Recurrent Pregnancy Loss and Concealed Long-QT Syndrome

Laura Kasak, PhD; Kristiina Rull, MD, PhD; Tao Yang, MD, PhD; Dan M. Roden, MD, PhD; Maris Laan, PhD

BACKGROUND: Recurrent pregnancy loss affects 1% to 2% of couples attempting childbirth. A large fraction of all cases remains idiopathic, which warrants research into monogenic causes of this distressing disorder.

METHODS AND RESULTS: We investigated a nonconsanguineous Estonian family who had experienced 5 live births, intersected by 3 early pregnancy losses, and 6 fetal deaths, 3 of which occurred during the second trimester. No fetal malformations were described at the autopsies performed in 3 of 6 cases of fetal death. Parental and fetal chromosomal abnormalities (including submicroscopic) and maternal risk factors were excluded. Material for genetic testing was available from 4 miscarried cases (gestational weeks 11, 14, 17, and 18). Exome sequencing in 3 pregnancy losses and the mother identified no rare variants explicitly shared by the miscarried conceptuses. However, the mother and 2 pregnancy losses carried a heterozygous non-synonymous variant, resulting in p.Val173Asp (rs199472695) in the ion channel gene KCNQ1. It is expressed not only in heart, where mutations cause type 1 long-QT syndrome, but also in other tissues, including uterus. The p.Val173Asp variant has been previously identified in a patient with type 1 long-QT syndrome, but not reported in the Genome Aggregation Database. With heterologous expression in CHO cells, our in vitro electrophysiologic studies indicated that the mutant slowly activating voltage-gated K+ channel (IKs) is dysfunctional. It showed reduced total activating and deactivating currents (P<0.01), with dramatically positive shift of voltage dependence of activation by ≈10 mV (P<0.05).

CONCLUSIONS: The current study uncovered concealed maternal type 1 long-QT syndrome as a potential novel cause behind recurrent fetal loss.

Key Words: exome • KCNQ1 • long-QT syndrome • miscarriage • recurrent pregnancy loss

Recurrent pregnancy loss (RPL) is a disease defined by the spontaneous demise of ≥2 pregnancies, affecting ≈1% to 2% of women.1,2 RPL has a long list of different causes, as both maternal and fetal, as well as combined factors, may be responsible. As a major known cause, up to 50% of products of conception of patients with RPL have gross genomic rearrangements.3 However, it is estimated that roughly half of RPL cases remain truly idiopathic. Because the familial history of miscarriage increases the risk to RPL ≈2-fold,4 it is likely that some of these cases have unknown monogenic origins.3,5 In addition to pregnancy loss being an obvious negative life event, there are emerging data that these women are at an increased risk of various health problems later in life. Reported comorbidities include type 2 diabetes mellitus,6 autoimmune and cardiovascular complications, such as atherosclerosis, cerebral infarction, heart failure, and pulmonary embolism, as well as psychiatric diseases.7-9 These observations make it especially important to identify the underlying genetic factors with pleiotropic effects that may predispose to pregnancy failure as well as to the long-term health of the woman. Until now, monogenic causes of RPL remain largely unexplored, and only a handful of studies using exome sequencing have been published.10

The current study aimed to clarify the potential genetic cause of idiopathic RPL in an Estonian couple
who had experienced in total 9 pregnancy losses, 3 early miscarriages and 6 fetal deaths after 10 weeks’ gestation.

METHODS

The authors declare that all supporting data are available within the article and its online Supplemental Material.

Ethical Approval

The study was approved by the Ethics Review Committee of Human Research of the University of Tartu, Tartu, Estonia (permission Nos. 117/9, 16.06.2003; 146/18, 27.02.2006; 150/33, 19.06.2006; 212/M-32, 09.03.2012; and 286/M-18, 15.10.2018) and was performed in compliance with the Declaration of Helsinki. A written informed consent to participate in the study was acquired from all adult research participants before recruitment.

Exome Sequencing and Variant Detection

DNA samples from the proband’s blood and 3 miscarriage events (III-5, III-9, and III-13; Figure 1A) were subjected to whole-exome sequencing (WES). The WES data generation and data analysis were performed as described in the study by Kasak et al.\textsuperscript{11} Briefly, wet-laboratory processing, base calling of the raw sequencing data, primary sequence analysis, and variant calling were performed at FIMM (Institute for Molecular Medicine Finland) Next Generation Sequencing Service (Helsinki, Finland). Whole exome enrichment was undertaken with the SeqCap EZ MedExome Target Enrichment Kit (Roche NimbleGen, Madison, WI) following the manufacturer’s protocol. Sequencing was performed on Illumina HiSeq 2500 sequencing system (San Diego, CA). Primary sequence analysis and variant calling were performed using the Variant Calling Pipeline (VCP3.7).\textsuperscript{12} Illumina paired-end reads were trimmed with Trimmomatic (version 0.36) and aligned against human genome build hg19 with the Burrows-Wheeler Aligner (version 0.6.2).\textsuperscript{13} Next, polymerase chain reaction duplicates were removed with Picard MarkDuplicates (version 2.9.0). Single-nucleotide variants and insertions/deletions were called with SAMtools (version 1.4)\textsuperscript{14} and Pindel (version 0.2.5b8),\textsuperscript{15} respectively. Mean target coverage ranged from 91× to 122×, with an average of 97% of bases covered at 20×.

Exome Data Filtering and Prioritization of Variants

Variant call format (VCF) files of the pregnancy loss samples were annotated using ANNOVAR (version 20191024).\textsuperscript{16} Among all coding variants (exonic and splicing), synonymous and common variants with minor allele frequency of >0.1% in Genome Aggregation Database\textsuperscript{17} and Exome Aggregation Consortium\textsuperscript{18} were removed (Figure S1). Only shared variants in all pregnancy loss samples were retained for the analysis.

To prioritize variants from the WES data of the proband, the population sampling probability (PSAP) pipeline\textsuperscript{19} developed for n-of-1 analyses was applied. It is a model-based framework to evaluate the significance of genotypes ascertained from a single case by determining the by-chance probability of sampling the detected genotypes in the unaffected population based on the pathogenicity scores and observed frequencies of variants. The variants were prioritized to

Nonstandard Abbreviations and Acronyms

| Acronym | Description |
|---------|-------------|
| LQTS1   | type 1 long-QT syndrome |
| PSAP    | population sampling probability |
| QTcB    | the heart-rate corrected QT interval based on the Bazett’s formula |
| QTc     | QT interval corrected for the heart rate |
| RPL     | recurrent pregnancy loss |
| WES     | whole-exome sequencing |

CLINICAL PERSPECTIVE

What Is New?

- The current study uncovered concealed maternal type 1 long-QT syndrome as a potential novel cause behind recurrent pregnancy loss.
- In vitro electrophysiological studies showed that the KCNQ1 p.V173D (c.518T>A) variant causes dysfunction and marked attenuation of the I_{Ks} channel.

What Are the Clinical Implications?

- This novel finding emphasizes the value of personalized medicine and the importance of in-depth assessment of all potentially causative genotype-to-phenotype links.
- Genotype-positive, phenotype-negative subjects should avoid exposure to any exogenous risk factors, such as competitive sports or QT-prolonging drugs.
- Early detection of KCNQ1 pathogenic variant carriers by testing among family members facilitates timely monitoring, treatment, and prevention of cardiac arrhythmias; clinical assessment of unexplained recurrent fetal losses should consider concealed maternal and/or fetal long-QT syndrome as a possible cause.

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satisfy the following criteria: (1) missense and loss-of-function variants; (2) low minor allele frequency (≤0.001 Exome Aggregation Consortium, Genome Aggregation Database, and 1000 Genomes Project) or a previously undescribed variant; and (3) the PSAP statistical significance value ≤0.005 and the Combined Annotation-Dependent Depletion score ≥20. PSAP pipeline was also applied for the extended assessment of individual fetal exomes.

Next, for both parts of the analysis (pregnancy loss samples and the proband), in-house blacklisted variants were removed. Visual inspection of the quality of sequencing reads was performed using the Integrative Genomics Viewer software for all prioritized rare and novel variants to ensure high quality of retained variants. All the retained variants were manually inspected using scientific literature and genome databases. Variants were classified according to the American College of Medical Genetics and Genomics guidelines.

Figure 1. Identification of the KCNQ1 variant.
A, Pedigree of the family. Circles denote female family members, squares denote male members, and triangles indicate spontaneous pregnancy losses. The proband is indicated with an arrow. Solid symbols indicate pedigree members, whose genomic DNA was subjected to exome sequencing. B, Sanger sequencing confirmation of the KCNQ1 p.V173D variant (rs199472695) in the family. C, Molecular position of the K\text{V}7.1 variant p.V173D in the first C-loop. D, The conservation of the amino acid residue affected by the KCNQ1 variant in different species. d, death; ECT indicates ectopic pregnancy; trim, trimester; wks, gestational weeks; WT, wild type and yrs, years.

KCNQ1 p.V173D Variant Validation

Primers (forward, TGCTATGGACATGAGCTGA; reverse, GGGAATCTGTGAGGGACCAA; sequencing, GCATGGCTGGGTTCAAACA) for amplification and sequencing of the KCNQ1 p.V173D variant (rs199472695) were designed in Primer3web (https://bioinfo.ut.ee/primer3/), tested by National Center for Biotechnology Information Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and GenomeTester (https://bioinfo.ut.ee/genometester/). DNA fragments (individuals I-2, II-1, II-2, II-3, III-1, III-2, III-3, III-5, III-9, III-10, III-11, III-12, III-13, III-14, and III-16) were amplified by standard polymerase chain reaction, sequenced with the BigDye Terminator v.3.1 Cycle Sequencing Kit, and run on an ABI 3730 DNA Analyzer (both Applied Biosystems, Carlsbad, CA). Sequences were analyzed with 4Peaks (https://nucleobies.com/4peaks/index.html).
**Functional Assessment of the KCNQ1 p.V173D Variant**

**Site-Directed Mutagenesis**

The p.V173D variant was performed on a plasmid containing a 710-bp region (encoding amino acids 1–237) of KCNQ1 with the QuikChange Lightning Multi-site kit (Agilent) using the primer 5′GCAGGTTTGCATGTCGCCCATCAAGG3′. The mutated region of KCNQ1 was subcloned using restriction enzymes Clal and Bsu36I into an expression vector (pIRES2-EGFP) containing full-length KCNQ1:internal ribosome entry site (IRES);green fluorescent protein (GFP), and the wild-type and mutant sequences were confirmed. Plasmids were transfected into CHO cells using Fugene 6 (Promega) following manufacturer’s instructions. Wild-type or p.V173D pIRES2-GFP KCNQ1 plasmids were cotransfected in equimolar ratios with a pIRES2-dsRed expression plasmid expressing wild-type KCNE1 (the I<sub>Ks</sub> accessory subunit). Two days after transfection, cells expressing both KCNQ1 in green and KCNE1 in red were selected with fluorescent lights for electrophysiologic functional studies.

**Electrophysiologic Functional Studies**

Whole-cell voltage clamp experiments were performed at room temperature (22 °C–23 °C) using a patch-clamp system: MultiClamp 700B amplifier, 1350 DigiData, and data acquisition software pClamp 10.7 (Molecular Devices Inc, Sunnydale, CA). Patch glass microelectrodes with 3 to 5 MΩ were used to patch cells. The pipette (intracellular) solution contained (in mmol/L): NaCl 145, KCl 4.0, MgCl<sub>2</sub> 1.0, CaCl<sub>2</sub> 1.8, glucose 10, and HEPES 10, with the pH of 7.2, adjusted with KOH. The extracellular solution contained (in mmol/L): NaCl 145, KCl 4.0, MgCl<sub>2</sub> 1.0, CaCl<sub>2</sub> 1.8, glucose 10, and HEPES 10, with the pH of 7.4, adjusted with NaOH. Data acquisition was performed using pClamp 10.7, sampling at 1 kHz, and low-pass filtered at 5 kHz. Activating current was elicited with 5-s depolarizing pulses from a holding potential of −80 to 80 mV at 20-mV increments, and deactivateing tail current was recorded on return to −40 mV. The voltage-clamp protocol is shown in Figure 2A. Pulses were delivered every 30 s. I-V relationships were analyzed by fitting the Boltzmann equation to the data: I = I<sub>max</sub>/[1 + exp[(V<sub>t</sub>−V<sub>1/2</sub>)/k]], where I<sub>max</sub> is the maximal current, V<sub>t</sub> is the test potential, V<sub>1/2</sub> is the membrane potential at which 50% of the channels are activated, and k is the slope factor. Current densities in picoamperes per picofarad (pA/pF) were obtained after normalization to cell surface area calculated by the Membrane Test in pClamp 10.7.

**Statistical Analysis**

Functional I<sub>Ks</sub> analysis data are expressed as mean±SEM. For comparisons of 2 groups of data, 1-way repeated-measures ANOVA incorporated in the Ultimate Software for Analysis & Graphing (OriginPro version 8.5.1; OriginLab Corp, Northampton, MA) was used to analyze the I<sub>Ks</sub> densities at individual testing membrane potentials. A P<0.05 or 0.01 is considered statistically significant.

**RESULTS**

**Clinical Case**

We investigated a nonconsanguineous Estonian family who had experienced 5 live births, intersected by 3 early pregnancy losses and 6 fetal deaths (Figure 1A and Table 1). Three miscarriages occurred in the second trimester, at 14+5, 17+0, and 18+0 gestational weeks. Detailed health evaluation of the couple for known clinical risk factors of RPL was performed after the third miscarriage (maternal and paternal age at assessment, 26 and 32 years, respectively). Both partners had a normal karyotype and negative repeated test results for genital tract infections (urogenital chlamydia, gonorrhea, ureaplasmosis, and mycoplasmosis). The proband (subject II-1, referring to second generation, first case; Figure 1A) has a normal menstrual cycle (27–29 days) and no major uterine anomalies (based on repeated ultrasonography and hysterosonogram) and endocrinological disorders (diabetes mellitus, hypothyroidism/hyperthyroidism, hyperprolactinemia, and hyperhomocysteinemia; Table S1). Maternal antiphospholipid syndrome (anticardiolipin antibodies, β-2 glycoprotein 1 antibodies, and lupus anticoagulants) and risk variants predisposing to thrombophilia, factor V Leiden (MIM: 612309, F5 p.R534Q, rs6025), and factor II prothrombin deficiency (MIM: 176930, F2 c.G20210A, rs1799963) were excluded. Autosomies, performed in 4 of 6 fetal death cases, were normal; and all these fetuses had a normal karyotype. Also, screening of pathogenic submicroscopic chromosomal variants did not result in any clinically relevant findings for either of the partners or 2 miscarriages (14–18 gestational weeks) excluded placental inflammation as a contributing factor, but signs of focal delayed placental maturation and circulatory complications were reported (Table 1). Four live-born sons of the couple have no congenital malformations (Table 2). However, their daughter (subject III-12) was diagnosed at the age of 3 years with congenital ventricular septal...
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Genetic Analysis of the Proband and 3 Miscarried Fetuses

The pedigree structure suggested a possible genetic predisposition to RPL on the maternal side of the family as early pregnancy complications had been reported also for the proband’s mother (I-1; ectopic pregnancy) and sister (II-3; miscarriage) (Figure 1A). An alternative scenario to explain the extensive series of pregnancy losses was pathogenic combinations of maternal and paternal rare recessive variants in all miscarried conceptuses. We performed WES using genomic DNA extracted from the maternal blood and 3 available miscarried products of conception (Figure 1A). No pathogenic variants were identified in any of these samples in genes previously reported in WES studies of RPL cases.10,24 In the joint assessment of the WES data sets of 3 aborted fetuses, also no shared homozygous or biallelic rare disease-causing coding or splicing variants were detected (Figure S1 and Table S2).

When considering only rare variants (minor allele frequency, <0.001) with high scores of possible functional effect (Combined Annotation-Dependent Depletion score ≥2025) and PSAP statistical significance threshold $P \leq 0.005$, most retained variants in the proband were recessive and classified on the basis of the American College of Medical Genetics and Genomics guidelines21 as variants of uncertain significance. A single likely pathogenic variant was identified: an ultrarare missense heterozygous change c.518T>A (p.V173D, rs199472695) in the KCNQ1 gene (MIM: 607542) (Figure 1B and Table S3).

Figure 2. KCNQ1 mutation p.V173D caused a loss of cardiac $I_{\text{Ks}}$ function.

A and B, Typical $I_{\text{Ks}}$ traces recorded in CHO cells in which either wild-type (WT) KCNQ1 or V173D were coexpressed with WT KCNE1. C and D, $I_{\text{Ks}}$ steady-state and tail current densities in the 2 groups of cells (n=10 each). The mutant channel p.V173D significantly reduced total $I_{\text{Ks}}$ steady-state and tail currents with a dramatic positive shift of the voltage dependence of activation, by ≈10 mV ($P \leq 0.05$). Current densities were expressed in pA/pF after normalization of current amplitude to cell capacitance. The voltage clamp protocol is shown in the insert.
In applying the same variant filtering pipeline for the individual fetal exomes, 2 of the 3 analyzed pregnancy loss cases (III-9 and III-13) also carried the KCNQ1 p.V173D substitution. No additional (likely) pathogenic variants were identified in fetal exomes (Table S4).

The KCNQ1 p.V173D variant is absent from all population-based human genetics public databases, but is in ClinVar (accession identifier: VCV000053057). It was reported in one individual diagnosed with type 1 long-QT syndrome (LQTS1; MIM: 192500),26 characterized by a prolonged QT interval in the ECG. This represents a high risk to syncope or sudden death from cardiac arrhythmia.27 LQTS1 condition is inherited in an autosomal dominant manner with reduced (30%–40%) penetrance and variable expressivity between as well as within families.29,30 More recently, channelopathy genes have also been considered as relevant candidates for unexplained stillbirth and pregnancy losses without fetal anomalies.31-33 In the index family, the KCNQ1 p.V173D variant was also identified not only in 2 of the 3 analyzed pregnancy losses (III-9 and III-13), but also in 2 live-born children of the couple (III-11 and III-12) and the sister of the proband (II-3; Figure 1A and 1B).

Cardiological Assessment of the KCNQ1 p.V173D Variant Carriers

In retrospective assessment of clinical data gathered during the monitored pregnancies of the proband, the only documented symptoms possibly indicating a cardiovascular phenotype were at 17 to 18 weeks of pregnancy III-13. The patient presented transient hypertension, swelling of feet and hands, palpitations, and sweating at night for a couple of weeks, and she was administered labetalol (nonselective β-blocker and selective α1-blocker). The symptoms were interpreted as psychosomatic reaction to fetal loss.

After the genetic diagnosis, follow-up clinical interviews and monitoring of cardiac function were performed for the family members carrying the pathogenic KCNQ1 p.V173D variant: the proband, her sister, and 2 children. During the cardiac health assessment, the proband (aged 41 years) and her sister (II-3; aged 38 years; diagnosed with chronic magnesium deficiency) were taking magnesium and potassium supplements. Both sisters have reported occasional palpitations, faintness, and presyncope during physical or emotional stress. The proband's ECG showed normal sinus rhythm (heart rate, 84 beats per minute; electrical axis, 62°), but no abnormal repolarization and depolarization (QTcB = 457 ms compared with the threshold of normal QTcB ≤450 ms in women34). In addition, the proband was assigned to a 24-hour Holter monitoring.

### Table 1. Recurrent Pregnancy Loss History of the Proband

| Pregnancy loss identifier | Maternal age, y | Gestational age | Karyotype | Pregnancy data | Fetal malformations | Placental phenotype | Microdeletions/ duplications* | KCNQ1 c.518T>A |
|---------------------------|----------------|----------------|-----------|----------------|---------------------|---------------------|---------------------------|------------------|
| III-3                     | 25             | 5 wk           | NA        | Empty sac pregnancy loss | NA                 | NA                  | NA                        | NA               |
| III-4                     | 25             | 10 wk, 5 d     | 46,XX     | Vanishing twin at 6–7 wk, fetal death at 14 wk, 5 d | Not detected       | Focal delayed maturation and circulatory disorders, no inflammation | Not detected       | TT (WT)          |
| III-5                     | 26             | 14 wk, 5 d     | 46,XX     | Biochemical pregnancy loss | NA                 | NA                  | NA                        | NA               |
| III-6                     | 26             | 4.5 wk         | NA        | Yolk sac pregnancy loss | NA                 | NA                  | NA                        | NA               |
| III-7                     | 26             | 6 wk           | NA        | Biochemical pregnancy loss | NA                 | NA                  | NA                        | NA               |
| III-8                     | 27             | 12 wk          | 46,XY     | Fetal death             | Not detected       | Focal thrombotic vasculopathy, abundant erythroblasts in placental vessels, may indicate fetal anemia, no inflammation | Not detected       | NA               |
| III-9                     | 28             | 17 wk          | 46,XX     | Fetal death             | Not detected       | Hypercoiling of umbilical cord (coiling index, 0.61), normal finding with some postmortem changes: minimal villous fibrosis, no inflammation | NA               | NA               |
| III-13                    | 39             | 18 wk          | 46,XX     | Fetal death             | NA                 | NA                  | NA                        | NA               |
| III-14                    | 40             | 11 wk, 3 d     | 46,XX     | Fetal death             | Not detected       | NA                  | NA                        | TT (WT)          |

NA indicates not assessed; and WT, wild type.

*On the basis of the analysis using chromosomal microarray.
that also revealed prolonged QT interval corrected for the heart rate (QTc) at nighttime. The longest QT interval corrected for the heart rate at night was measured 534 ms, whereas the longest daytime QT interval corrected for the heart rate was 508 ms (including daily exercises; Data S1). Currently, the patient has been referred to a thorough cardiological workup. To date, no ECG pathological feature has been detected for the proband’s sister (detailed report unavailable because of residency abroad).

The proband’s daughter (III-12) was diagnosed at the age of 3 years with a ventricular septal defect that was confirmed to have closed on its own by the age of 7 years at the follow-up cardiology visit. She has complained of occasional chest pain at resting state, occurring more frequently during school period. Although she had normal ECG (aged <15 years: reference threshold of normal QTcB <440 ms), a minor sinus arrhythmia was detected (heart rate, 68 beats per minute; axis, 57°; PR, 133 ms; QRS, 69 ms; QTcB, 428 ms; normal depolarization and repolarization; Data S1). Therefore, a follow-up cardiac assessment in 5 years was suggested by the managing pediatric cardiologist. The proband’s youngest son, III-11 (aged 11 years), received a pediatric cardiologist’s assessment after the genetic diagnosis of LQTS1. Also in his case, the ECG was normal, but in lying position a minor sinus arrhythmia was reported (heart rate, 66 beats per minute; axis, 79°; PR, 120 ms; QRS, 82 ms; QTcB, 428 ms; normal depolarization and repolarization; standing: heart rate, 71 beats per minute; QTcB, 438 ms) and a follow-up visit was scheduled in 1 year.

### Table 2. Clinical Characteristics of the Proband’s Live-Born Children

| Live birth | Newborn | Childhood | Genetics |
|------------|---------|-----------|----------|
| Identifier | Maternal age, y | Gestational week | Pregnancy | Sex | Weight, g* | Health problems | QTcB, ms | KCNQ1 c.518T>A |
| III-1      | 20      | 39        | Uncomplicated | Boy | 3300 | Atopic dermatitis, asthma, minor scoliosis | 3300 | TT (WT) |
| III-2      | 24      | 38        | Uncomplicated | Boy | 3600 | Minor sport injuries | 3600 | TT (WT) |
| III-10     | 29      | 37        | Administration of enoxaparin1 | Boy | 3364 | Atopic dermatitis, umbilical hernia (surgery at the age of 8 y) | 3364 | TT (WT) |
| III-11     | 30      | 40        | Administration of enoxaparin1 | Boy | 3395 | Appendicitis (surgery at the age of 4 y), minor sport injuries | 442/438 (11 y)† | TA |
| III-12     | 33      | 38        | Administration of enoxaparin1 | Girl | 3344 | Napkin dermatitis, congenital ventricular septal defect (asymptomatic, detected accidentally by auscultation at the age of 3 y [2-degree murmur], spontaneous closure by the age of 7 y) | 420/428 (7/8 y)§ | TA |

WT indicates wild type; and QTcB, corrected QT interval estimated using the Bazett formula.34

*All normal vaginal deliveries.

†Because of focal delayed maturation of villi and circulatory disorders for pregnancy loss case at 14 weeks 5 days (III-5) and focal thrombotic vasculopathy for pregnancy loss at 17 weeks (III-9), low-molecular-weight heparin enoxaparin as a preventive management measure of recurrent abortions was administrated during subsequent pregnancies.

‡Lying/sitting position.

§Two assessments at the ages of 7 and 8 years.

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In Vitro Functional Effect of the KCNQ1 p.V173D Variant

To establish whether the KCNQ1 p.V173D variant confers identifiable functional defects, we performed in vitro electrophysiological studies using the whole cell patch-clamp technique. The KCNQ1 variant p.V173D was engineered in a recombinant K(V)7.1 potassium channel plasmid vector and heterologously coexpressed with KCNE1 cDNA in CHO cells to assess its functional consequence (see Methods for details).

Compared with the wild-type channel (Figure 2), the mutant channel p.V173D statistically significantly reduced total activating and deactivating currents (P<0.01), with dramatically positive shift of voltage dependence of activation by ≈10 mV (P<0.05). Thus, K(V)7.1-V173D causes a loss of function and marked attenuation of I(Ks), classifying KCNQ1 p.V173D (c.518T>A) as a pathogenic variant. Furthermore, we examined the responses of wild type–I(Ks) and the KCNQ1 variant p.V173D–I(Ks) to β-adrenergic receptor agonist isoproterenol (1 µmol/L). As shown in Figure S2, wild type–I(Ks) was dramatically increased by isoproterenol, a well-recognized effect in agreement with previous studies.35,36 However, the variant p.V173D–I(Ks) was slightly enhanced by isoproterenol.

DISCUSSION

The current study uncovered concealed maternal LQTS1 as a potential novel cause behind recurrent fetal loss. Exome sequencing analysis of the
KV7.1 consists of 6 transmembrane segments, termed (Figure 1C and 1D). According to VarSite server, 41 a is the only naturally occurring variant at this position in the first C-loop between segments S2 and S3 and where it coassembles with KCNE1 to form the slowly activating voltage-gated potassium ion channel $I_{Ks}$ in the heart by coassembly with KCNE1. It has been reported that 66% of LQTS1 carriers inherited the variant from their mother and 39% (versus expected 25%) from their maternal grandmother.50 The residue V173 is located in the first C-loop between segments S2 and S3 and is the only naturally occurring variant at this position (Figure 1C and 1D). According to VarSite server,41 a Val to Asp residue change has a high “disease propensity” value of 2.81. The propensities measure how much more frequently a variant is seen in diseases than in the natural variant data obtained from Genome Aggregation Database17 (values range from 0.25 [I>V] to 3.27 [C>R]). Supporting the critical function of this KV7.1 region, ClinVar (accessed March 2021) lists 38 likely pathogenic/pathogenic variants for 27 residues between amino acids 169 and 196 (S2–S3 C-loop). In addition, $K_{v7.1}$ interacts with calmodulin through residues in the S2 to S3 loop. This interaction is important for channel assembly, trafficking, and channel gating.42 The p.V173D change could affect the binding of calmodulin and thus impair channel function. The functional assessment in vitro supported the pathogenicity of the variant, leading to the dysfunctional $I_{Ks}$ channel. Compared with the wild-type–$I_{Ks}$ channel, the current of the variant–expressed p.V173D–$I_{Ks}$ channel is less sensitive to β-receptor stimulator isoproterenol. The β-receptor stimulated $I_{Ks}$ increase is associated with intracellular cAMP-Protein kinase A (PKA) mediated phosphorylation of the channel protein.43 The mechanism underlying lower sensitivity of the V173D channel to isoproterenol remains to be studied.

In addition to the proband, the KCNQ1 p.V173D variant was also identified in 2 of the 3 second-trimester miscarriages (III-9 and III-13), 2 live-born children (III-11 and III-12), and the sister of the proband (II-3; Figure 1A and 1B). Therefore, it can be concluded that a fetus carrying a pathogenic KCNQ1 variant is not per se at risk to miscarriage. Consistent with our data, a retrospective study by Cuneo et al (2020), targeting 148 pregnancies of patients with LQTS, showed that maternal, not fetal, pathogenic variants in LQTS genes, especially in the KCNQ1, confer increased risk for fetal deaths.44 This analysis showed that pregnancies were significantly more likely to end in miscarriage or stillbirth with maternal rather than paternal LQTS condition (24.4% versus 3.5% of gestations, respectively). Interestingly, 2 of 3 women who reported ≥2 pregnancy losses in that study also carried pathogenic variants located in one of the C-loops of the encoded protein, as the KCNQ1 p.V173D variant described in the current report (Table 4). Important for the clinical management of pregnant women with cardiac arrhythmias, there is evidence that pathogenic variants in LQTS genes may represent a shared cause of (late) fetal deaths and stillbirths. For example, the KCNQ1 c.1189C>T, p.R397W variant has been reported in a case with fetal intrauterine death at 16 gestational weeks,31 as well as in a stillbirth case at 27 gestational weeks.32 Similarly, the KCNQ1 c.1766G>A, p.G589D variant was identified in women who have experienced ≥1 miscarriage(s) and also a stillbirth (Table 4).44

Table 3. Relevance of Maternal KCNQ1 Mutations to the Risk of RPL

| KCNQ1 function | Relevance to pregnancy complications |
|----------------|--------------------------------------|
| K+ homeostasis maintenance needed for electrolyte and hormone transport | Women with LQTS are at increased risk for fetal death and IUGR caused by placental or myometrial dysfunction |
| KCNQ1 forms the slowly activating voltage-gated potassium ion channel $I_{Ks}$ in the heart by coassembly with KCNE1 | KCNQ1 is important for contractile function of the uterine smooth muscle and vascular tone regulation in pregnancy |
| Depends on the tissue and available subunits (KCNE1–5) that drastically modify the channel kinetics | Stillbirth |
| KCNQ1 expression in human (ProteinAtlas and Wang et al40) | Sudden infant death syndrome |
| One heart-specific isoform | Trophoblast differentiation |
| Other isoform ubiquitously expressed | |

Diseases related to loss of function in human (OMIM)

- Long QT syndrome 1 (LQTS1): type 1 long-QT syndrome
- Paternal LQTS: autosomal dominant; maternal LQTS: autosomal recessive
- IUGR (intrauterine growth restriction): increased risk of perinatal complications
- Familial atrial fibrillation: causes palpitations, syncpe, thromboembolic stroke, and congestive heart failure (AD)
- Short-QT syndrome: causes syncpe and sudden death (AD)
- Jervell and Lange-Nielsen syndrome: characterized by congenital deafness and prolongation of the QT interval (AR)
- Trophoblast differentiation

Relevance to pregnancy complications

| Risk of RPL | Clinical outcomes |
|-------------|-------------------|
| Risk of RPL | Increased risk for fetal death and IUGR |
| Risk of RPL | Increased risk for stillbirth |
| Risk of RPL | Increased risk for SIDS |

Other isoform ubiquitously expressed

- KCNQ1 is also important for contractile function of the uterine smooth muscle and vascular tone regulation in pregnancy
- Stillbirth
- Sudden infant death syndrome
- Trophoblast differentiation

AD indicates autosomal dominant; AR, autosomal recessive; IUGR, intrauterine growth restriction; LQTS1, type 1 long-QT syndrome; OMIM, Online Mendelian Inheritance in Man; and RPL, recurrent pregnancy loss.
in people aged ≥75 years. However, certain thyroid disorders and several drugs used as supportive care therapy in patients with cancer are associated with prolonged QT interval. It cannot be excluded that thyroid cancer or chemotherapy could have brought about an arrhythmia, causing her death at such a young age.

The occurrence of asymptomatic carriers in this family is not unexpected as 37% of KCNQ1 mutation carriers do not have abnormal ECGs or experience any symptoms of LQTS-related arrhythmias. In patients with C-loop missense mutations compared with other pathogenic variant carriers, 2-fold higher rate of life-threatening cardiac events has been reported. However, although associated with a higher risk of severe cardiac events, C-loop pathogenic variant carriers have a pronounced response to β-blocker therapy. This knowledge of allelic heterogeneity in response to therapy is valuable to ensure proper clinical management to prevent severe symptoms.

K_7.1 K⁺ channel protein encoded by KCNQ1 wears many hats by performing various cellular tasks in the inner ear, kidney, intestine, colon, thyroid, brain, as well as airways to regulate the electric activity or maintain K⁺ homeostasis needed for electrolyte and hormone transport (Table 3). Thus, it is anticipated that KCNQ1 mutations may lead to complex and pleiotropic consequences. There are limited data on the role of K⁺ channels and specifically on K_7.1 in the normal function and physiology of female reproductive organs.

The current study data suggest that mutations affecting K_7.1 function may affect placental or uterine function. Lundquist et al (2006) have shown that KCNQ1 is expressed in the uterus together with KCNE1a in the relative absence of other KCNE genes, suggesting the possible generation of an I_Ks channel complex that is involved in uterine physiology.

Future studies are needed to fully understand the contribution of proteins regulating K⁺ homeostasis in pregnancy success and failure.

As an additional observation, in the index family, 4 of 5 pregnancy losses with known sex were female and vice versa of 5 live-born children of the proband are boys. Progesterone and testosterone are known to have protective effects against arrhythmias, attributable to vasorelaxant effect, whereas estradiol exerts a proarrhythmic effect. Testosterone levels in amniotic fluid are significantly higher in male fetuses during weeks 12 and 18. This has been proposed as the cause of female predominance in LQTS and could also explain the sex differences in miscarriages and live-born children in this family.

Table 4. **KCNQ1 Genotypes Linked to Fetal Deaths**

| Nucleotide change* | Protein change* | rs number | gnomAD MAF | Location in protein | Fetal losses, n | Gestational weeks | ACMG† | Ref | ClinVar: reported in LQTS |
|-------------------|-----------------|-----------|------------|---------------------|-----------------|------------------|--------|-----|--------------------------|
| Maternal long-QT genotype (fetal genotype not available) |
| c.518T>A | p.V173D | rs199472695 | NA | S2-S3 C-loop | 9 | 10.8±5.0 | LP | This study | Yes |
| c.551A>C | p.Y184S | rs199473397 | NA | S2-S3 C-loop | 3 | 10.1±3.4 | Pathogenic | 44 | Yes |
| c.760G>A | p.V254M | rs120074179 | NA | S4-S5 C-loop | 2 | 10.1±3.4 | Pathogenic | 44 | Yes |
| c.1766G>A | p.G589D | rs120074190 | 4.96×10⁻⁵ | C-terminus | 3 women with 3, 1, 1 fetal losses, respectively | 3.98×10⁻⁶ | Pathogenic | 44 | Yes |
| c.1771C>T | p.R591C | rs199473483 | 3.98×10⁻⁶ | C-terminus | 1 | 10.1±3.4 | LP | 44 | Yes |
| Fetal long-QT genotype in second-trimester miscarriages (maternal genotype not available) |
| c.847G>A | p.A283T | NA | NA | SS-S6 linker | NA | 15.7 | LP | 31 | No |
| c.1189C>T | p.R397W | rs199472776 | 1.88×10⁻⁴ | C-terminus | NA | 16.0 | LP | 31 | Yes |

ACMG indicates American College of Medical Genetics and Genomics; gnomAD, Genome Aggregation Database; LP, likely pathogenic; LQTS, long-QT syndrome; MAF, minor allele frequency; NA, not applicable; and Ref, literature reference.

*According to transcript NM_000218.2, ENST00000155840.
†In gnomAD, 14 carriers (8 females) among 141,171 subjects vs 3 of 60 women with type 1 LQTS, who all had experienced fetal losses and/or stillbirths.
‡One woman had experienced a miscarriage and a stillbirth.
§Also reported in a stillbirth case (gestational week 27+4).
In summary, the current study uncovered a potential novel cause behind RPL and fetal death. WES revealed a rare maternal pathogenic variant p.V173D in KCNQ1 as a possible cause to explain her extensive series of miscarriages. KCNQ1 variants have been predominantly analyzed in patients with the diagnosis of LQTS, and reproductive history is rarely if at all reported in these individuals. To date, no familial studies on the link between RPL and LQTS1 have been performed, although first-degree relatives of women with RPL have been shown to exhibit an increased risk of cardiovascular disease.6,9,60,61 Our study emphasizes the value of personalized medicine and the importance of in-depth assessment of all potentially causative genotype-to-phenotype links. The findings of the current study have had an impact not only on the proband, but also on the long-term clinical monitoring and counseling of a large number of family members. Genotype-positive, phenotype-negative children should be watched carefully and not be exposed to any exogenous risk factors, such as competitive sports62 or QT-prolonging drugs.53,64 In the era of WES, early detection of pathogenic variant carriers facilitates timely monitoring, treatment, and prevention of serious cardiac arrhythmias and, in extreme cases, sudden death. Globally, identification of novel genetic determinants of RPL reduces the number of idiopathic cases and is expected to have major individual patient impact with respect to counseling and treatment.

ARTICLE INFORMATION
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Clinical Phenotyping

Proband
Clinical assessment of the proband was performed at the Women’s Clinic of Tartu University Hospital (TUH, Table S1). Laboratory test were performed at the United Laboratories, TUH.

Table S1. Maternal clinical characteristics

|                      | Maternal age at assessment (years) | Results                          | Reference               |
|----------------------|-----------------------------------|----------------------------------|-------------------------|
| **Lifestyle factors**|                                   |                                  |                         |
| Body mass index (non-pregnant state) | 25-40                             | 22-24 kg/m²                      | 19-25 kg/m²             |
| Smoking              | never                             |                                  |                         |
| Diet                 | no restrictions                   |                                  |                         |
|                      |                                   |                                  |                         |
| **Anatomical factors**|                                   |                                  |                         |
| Ultrasonography      | 26-40, every 1-2 year             | normal                           |                         |
| Hysteroscopy         | 26                                | normal                           |                         |
|                      |                                   |                                  |                         |
| **Hormonal factors** |                                   |                                  |                         |
| Thyroid stimulating hormone | 26; 38                           | 1.80; 2.13 mU/L                  | 0.27 ... 4.20 mU/L     |
| Free thyroxine       | 26; 38                            | 17.5; 14.6 pmol/L                | 12.0 ... 22.0 pmol/L   |
| Prolactin            | 26; 38                            | 334; 256 mU/L                    | 102–496 mU/L           |
| Progesterone (luteal phase) | 26; 33; 38                      | 34.5, 30.9; 41.6 nmol/L          | 5.30 ... 86.00 nmol/L |
|                      |                                   |                                  |                         |
| **Autoantibodies**   |                                   |                                  |                         |
| Anticardiolipin antibodies | 26                              | 4 IU/mL                          | <12 IU/mL              |
| Anticardiolipin antibodies IgM | 38                          | 0.9 kU/L                         | <10 kU/L               |
| Anticardiolipin antibodies IgM | 38                          | 1.1 kU/L                         | <10 kU/L               |
| Beta-2 glucoprotein antibodies | 33         | negative                        | negative               |
| Beta-2 glucoprotein antibodies IgM | 38         | <2.9 kU/L                        | <10 kU/L               |
| Beta-2 glucoprotein antibodies IgG | 38      | 0.7 kU/L                         | <10 kU/L               |
| TPO- IgG             | 26, 38                            | 17; 19 kU/L                      | <34 kU/L               |
| Lupus antibodies     | 38                                | negative                         | negative               |
| Blood coagulation    |                                   |                                  |                         |
| Protein S antigen, free | 26                              | 94                               | 62-146%                |
| Protein C activity   | 26                                | 133                              | 75-135%                |
| Antithrombin III activity | 26                           | 125                              | 75-135%                |
| Other tests          |                                   |                                  |                         |
| Homocysteine         | 26; 38                            | 7.5; 9.3 µmol/L                  | <12.0 µmol/L           |
| Test                              |every pregnancy| < 5.1 mmol/l| < 5.1 mmol/L |
|----------------------------------|----------------|-------------|-------------|
| Fasting glucose                 |                |             |             |
| Vitamin D 26; 38; 75; 81 nmol/L| 26; 38         | 75; 81 nmol/L| > 50 nmol/L |
| Chlamydia every pregnancy        | negative       |             | negative    |
| Gonorrhea every pregnancy        | negative       |             | negative    |
| Trichomonosis 26; 38             | negative       |             | negative    |
| Mycoplasmosis every pregnancy    | negative       |             | negative    |
| HIV                              | negative       |             | negative    |
| Genetic factors                  |                |             |             |
| Karyotype of both partners 26/32| partner        | 46 XX/46 XY | 46 XX/46 XY |
| Factor V (Leiden)  p.Arg506Gln, rs60254| 26 | Major allele |
| Factor II (prothrombin) c.G20210A, rs179996341| 26 | Major allele |

**Electrocardiograms**

ECG report of the proband was initially assessed by Dr. Anne Kirss, a specialist in internal diseases and obstetric medicine at the Women’s Clinic of Tartu University Hospital. Additionally, the ECG reports were re-analyzed using Medilog Darwin Enterprise software ver. 2.9.2 (Schiller AG, Switzerland) and evaluated by Dr. Piibe Muda, a cardiologist specialized to rhythmology (Dept. of Clinical Physiology, Cardiology Clinic of Tartu University Hospital).

The proband has been subjected to ECG twice:

- at the age of 37 (heart rate 60 beats per minute, sinus rhythm, no abnormal re- and depolarization)
- at the age of 41 (see pp.3-6 detailed clinical reports, including Holter monitoring).

QTc was estimated using the Bazett formula. For females, normal range of QTcB is considered <450 ms, borderline 451-470 and prolonged >470 ms. For children under the age of 15, normal range of QTcB is considered <440 ms, borderline 441-460 and prolonged >460 ms.
24-hour Holter-monitoring:

Normal sinus rhythm, no pauses, daily average 76 beats per minute. A few extrasystols. Borderline QT intervall. QTc is prolonged at night, the longest QTc 534 ms (2:10 am). The longest QTc 508 ms at day time (incl. after daily exercises).
Details of Holter monitoring
Details of pregnancy loss pathology reports
All pathology examinations were performed at the Pathology Department of Tartu University Hospital and revised by Dr. Liis Salumäe.

Case III-5
Fetal death at 14 weeks and 5 days
Vanishing twin at 6-7 gestational weeks
Placenta: weight 20 g, focal delayed maturation and circulatory disorders, no inflammation, placental age corresponds to 13-14 gestational weeks
Fetus: weight 44 g, length 16.5 cm, female, no malformations

Case III-9
Fetal death at 17 gestational weeks
Placenta: weight 56 g, stroma of chorionic villi contain abundant Hoffbauer cells, focal thrombotic vasculopathy, abundant erythroblasts in placental vessels that may indicate fetal anemia, no inflammation.
Fetus: weight 56 g (corresponds to 15-16 weeks), length 15.4 cm (corresponds to 16-17 weeks), rump length 10.5 cm, foot 1.7 cm, female, no malformations. Lung tissue contains abundant erythroblasts, which may indicate fetal anemia.
**Case III-13**
Fetal death at 18 gestational weeks
Placenta and umbilical cord: weight 91.9 g, size 12x9.5x1.5 cm, marginal insertion of umbilical cord, length of umbilical cord 52 cm, coiling index 0.61 (normal range 0.07-0.3). Some typical postmortem changes: minimal villous fibrosis, no inflammation.
Fetus: weight 174 g (corresponds to 17-18 weeks), length 21.5 cm (corresponds to 18 weeks), rump length 15 cm, foot 2.6 cm, head circumference 14 cm, abdominal circumference 13 cm.
Visual examination: left hand mild clinodactyly, low-lying ears.
Brain-liver ratio=4.7 (normal 3), indicates asymmetric fetal growth restriction. No malformations.

**Detailed health data for live born children**
Data about pregnancy course and deliveries were obtained from the medical case database of Tartu University Hospital and personal contact. Health data for children was obtained from interviews with the proband and electronic medical case history. Cardiac assessment of two children (III-11, III-12), both heterozygous carriers of the KCNQ1 c.518T>A variant, was performed by Dr. Kristel Köbas, pediatric cardiologist, Children’s Clinic of Tartu University Hospital.

**III-1**
Uncomplicated pregnancy and vaginal birth at 39 weeks, birth weight 3300 g, male.
According to maternal data, there was fetal hypoxia and meconial staining of amniotic fluid. Atopic dermatitis and asthma since 1 months after birth, D-vitamin deficiency during infancy and minor scoliosis.

**III-2**
Uncomplicated pregnancy and vaginal birth at 38 weeks, birth weight 3600 g, male.
No health problems during infancy and childhood except sport injuries: ear injury (at the age of 11 years), fracture of left big toe (at the age of 16 years).

**III-10**
Uncomplicated pregnancy and vaginal birth at 37 weeks, birth weight 3364 g, male.
During pregnancy, low molecular-weight heparin enoxaparin was administered due to focal delayed maturation of villi and circulatory disorders for pregnancy loss case at 14 weeks and 5 days (III-5) and focal thrombotic vasculopathy for pregnancy loss at 17 weeks (III-9). Atopic dermatitis during infancy, scarlet fever (at the age of 6), injury of elbow (at the age of 4), surgical repair of umbilical hernia (at the age of 8).

**III-11**
Uncomplicated pregnancy and vaginal birth at 40 weeks, birth weight 3395 g, male.
During pregnancy, low molecular-weight heparin enoxaparin was administered. During infancy bronchitis and asthma, surgery due to appendicitis (at the age of 4 years), sport injuries.
Heterozygous carrier of KCNQ1 c.518T>A variant.

Cardiac assessment at the age of 11:

No complaints, able to perform all physical activities and sports. No extra physical training.
Weight 35 kg, height 155 cm, blood pressure systolic 106 mmHg, diastolic 66 mmHg.
Lying position: mild sinus arrhythmia, heart rate 64 beats per minute
Standing position: normal rhythm, heart rate 80 beats per minute
No pathological murmurs.
ECG lying: sinus rhythm heart rate 66 beats per minute, axis +79 degree, PR 120ms, QRS 82ms, slightly prolonged QTcB 442 ms. De- and repolarization normal.
ECG standing: sinus rhythm heart rate 71 beats per minute, axis +73 degree, PR 115 ms, QRS 72ms, normal QTcB 438 ms.
Echocardiography: Four normal size chambers, no septal defects. The myocardial thickness of left ventricle normal, fractional shortening 39% (normal), ejection fraction 70% (normal). Normal function of valves. Physiological regurgitation at tricuspid valve and pulmonal valve. Pulmonary pressure normal.
Conclusion: No pathology.
Lying position: sinus rhythm, heart rate 66 beats per minute. Normal electrical axis 79 degrees, PR 120 ms, QRS 82 ms, slightly prolonged QTcB 442 ms. Repolarization normal, no hypertrophia.
Standing position: sinus rhythm, heart rate 71 beats per minute. Normal electrical axis 73 degrees, PR 115 ms, QRS 72 ms, normal QTcB 438 ms.
Ill-12

Uncomplicated pregnancy and vaginal birth at 38 weeks, birth weight 3344 g, female. During pregnancy, low molecular-weight heparin enoxaparin was administered. Napkin dermatitis in infancy.

Congenital ventricular septal defect “swiss cheese” type - multiple small muscular defects. The defect was detected accidentally by auscultation at the age of 3 (2-degree murmur), spontaneous closure by age 7. KNOWLEDGE: Heterozygous carrier of KCNQ1 c.518T>A variant

Cardiac assessment at the age of 7:
No complaints, able to perform all physical activities. Weight 19 kg, height 123 cm, blood pressure systolic 95 mmHg, diastolic 61 mmHg. Mild systolic murmur and mild respiratory arrhythmia. ECG: sinus rhythm, heart rate 78 beats per minute, axis +46 degree. De- and repolarization normal. Echocardiography: Four normal size chambers, no septal defects. The myocardial thickness of left ventricle normal, fractional shortening 37% (normal), ejection fraction 67% (normal). Normal function of valves. Physiological regurgitation at tricuspid valve and pulmonal valve. Pulmonal pressure normal. Extra chordae tendineae were detected at left ventricle that may explain mild murmur.

Conclusion: No pathology

Cardiac assessment at the age of 8:
Occasional chest pain at resting state, occurring more frequently during school period. She is able to perform all physical activities without any complaints. Weight 24 kg, height 133 cm, blood pressure systolic 101 mmHg, diastolic 66 mmHg. No pathological murmurs at auscultation. ECG: sinus rhythm, heart rate 68 beats per minute, PE 133 ms, QRS 69 ms, QTcB 427 ms, axis +57 degree. De- and repolarization normal. Echocardiography: Four normal size chambers, no septal defects. The myocardial thickness of left ventricle normal. Normal function of valves. Physiological regurgitation at mitral valve. Pulmonal pressure normal.

Conclusion: No pathology

24-hour Holter: Normal sinus rhythm. 24-h average heart rate 76 beats per minute, minimal 47 beats per minute during sleep, maximum 153 beats per minute during exercise.
Sinus rhythm, heart rate 68 beats per minute. Normal electrical axis 57 degrees, PR 133 ms, QRS 69 ms, normal QTcB 427 ms. De- and repolarization normal.
Gathered health data of the proband’s sister, who is living abroad (II-3)
The proband’s sister (age 38) has experienced one empty sac pregnancy loss followed by two uncomplicated pregnancies and vaginal deliveries (at age 32 and 34). She has had appendectomy at the age of 10 and tonsillectomy at the age of 23. She was diagnosed with myocarditis (age 24) and thyroid gland cyst (27). During recent years, she has had complaints for occasional palpitations and faintness; however, no pathology was found at electrocardiogram (age 38, detailed report unavailable due to residency abroad). She was also diagnosed with magnesium deficiency (detailed data is not available) and oral supplementation of both magnesium and potassium improved her status.
| Chr | Start (hg19) | End   | Ref | Alt | Rs number | Gene.refGene | ExonicFunc. | gnomAD_genomes_ALL | ExAC_ALL | 1000g2015aug_all | ACMG  |
|-----|--------------|-------|-----|-----|-----------|-------------|-------------|------------------|-----------|------------------|-------|
| 1   | 77515945     | 77515945 | A   | G   | rs779329661 | ST6GALNAC5  | missense    | 6.5E-05          | 8.2E-06   | NA               | VUS   |
| 1   | 170952626    | 170952626 | T   | C   | rs201549824 | MROH9       | missense    | 7.0E-04          | 4.0E-04   | NA               | VUS   |
| 1   | 183876214    | 183876214 | G   | A   | rs752600877 | RGL1        | missense    | 6.5E-05          | 2.0E-05   | NA               | VUS   |
| 1   | 201181256    | 201181256 | C   | G   | NA        | IGFN1       | missense    | NA              | NA        | NA               | VUS   |
| 2   | 65543962     | 65543962 | G   | A   | rs141237945 | SPRED2      | missense    | 7.0E-04          | 7.8E-05   | 2.0E-04          | VUS   |
| 2   | 85012801     | 85012801 | C   | T   | rs531681722 | DNAH6       | missense    | 1.0E-04          | NA        | NA               | VUS   |
| 3   | 48667506     | 48667506 | G   | A   | rs201569807 | SLC26A6     | missense    | 9.0E-04          | 5.0E-04   | NA               | VUS   |
| 3   | 151161276    | 151161276 | C   | T   | rs770724810 | IGSF10      | missense    | NA              | 1.6E-05   | NA               | VUS   |
| 4   | 14488046     | 14488046 | G   | A   | rs773401747 | TRIO        | missense    | 6.0E-04          | 2.0E-04   | NA               | LB    |
| 5   | 36049244     | 36049244 | A   | G   | rs200492917 | UGT3A2      | missense    | 2.0E-04          | 2.0E-04   | NA               | VUS   |
| 5   | 41621112     | 41621112 | C   | A   | rs371572169 | MDFI        | stopgain nonframeshift deletion | 2.0E-04 | 3.0E-04 | 6.0E-04          | VUS   |
| 6   | 43307372     | 43307372 | GGTGGTGGGA | -  | rs563671976 | ZNF318      | stopgain nonframeshift deletion | 2.0E-04 | 3.0E-04 | 6.0E-04          | VUS   |
| 6   | 109752445    | 109752445 | A   | G   | rs199983515 | PPI6        | missense    | 3.0E-04          | 8.0E-04   | 6.0E-04          | VUS   |
| 7   | 142749654    | 142749654 | A   | G   | rs373694316 | OR6V1       | missense    | 9.7E-05          | 5.8E-05   | NA               | VUS   |
| 8   | 100693345    | 100693345 | G   | C   | rs139664531 | HEMGN       | missense    | 7.0E-04          | 6.0E-04   | NA               | LB    |
| 9   | 102668000    | 102668000 | G   | A   | rs773149802 | MMP1        | missense    | NA              | 8.2E-06   | NA               | VUS   |
| 12  | 6939652      | 6939652  | C   | A   | NA        | P3H3        | missense    | 6.5E-05          | NA        | NA               | VUS   |
| 12  | 52828048     | 52828048 | C   | T   | rs146288298 | KRT75       | missense    | 4.0E-04          | 4.0E-04   | 2.0E-04          | VUS   |
| 13  | 20220901     | 20220901 | G   | C   | NA        | MPHOSPH8    | missense    | NA              | NA        | NA               | VUS   |
| 13  | 78143586     | 78143586 | G   | C   | rs1280854840 | SCEL        | missense    | 1.0E-04          | NA        | NA               | VUS   |
| 14  | 71199844     | 71199844 | G   | A   | rs149935990 | MAP3K9      | missense    | 1.0E-04          | 1.0E-04   | NA               | VUS   |
| 14  | 95053962     | 95053962 | A   | C   | rs1274092862 | SERPINA5    | missense    | 5.0E-04          | NA        | NA               | VUS   |
| 16  | 136766       | 136766   | C   | T   | rs188724206 | NPRL3       | missense    | 6.5E-05          | 1.0E-04   | NA               | VUS   |
| 16  | 10637443     | 10637443 | G   | C   | rs146745761 | EMP2        | missense    | 2.0E-04          | 9.1E-05   | NA               | VUS   |
|   |   |   | |   |   | |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|---|
| 16 | 67866375 | 67866375 | G | A | rs892367026 | CENPT | missense | NA | NA | NA | VUS |
| 19 | 14518820 | 14518820 | G | A | rs201291607 | ADGRE5 | missense | NA | NA | NA | VUS |
| 19 | 58370042 | 58370042 | C | T | NA | ZNF587 | missense | NA | NA | NA | VUS |
| 20 | 18286410 | 18286410 | G | A | NA | ZNF133 | missense | NA | NA | NA | VUS |
| 20 | 36841679 | 36841679 | G | A | rs867311806 | KIAA1755 | missense | NA | NA | NA | VUS |
| 22 | 50298065 | 50298065 | G | A | rs748537273 | ALG12 | missense | NA | NA | NA | VUS |
| 22 | 50873472 | 50873472 | G | A | rs144614869 | PPP6R2 | missense | NA | NA | NA | VUS |
|   | 48752372 | 48752372 | G | A | rs142117152 | TIMM17B | missense | NA | NA | NA | VUS |
|   | 68725840 | 68725840 | G | A | rs201772031 | FAM155B | missense | NA | NA | NA | VUS |

LB, likely benign; VUS, variant of uncertain significance
| Chr | Start (hg19) | Ref | Alt | Rs number | Gene. wgEncode Gencode Gene| ExonicFunc. wgEncode Gene| ExonicFunc. Gen| gnomAD exome_ALL | gnomAD exome_NFE | ExAC_ALL | 1000g 2015aug_ | CADD13_ | PSAP score | ACMG |
|-----|-------------|-----|-----|-----------|-----------------------------|-----------------------------|-----------------|----------------|----------------|----------|-------------|----------|------------|------|
| 1   | 11718846    | T   | C   | rs1259964802 | FBXO44 | missense | NA | NA | NA | NA | NA | 26 | 0.00351 | VUS |
| 2   | 74699557    | T   | A   | rs75197688   | MRPL53 | missense | 8.15E-06 | 0 | NA | NA | NA | 24 | 0.00074 | VUS |
| 2   | 113147677   | G   | A   | rs572623209  | RGPD8 | stopgain | 6.00E-04 | 0.0003 | 5.00E-04 | 2.00E-04 | 35 | 0.00147 | VUS |
| 2   | 241570199    | A   | G   | rs201512343 | PRK35 | missense | 4.06E-06 | 8.96E-06 | NA | NA | 26 | 0.00450 | VUS |
| 3   | 48667506    | G   | A   | rs201569807  | SLC26A6 | missense | 7.00E-04 | 0.0007 | 5.00E-04 | NA | 28 | 0.00349 | VUS |
| 3   | 57456183    | G   | A   | rs765440022  | DABH12 | stopgain | 1.00E-04 | 0.0002 | 9.25E-05 | NA | 39 | 0.00401 | VUS |
| 3   | 159943576    | T   | C   | rs774658584  | C3orf80 | missense | 7.00E-04 | 9.77E-05 | 1.00E-04 | NA | 27 | 0.00140 | VUS |
| 5   | 36049244    | A   | G   | rs200492917  | UGT1A2 | missense | 2.00E-04 | 0.0003 | 2.00E-04 | NA | 28 | 0.00119 | VUS |
| 6   | 41621112    | C   | A   | rs371572169  | MDFI | stopgain | 5.32E-05 | 2.70E-05 | 6.61E-05 | NA | 38 | 0.00009 | VUS |
| 7   | 43594284    | C   | T   | rs202007423  | HECW1 | missense | 0.001 | 0.0001 | 6.00E-04 | NA | 33 | 0.00464 | VUS |
| 8   | 66619295    | C   | A   | rs1316653370 | MTF1 | missense | 0 | 0 | NA | NA | 25 | 0.00297 | VUS |
| 11  | 2591898    | T   | A   | rs199472695  | KCNQ1 | missense | NA | NA | NA | NA | 29 | 0.00330 | LP* |

**Table S3.** Top variants called with high confidence in the proband (II-1) by the PSAP pipeline (all in heterozygous state)
| 19 | 18392033 | G   | C   | rs1293425649 | JUND     | missense | NA   | NA   | NA   | NA   | 24 | 0.00146 | VUS |
| 19 | 55597880 | G   | T   | rs200225675 | EPS8L1   | stopgain  | 2.00E-04 | 0.0005 | 4.00E-04 | NA   | 42 | 0.00105 | VUS |
| 21 | 33739048 | G   | A   | rs187485893 | URB1     | missense  | 5.00E-04 | 0.0006 | 6.00E-04 | 2.00E-04 | 34 | 0.00455 | VUS |
| 22 | 29927873 | G   | A   | rs199572850 | THOC5    | missense  | 8.00E-04 | 0.0001 | 7.00E-04 | 3.99E-04 | 34 | 0.00079 | VUS |
| X  | 117861610| G   | A   | NA   | IL13RA1   | stopgain  | NA   | NA   | NA   | NA   | 35 | 0.00004 | VUS |

*ACMG tags: PM1, PM2, PP2, PP3
LB, likely benign; LP, likely pathogenic; VUS, variant of uncertain significance
**Table S4. Merged output of the top variants called with high confidence in the pregnancy loss samples (III-5, III-9, III-13) by the PSAP pipeline (all in heterozygous state)**

| Chr | Start (hg19) | Ref | Alt | Gene. wgEncode Genencode BasicV19 | Rs number | ExonicFunc. wgEncode Genencode BasicV19 | gnomAD. exome_ALL | gnomAD. exome_NFE | ExAC. ALL | 1000g 2015aug. all | CADD13. PHRED | PSAP | ACMG | Number of carrier pregnancy losses |
|-----|-------------|-----|-----|----------------------------------|-----------|----------------------------------------|-------------------|------------------|------------|----------------|-----------------|-------|-------|----------------------------------|
| 1   | 11718846    | T   | C   | FBXO44                           | rs1259964802 | missense                              | NA                | NA               | NA         | NA             | NA              | 26    | 0.00351 | VUS                            | 2          |
| 1   | 16111074    | C   | A   | FBLIM1                           | rs772470861 | missense                              | 4.08E-06          | 9.04E-06         | 8.24E-06   | NA             | NA              | 34    | 0.00033 | VUS                            | 1          |
| 1   | 101005357   | C   | T   | GPR88                            | rs1255606770 | missense                              | 8.54E-06          | 0                | NA         | NA             | NA              | 24    | 0.00070 | VUS                            | 1          |
| 1   | 113253469   | C   | T   | PPM1J                            | rs146793493 | missense                              | 6.91E-05          | 8.96E-05         | 7.42E-05   | NA             | NA              | 33    | 0.00253 | VUS                            | 1          |
| 1   | 120502048   | G   | A   | NOTCH2                           | NA         | missense                              | 8.14E-06          | 0                | NA         | NA             | NA              | 29    | 0.00474 | VUS                            | 2          |
| 1   | 150621165   | C   | T   | GOLPH3L                          | rs186291546 | missense                              | 5.00E-04          | 0.0008          | 4.00E-04   | NA             | NA              | 34    | 0.00196 | VUS                            | 1          |
| 1   | 156767115   | C   | G   | PRCC                             | NA         | missense                              | NA                | NA               | NA         | NA             | NA              | 26    | 0.00087 | VUS                            | 2          |
| 2   | 74699557    | T   | A   | MRPL53                           | rs751976686 | missense                              | 8.15E-06          | 0                | NA         | NA             | NA              | 24    | 0.00074 | VUS                            | 2          |
| 2   | 113147677   | G   | A   | RGPD8                            | rs572623209 | stopgain                              | 6.00E-04          | 0.0003          | 5.00E-04   | 2.00E-04       | 35    | 0.00147 | VUS                            | 1          |
| 2   | 219825198   | C   | T   | CDK5R2                           | rs1447466372 | missense                              | NA                | NA               | NA         | NA             | NA              | 33    | 0.00017 | VUS                            | 2          |
| 2   | 228560627   | T   | G   | SLC19A3                          | rs1473191700 | missense                              | 4.88E-05          | 0                | NA         | NA             | NA              | 27    | 0.00219 | VUS                            | 1          |
| 2   | 241570199   | A   | G   | GPR35                            | rs201512343 | missense                              | 4.06E-06          | 8.96E-06         | NA         | NA             | NA              | 26    | 0.00450 | VUS                            | 2          |
| 3   | 46621330    | G   | C   | TDGF1                            | rs148619685 | missense                              | 6.00E-04          | 0.0009          | 6.00E-04   | 2.00E-04       | 22    | 0.00345 | LB                              | 1          |
| 3   | 48667506    | G   | A   | SLC26A6                          | rs201569807 | missense                              | 7.00E-04          | 0.0007          | 5.00E-04   | NA             | 28    | 0.00349 | VUS                            | 3          |
| 3   | 130743495   | G   | A   | ASTE1                            | rs140373702 | missense                              | 5.00E-04          | 0.0009          | 7.00E-04   | NA             | 27    | 0.00334 | VUS                            | 2          |
| 3   | 159943576   | T   | C   | C3orf80                          | rs774658584 | missense                              | 7.00E-04          | 9.77E-05        | 1.00E-04   | NA             | 27    | 0.00140 | VUS                            | 3          |
| 4   | 76551105    | T   | G   | CDKL2                            | NA         | missense frameshift deletion          | NA                | NA               | NA         | NA             | NA              | 26    | 0.00179 | VUS                            | 2          |
| 5   | 34824200    | AAGC | - | RAI14                            | NA         | missense frameshift deletion          | NA                | NA               | NA         | NA             | NA              | 33    | 0.00155 | VUS                            | 2          |
| 5   | 36049244    | A   | G   | UGT3A2                           | rs200492917 | missense                              | 2.00E-04          | 0.0003          | 2.00E-04   | NA             | 28    | 0.00119 | VUS                            | 3          |
| 5   | 39376818    | C   | T   | DAB2                             | rs760327528 | missense                              | 1.63E-05          | 3.59E-05        | 2.48E-05   | NA             | 33    | 0.00431 | VUS                            | 2          |
| 6   | 31683151    | G   | T   | LY6G6D                           | rs536760002 | missense                              | 4.69E-05          | 9.51E-05        | 8.17E-05   | 2.00E-04       | 25    | 0.00050 | VUS                            | 2          |
| 6   | 41621112    | C   | A   | MDFI                             | rs371572169 | stopgain                              | 5.32E-06          | 0.0007          | 8.24E-06   | NA             | 34    | 0.00046 | VUS                            | 1          |
| 6   | 42179538    | G   | T   | MRPS10                           | rs768228186 | missense                              | 4.07E-06          | 0                | 8.24E-06   | NA             | 34    | 0.00046 | VUS                            | 1          |
| SNP          | Gene   | rsID   | Type    | Effect | p-value | Odds Ratio | HWE  | MAF  | NA | VUS | VUS  |
|--------------|--------|--------|---------|--------|---------|------------|------|------|----|-----|------|
| rs198809991  | HECW1  |        | missense|        | 0.001   | 6.00E-04   | NA   | NA   | NA | NA  | VUS  |
| rs1471734047 | SLC26A3|        | missense| NA     | NA      | NA         | NA   | NA   | NA | NA  | VUS  |
| rs1350123170 | ARFGEF1|        | missense| NA     | NA      | NA         | NA   | NA   | NA | NA  | VUS  |
| rs145500903  | GPR123 |        | missense| 2.00E-04| 0.0003  | 3.00E-04   | NA   | NA   | NA | NA  | VUS  |
| rs199472695  | KCNQ1  |        | missense| NA     | NA      | NA         | NA   | NA   | 29| 0.00330 | LP |
| rs199819045  | CCDC86 |        | stopgain| 2.00E-04| 0.0004  | 3.00E-04   | NA   | NA   | 36| 0.00144 | VUS |
| rs147348885  | SPDYC  |        | missense| 2.00E-04| 0.0002  | 1.00E-04   | NA   | NA   | 28| 0.00086 | VUS |
| rs772121984  | DPAGT1 |        | missense| 4.54E-05| 1.05E-05| 2.67E-05   | NA   | NA   | 28| 0.00448 | VUS |
| rs1344550169 | USP2   |        | missense| 1.22E-05| 0       | NA         | NA   | NA   | 24| 0.00454 | VUS |
| rs759817484  | FOXN4  |        | missense| NA     | NA      | NA         | NA   | NA   | 26| 0.0072  | VUS |
| rs750118817  | YLPM1  |        | missense| 4.07E-06| 0       | 8.30E-06   | NA   | NA   | 35| 0.00280 | VUS |
| rs188724206  | MPG    |        | missense| 7.41E-05| 0.0001  | 1.00E-04   | NA   | NA   | 34| 0.00190 | VUS |
| rs188724206  | NPRL3  |        | missense| 7.41E-05| 0.0001  | 1.00E-04   | NA   | NA   | 34| 0.00209 | VUS |
| rs559217484  | TXNDC11|        | missense| 2.85E-05| 2.69E-05| 3.37E-05   | 2.00E-04| NA | 34| 0.00180 | VUS |
| rs199567405  | DPEP3  |        | missense| 2.00E-04| 5.38E-05| 3.00E-04   | 2.00E-04| NA | 35| 0.00027 | VUS |
| rs199792911  | MMP28  |        | missense| 1.00E-04| 0.0002  | 1.00E-04   | NA   | NA   | 35| 0.00079 | VUS |
| rs201057482  | RAPGEFL1|       | missense| 2.03E-05| 1.79E-05| 1.65E-05   | 2.00E-04| NA | 21| 0.00368 | VUS |
| rs150364683  | HEATR6 |        | missense| 6.00E-04| 0.0006  | 5.00E-04   | NA   | NA   | 35| 0.00456 | VUS |
| rs200298746  | MXRA7  |        | missense| 1.00E-04| 0.0002  | 2.00E-04   | NA   | NA   | 26| 0.00355 | VUS |
| rs1020866073 | NEDD4L |        | NA      | NA     | NA      | NA         | NA   | NA   | 35| 0.00076 | VUS |
| rs201914248  | ABHD17A|        | missense| 3.00E-04| 0.0002  | 2.00E-04   | NA   | NA   | 23| 0.00103 | VUS |
| rs534549628  | OAZ1   |        | missense| 7.00E-04| 5.08E-05| 2.00E-04   | 2.00E-04| NA | 35| 0.00023 | VUS |
| rs749498595  | ZNF414 |        | missense| 1.00E-04| 0.0002  | 1.00E-04   | NA   | NA   | 26| 0.00216 | VUS |
| rs201291607  | DDX39A |        | missense| 2.44E-05| 2.69E-05| 2.49E-05   | 2.00E-04| NA | 22| 0.00400 | VUS |
| rs1293425649 | JUND   |        | missense| NA     | NA      | NA         | NA   | NA   | 24| 0.00146 | VUS |
| rs141103699  | DMRTC2 |        | stopgain| 1.00E-04| 0.0002  | 7.42E-05   | NA   | NA   | 37| 0.00041 | VUS |
| Chr | Genomic Position | Ref/Alt | Gene | rsID | Mutation Type | Z score | p value | OR | 95% CI         | Age | VUS | Notes |
|-----|------------------|---------|------|------|--------------|---------|---------|----|----------------|-----|-----|-------|
| 19  | 55597880         | G       | T    | EPS8L1 | rs200225675 | stopgain | 2.00E-04 | 0.0005 | 4.00E-04 | NA  | 42  | 0.00105 | VUS 1 |
| 21  | 30959710         | C       | T    | GRIK1  | rs753443587 | missense | 4.07E-06 | 0.0008 | 4.00E-04 | NA  | 29  | 0.00265 | VUS 2 |
| 21  | 42807881         | G       | A    | MX1    | rs150271063 | missense | 4.00E-04 | 0.0008 | 4.00E-04 | NA  | 31  | 0.00344 | VUS 2 |
| 22  | 29927873         | G       | A    | THOC5  | rs199572850 | missense | 8.00E-04 | 0.0001 | 7.00E-04 | 3.99E-04 | 34  | 0.00079 | VUS 2 |
| X   | 48752372         | G       | A    | TIMM17B| rs142117152 | missense | 6.00E-04 | 0.0009 | 0.001 | NA  | 27  | 0.00224 | VUS 3 |

LB, likely benign; LP, likely pathogenic; VUS, variant of uncertain significance
Figure S1. Overview of variant prioritizing of WES data in the proband by PSAP analysis and in the pregnancy loss samples by filtering all variants. VCF files contained approximately 170,000 variants.

(A) An updated version of the population sampling probability (PSAP) pipeline was applied in order to prioritize potential causative variants from the WES data of the proband. A new feature in this implementation of PSAP is ethnicity-specific models. PSAP analysis pipeline resulted in 14,586 variants. The variants were prioritized to satisfy the following criteria: (i) missense and LoF variants, (ii) low minor allele frequency (MAF ≤0.001 ExAC, gnomAD and 1000GP) or a previously undescribed variant; (iii) the PSAP statistical significance value ≤0.005; the CADD score ≥20.

(B) VCF files of the pregnancy loss samples were annotated using ANNOVAR (version 20191024). Among all coding variants (exonic and splicing) synonymous and common variants with MAF of more than 0.1% in gnomAD and ExAC were removed. Only shared variants in all pregnancy loss samples were retained.

Next, for both parts of the analysis (A-B), in-house blacklisted variants were removed, i.e., variants corresponding to false signals generated by incomplete reference genome assembly, location in low-complexity regions, bioinformatic misprocessing or due to sequencing kits (see reference demonstrating the significance of removing variant blacklists). Visual inspection of the quality of sequencing reads was performed using The Integrative Genomics Viewer (IGV) software for all prioritized rare and novel variants in order to ensure high quality of retained variants. All the retained variants were manually inspected using scientific literature and genome databases (Table S2-4). Variants were classified according to the ACMG guidelines.
Figure S2. WT- and V173D-I_{Ks} responses to isoproterenol (ISO, 1 µM). Panel A and B show current traces of WT- and V173D-I_{Ks} before and after ISO. Panel C is a summary of ISO-increased steady-state I_{Ks} (%) in WT and V173D by 5-sec pulsing to +60 mV from holding potential of -80 mV (n=6 each).