interests: Authors state no conflict of interest.
Informed consent: Informed written consent for use of samples for research was given at the time of consent for post-mortem examination.
Ethical approval: Ethical approval was given by the East Midlands–Derby Research Ethics Committee (Ref 17/EM/0362).

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Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/jpm-2022-0227).