




ORIGINAL ARTICLE

Loss of Nexilin function leads to a recessive lethal fetal cardiomyopathy characterized by cardiomegaly and endocardial fibroelastosis

Josefin Johansson¹  | Carina Frykholm¹ | Katharina Ericson² |
Kalliopi Kazamia^{3,4,5} | Amanda Lindberg¹ | Nancy Mulaiese¹ | Geir Falck⁶ |
Per-Erik Gustafsson⁷ | Sarah Lidéus¹ | Sanna Gudmundsson^{8,9} | Adam Ameer¹  |
Marie-Louise Bondeson¹ | Maria Wilbe¹ 

¹Department of Immunology, Genetics and Pathology, Uppsala University, Science for Life Laboratory, Uppsala, Sweden

²Department of Clinical Pathology, Akademiska University Hospital, Uppsala, Sweden

³Department of Women's and Children's Health, Karolinska Institute, Stockholm, Sweden

⁴Children's Heart Center Stockholm-Uppsala, Karolinska University Hospital, Stockholm, Sweden

⁵Children's Heart Center Stockholm-Uppsala, Akademiska University Hospital, Uppsala, Sweden

⁶Department of Internal Medicine, Bollnäs Hospital, Bollnäs, Sweden

⁷Department of Cardiology, Gävle Hospital, Gävle, Sweden

⁸Program in Medical and Population Genetics, Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, Massachusetts, USA

⁹Division of Genetics and Genomics, Boston Children's Hospital, Boston, Massachusetts, USA

Correspondence

Maria Wilbe, Department of Immunology, Genetics and Pathology, Uppsala University, Science for Life Laboratory, Uppsala, Sweden, Husargatan 3, Box 815, SE-751 08 Uppsala, Sweden.

Email: maria.wilbe@igp.uu.se

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Abstract

The Nexilin F-Actin Binding Protein (Nexilin) encoded by *NEXN* is a cardiac Z-disc protein important for cardiac function and development in humans, zebrafish, and mice. Heterozygote variants in the human *NEXN* gene have been reported to cause dilated and hypertrophic cardiomyopathy. Homozygous variants in *NEXN* cause a lethal form of human fetal cardiomyopathy, only described in two patients before. In a Swedish, four-generation, non-consanguineous family comprising 42 individuals, one female had three consecutive pregnancies with intrauterine fetal deaths caused by a lethal form of dilated cardiomyopathy. Whole-exome sequencing and variant analysis revealed that the affected fetuses were homozygous for a *NEXN* variant (NM_144573:c.1302del;p.(Ile435Serfs*3)). Moreover, autopsy and histology staining declared that they presented with cardiomegaly and endocardial fibroelastosis. Immunohistochemistry staining for Nexilin in the affected fetuses revealed reduced antibody staining and loss of striation in the heart, supporting loss of Nexilin function. Clinical examination of seven heterozygote carriers confirmed dilated

Josefin Johansson and Carina Frykholm shared first authors.

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cardiomyopathy (two individuals), other cardiac findings (three individuals), or no cardiac deviations (two individuals), indicating incomplete penetrance or age-dependent expression of dilated cardiomyopathy. RNA sequencing spanning the variant in cDNA blood of heterozygote individuals revealed nonsense-mediated mRNA decay of the mutated transcripts. In the current study, we present the first natural course of the recessively inherited lethal form of human fetal cardiomyopathy caused by loss of Nexilin function. The affected family had uneventful pregnancies until week 23–24, followed by fetal death at week 24–30, characterized by cardiomegaly and endocardial fibroelastosis.

KEYWORDS

cardiomyopathy, lethal, Nexilin, NEXN

1 | INTRODUCTION

Cardiomyopathy (CMP) is a heterogeneous group of diseases with structural and/or functional impairment of the myocardium. Dilated cardiomyopathy (DCM) is one CMP subtype, defined as enlargement of the left or both ventricles and impaired systolic function that cannot be explained by aberrant loading conditions or coronary artery disease (Elliott et al., 2007; Pinto et al., 2016). DCM affects more than 1:2500 to 1:250 individuals worldwide, highly influenced by ethnicity, gender, age, and lifestyle factors (Codd et al., 1989; Hershberger et al., 2013; Maron et al., 2006; Mckenna & Judge, 2021). Approximately 20–50% of DCM cases are familial (Hershberger et al., 2009; Hershberger et al., 2013) and other causes include infection, metabolic disease, and autoimmunity (Maron et al., 2006). More than 100 genes have been associated with familial DCM, most often with an autosomal dominant inheritance (Mcnally & Mestroni, 2017; Morales & Hershberger, 2013). Many genetic variants causing familial DCM present with incomplete penetrance or age-dependent expression within the same family, ranging from asymptomatic to cardiac death (Baig et al., 1998; Mahon et al., 2005; Mckenna & Judge, 2021).

Fetal cardiomyopathies are rare, and to our knowledge, familial lethal fetal cardiomyopathies have only been reported in Barth syndrome caused by biallelic missense, splice, and stop gain variants in the *TAZ* gene (OMIM #302060), and six other families (Haug et al., 2001; Lieberwirth et al., 2021; Martins et al., 1996; Sardesai et al., 2001). Two of these families presented with a genetic diagnosis in *SLC30A5* (OMIM #607819) (homozygous NM_022902.4: c.832_836del;p.(Ile278Phefs*33) and NM_022902:c.1981_1982del;p.(His661Tyrfs*10)).

Previously, heterozygous variants in the *NEXN* gene have been reported to cause DCM and hypertrophic CMP with various ages of onset (0–67 years), inherited in an autosomal dominant mode (Andersen et al., 2019; Bruyndonckx et al., 2021; Gigli et al., 2019; Hassel et al., 2009; Kean et al., 2019; Klauke et al., 2017; Richard et al., 2019; Verdonschot et al., 2018; Waldmüller et al., 2015; Wang et al., 2010; Zhang et al., 2020). Only three DCM families with a recessive inheritance pattern in this gene has been described before,

associated with a homozygous three base pair deletion (NM_144573: c.1582_1584del;p.[Glu529del]) (Al-Hassnan et al., 2013), a homozygous stop gain variant (NM_144573:c.1174C>T; p.[Arg392*]) (Bruyndonckx et al., 2021), and compound heterozygote variants in *NEXN* (nonsense NM_144573:c.1756A>T;p.(Lys586*), frameshift NM_144573:c.1909_1912del;p.(Tyr637Alafs*684)) (Rinaldi et al., 2020). To our knowledge, lethal fetal cardiomyopathies caused by biallelic variants in *NEXN* have only been reported in two patients before, where the affected fetus had an emergency cesarean section or terminated pregnancy (Bruyndonckx et al., 2021; Rinaldi et al., 2020). The natural course of lethal fetal cardiomyopathies caused by biallelic variants in *NEXN* has not been described before.

The Nexilin F-Actin Binding Protein (Nexilin), encoded by the *NEXN* gene, is a filamentous actin (F-actin) binding protein important for cell adhesion (Ohtsuka et al., 1998; Wang et al., 2005), migration (Wang et al., 2005), and stabilization of Z-discs within the cardiac muscle sarcomere (Hassel et al., 2009). Cardiac-selective expression of *NEXN* negatively regulates cardiac differentiation and is suggested to lead to atrial septal defects by inhibiting the transcription factor *GATA4*, known to be important for cardiac development (Yang et al., 2014). Ectopic expression of missense variants and non-frameshift deletions in *NEXN* (p.Tyr652Cys, p.Pro611Thr, p.Gly650del) resulted in DCM symptoms in zebrafish, reversed by injection of wildtype Nexilin, indicating a dominant-negative effect (Hassel et al., 2009). In addition, Nexilin is central for smooth muscle cell structure and function (Zhu et al., 2018) and influences glucose transport in these cells (Lee et al., 2013). *NEXN* has been reported to be a susceptibility gene for coronary artery disease (Wu et al., 2013) and lung cancer (Yuan et al., 2016). Biallelic *Nexn* variants in mice result in severe DCM, leading to early lethality in global and cardiomyocyte-specific knock-out (*Nexn* $-/-$) mice (Aherrahrou et al., 2016; Liu et al., 2019) and survival until adulthood in homozygous p.Gly645del mice (Liu et al., 2019). Heterozygous mice of the same studies displayed mild DCM after birth, but this effect could not be observed in follow-up studies at 3 or 10 months of age (Aherrahrou et al., 2016; Liu et al., 2019). Loss of *Nexn* in adult mouse cardiomyocytes leads to impaired cardiac function, cardiac stress, and DCM, indicating an

important role not only in heart development but also for the maintenance of cardiac architecture in adults (Spinozzi et al., 2020).

The current study describes the first lethal fetal dilated cardiomyopathy (IfDCM) caused by a biallelic c.1302del;p.(Ile435Serfs*3) variant in the *NEXN* gene (NM_144573). The variant segregated with disease in a non-consanguineous four-generation family, where heterozygous carriers presented with incomplete penetrance or age-dependent expression of DCM. Functional analysis of the identified variant showed loss of Nexilin function by reduced RNA and protein expression (nonsense-mediated decay of mutated transcripts), loss of striation in the heart as well as fibrosis. In summary, we confirm *NEXN* as a new gene for a recessively inherited lethal form of fetal cardiomyopathy characterized by cardiomegaly and endocardial fibroelastosis.

2 | METHODS

2.1 | Ethical consent

Prior to the initiation of the study, the local ethics committee for human research in Uppsala, Sweden, approved this study (Dnr 2015/093). Informed consent was obtained from all participants and legal guardians (for participants below 18 years of age). All clinical investigations and genetic analyses were conducted in accordance with the guidelines of the Declaration of Helsinki.

2.2 | Clinical characteristics and variant identification

2.2.1 | Patient material

A Swedish, non-consanguineous, four-generation family of 42 individuals was recruited by Clinical genetics, Uppsala University Hospital, Sweden. Seventeen individuals were included in the clinical and genetic study (Figure 1). Individuals IV:5, IV:6, and IV:7 were affected by IfDCM and individuals III:2 and II:3 presented with late-onset DCM.

2.2.2 | Clinical genetic testing and ultrasound investigations

Clinical genetics testing was performed on all three fetuses described in Supplementary material. Detailed fetal echocardiograms of fetus IV:6 and IV:7 were performed by pediatric cardiologists and retrospectively reviewed.

2.2.3 | Autopsy and microbiological investigation

Autopsies on fetus IV:5, IV:6, and IV:7 were performed by a perinatal pathologist at the Department of Clinical Pathology, Uppsala

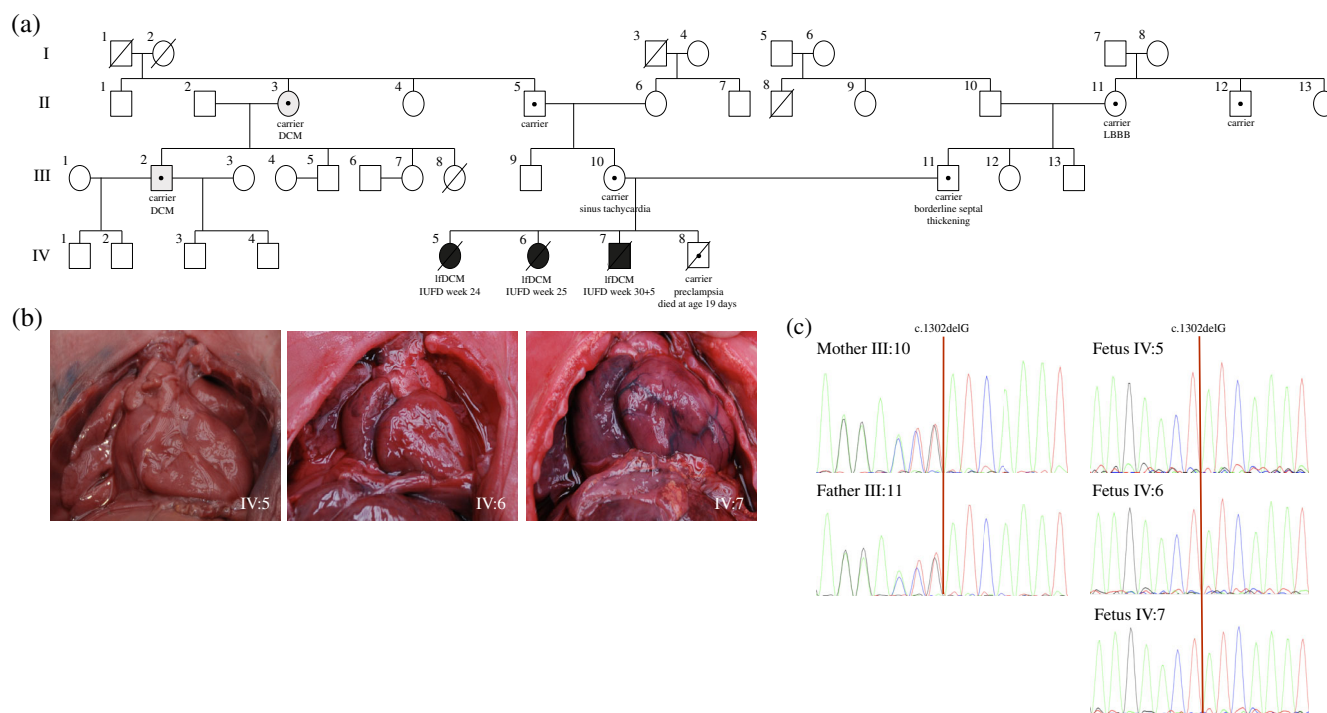


FIGURE 1 A four-generation family affected with three recurrent intrauterine fetal deaths (IUFD) were investigated for a pathogenic variant in *NEXN* (NM_144573:c.1302del). (a) This family consists of 42 individuals. Seventeen individuals were included in the clinical and genetic study. Heterozygote carriers with confirmed dilated cardiomyopathy (DCM) are highlighted in gray. Homozygous fetuses with lethal fetal dilated cardiomyopathy (IfDCM) are highlighted in black. LBBB = left bundle branch block. (b) Pictures of the fetal hearts (IV:5–7) with cardiomegaly. (c) The *NEXN* variant segregated in the family with obligate carriers being heterozygous and affected fetuses homozygous. Sequencing result using the reverse primer is shown

University Hospital, Sweden. A standard autopsy protocol for photographing, external examination, and dissection was followed. Tissue specimens from the organs were collected for histologic examination using a standard sampling protocol. A microbiological examination was performed on the liver and lung samples of fetus IV:6 for Enterovirus RNA, Parvo B19-DNA, CMV-DNA, HSV 1-DNA, HSV 2-DNA, and *Toxoplasma gondii*.

2.2.4 | Whole-exome sequencing

DNA was extracted from blood using the automated QIASymphony system (QIAGEN). Exome sequencing was performed on 100 ng of DNA from the mother III:10 and one affected fetus IV:7 at Uppsala Genome Center using Ampliseq (Life Technologies) and Ion Proton (Life Technologies). DNA library preparation was performed using the Ion AmpliSeq Exome Library Preparation protocol (Revision A.0, Life Technologies). Reads were aligned to the human genome assembly hg19, and variant detection was performed using Torrent Suite Software version 4.0. Variant annotation was obtained from dbSNP137 and ANNOVAR, and the data were compared to reference genomes in our in-house database CanvasDB (Ameur et al., 2014) (github.com/UppsalaGenomeCenter/CanvasDB). Only variants following the suspected recessive inheritance pattern with rare allele frequencies <1% in CanvasDB and gnomAD (<https://gnomad.broadinstitute.org>) (Karczewski et al., 2020) were considered. The predicted variant effect on the encoded protein was assessed using MutationTaster (<http://www.mutationtaster.org/>) and MetaDome (<https://stuart.radboudumc.nl/metadome/>) (Wiel et al., 2019).

2.2.5 | Cardiomyopathy NGS gene panel

To certify that no additional variant segregated with the lethal fetal cardiomyopathy in this family, exome sequencing was performed of individual III:2. In brief, 50 ng genomic DNA was used for target enrichment using Twist Comprehensive exome (Twist Target Enrichment Protocol, Twist Bioscience). Enriched DNA was sequenced on Illumina NextSeq550 with 150 bp paired-end reads. Alignment of raw data to hg19 and variant calling was performed using Bcbio-nextgen 1.1.5-b pipeline tool (<https://github.com/chapmanb/bcbio-nextgen>). Filtering of variants from 121 genes (Supplementary material) associated with cardiomyopathy conditions was performed using MOON software (www.diploid.com/moon). The filtering was performed with recessive, dominant, and X-linked modes using the following criteria: depth ≥ 8 , genotype quality ≥ 40 , and MAF frequency $\leq 1\%$ in gnomAD and Diploid. Only coding variants and flanking exon/intron boundaries ± 10 bp were considered.

2.2.6 | Segregation analysis

To analyze the segregation pattern for the NEXN variant (NM_144573:c.1302del;p.(Ile435Serfs*3)), genomic DNA from

16 individuals was PCR amplified using standard PCR conditions following Sanger sequencing, performed in both forward and reverse directions. Primers used for PCR and sequencing were forward 5'-CAC AGG AAT TCA AGG CCA GC-3' and reverse 5'-GCA AGA AGG AGA GCA ATT GACC-3', creating a PCR product of 267 bp. Sequence analysis was performed using CodonCode Aligner 5.0.1 software (CodonCode Corporation, www.codoncode.com). To investigate the region for recombination and to identify a potential founder effect, the region harboring the NEXN variant (NM_144573:c.1302del;p.(Ile435Serfs*3)) was analyzed for homozygosity by using SNPs in exome sequencing data of one of the affected fetuses (IV:7). To confirm the shared haplotype block, exome sequencing data of the mother (III:10) was analyzed for the same variants in heterozygote form.

2.2.7 | Clinical examinations of dilated cardiomyopathy in heterozygous carriers

Since heterozygote variants in NEXN have been associated with DCM previously (Andersen et al., 2019; Hassel et al., 2009; Kean et al., 2019; Wang et al., 2010; Zhang et al., 2020), seven heterozygous carriers (II:3, II:5, II:11, II:12, III:2, III:10, and III:11) of the NEXN variant were clinically examined for DCM at age 32–67 years by two cardiologists. The examination comprised echocardiography including estimation of ejection fraction, electrocardiography (ECG), blood pressure, and N-terminal prohormone of brain natriuretic peptide (NT-pro-BNP) measurement.

2.3 | Functional analysis of the NEXN variant

2.3.1 | RNA studies

RNA was extracted from blood of two heterozygote carriers (III:10, III:11) and controls using Trizol Reagent Ultra Pure (ThermoFisher Scientific), according to standard protocols. cDNA synthesis was performed with 150 ng of the total RNA as a starting material, according to Maxima H Minus First Strand cDNA Synthesis Kit using Oligo(dT) primers (ThermoFisher Scientific). Primers used for PCR were forward 5'-TTT TGA AGA AGC AAG GCG GC-3' and reverse 5'-GGA GCC TCG CTT TTT CTT GC-3', located in exon 8 and 12 (NM_144573) to create a PCR product of 678 bp (NM_144573, NM_001172309). PCR and Sanger sequencing were performed according to standard conditions, and sequence analysis was performed using CodonCode Aligner 5.0.1 software (CodonCode Corporation, www.codoncode.com).

2.3.2 | Immunohistochemistry

Heart tissue from the affected fetuses (IV:5, IV:6, and IV:7) as well as two control intrauterine fetal death (IUFD) fetuses of the same age and with no suspected heart disease were used. The formalin-fixed

and paraffin-embedded heart tissues were cut into 4 μ m sections. The specimens were stained with a polyclonal antibody, NEXN-TA590405, specific for the N terminus region of the target protein (Origene, 1:150 dilution). The peptide sequence for the antibody is gddslitvvpvksyktsgmkknfedelekereekerikyeedkriryeeqrpslkeakclslvmddeieseakke slspgklktfeelerqrqnrkk, corresponding to amino acids 43–142 of the 217 long GeneBank sequence AAH55084. Microscopic examinations were performed using light microscope (Olympus BX46) and pictures were taken at magnitude of 20 \times and 40 \times .

2.3.3 | Histological studies

Formalin-fixed and paraffin-embedded heart tissue were cut into 4 μ m sections from the same individuals as above and stained for histological examination. Standard hematoxylin and eosin (H&E) staining was used for histologic evaluation. To detect collagen fibers, Masson's Trichrome (MAT) and Sirius Red (SR) staining were used. Verhoeff-Van Gieson (VVG) staining was used to detect the elastin fibers. Microscopic examinations were performed using light microscopy (Olympus BX 46), and pictures were taken at magnitude 4 \times , 10 \times , 20 \times , and 40 \times . Measurements were performed to quantify collagen and elastin using ImageJ with 10 measurements/per image for MAT, SR, and VVG images generated with light microscope (4 \times and 20 \times). The mean values for each individual and group (cases or controls) were calculated, and bar plots were generated using Rstudio.

3 | RESULTS

3.1 | Clinical characteristics and variant identification

3.1.1 | Family history, clinical genetic testing, and ultrasound investigations

A family of Swedish descent was recruited to the research project because of recurrent intrauterine fetal deaths (IUFD) caused by DCM (IV:5–7, Figure 1a). The mother (III:10) had a BMI of 39 but no diabetes or hypertension. She had exertional asthma, fibromyalgia, and had previously undergone surgery for gallstones. The parents had a total of four pregnancies described below. Screening of blood from the mother showed no autoimmunity (lupus anticoagulant ANA, anti-cardiolipin, anticoagulant activated protein C, anti-SSA, anti-Sjögren's syndrome type B antibodies).

The first pregnancy (IV:5) at maternal age of 21 years was uneventful until week 24 + 0 when IUFD was noted. The test results for FISH analysis and QF-PCR on amnion cells on chromosomes 13, 18, and 21 were normal and the gender was female (XX). Karyotyping was not possible due to bacterial contamination. The fetus was sent for autopsy.

The second pregnancy (IV:6) at maternal age of 22 years was referred for regular ultrasound investigation due to the previous

history of IUFD. The pregnancy was uneventful until pregnancy week 23 when the reduction of fetal movements was noted. Fetal cardiac ultrasound of the female fetus (IV:6) was performed at 23 weeks of gestation by a pediatric cardiologist and showed generalized hydrops with significant bilateral pleural and pericardial effusion and ascites. The fetal heart was enlarged with an abnormal cardiothoracic index over 0.6. A primary DCM was suspected. The right ventricle was hyperechogenic suggesting the presence of endocardial fibroelastosis (EFE) and showed an almost complete akinesia. A mild regurgitation of the tricuspid valve with a Vmax of 2 m/s and a moderate regurgitation of the pulmonary valve were detected. The left ventricle was dilated and hypocontractile. No mitral regurgitation was shown. Doppler studies showed increased mean pulsatility index into the umbilical artery with almost zero end-diastolic flow. No structural cardiac or extracardiac abnormalities were detected. No intrauterine growth retardation was seen. Genetic testing of the *PRKGA2* gene was performed without remarks. The test results for amniocentesis, QF-PCR, and FISH for chromosomes 13, 18, and 21 were normal, and the gender was female (XX). Fetal death occurred at 25 weeks of gestation. The fetus was sent for autopsy.

The third pregnancy (IV:7) at maternal age of 23 years was closely followed up with repeated fetal scans every second week from gestation week 19. The first fetal echocardiography performed by a pediatric cardiologist (week 19) was unremarkable. Fetal echocardiography at 23 weeks of gestation revealed a mild regurgitation of the tricuspid valve, dilated right atrium, mildly dilated left atrium, and contractility reduction. The size and systolic function of the fetal heart was normal with cardiothoracic index of 0.5 and fractional shortening (FS) 28% for both ventricles. No ascites, pleural, or pericardial effusions were detected. Doppler studies of the umbilical artery, umbilical vein, and ductus venosus were normal with pulsatility index in the umbilical artery PI 1.23. Subsequent fetal echocardiography at 25 weeks of gestation revealed marked enlargement of the fetal heart with C/T index of 0.65. Both ventricles were significantly dilated with hyperechogenic and poorly contracting myocardium predominately in the right ventricle. The estimated Z-scores for left ventricular end-diastolic diameter (LVEDD) and right ventricular end-diastolic diameter (RVEDD) were +3.2 and +3.1, respectively. A moderate holosystolic regurgitation of tricuspid valve was seen. No signs of hydrops were detected. Doppler studies revealed abnormal findings, including absent end-diastolic flow in the umbilical artery and umbilical venous pulsations. The calculated fetal cardiovascular profile score (CVPS) of 5 indicated a high risk for IUFD. Repeated ultrasound evaluation at 27 and 30 weeks of gestation showed significant progression of fetal heart failure. Fetal scans revealed the presence of marked subcutaneous edema, ascites, bilateral pleural effusion, and pericardial effusion, striking enlargement of the heart with biventricular dilatation (C/T index >0.7), pronounced systolic dysfunction, signs of EFE, holosystolic regurgitation of the tricuspid valve, and abnormal doppler profiles with absent/reversed end-diastolic flow in umbilical artery and umbilical venous pulsations. Based on those findings, the estimated CVPS at week 30 was two. Genetic testing started by sequencing a dilated cardiomyopathy gene panel (27 genes) including the

mitochondrial genome, but no pathogenic variants were found. Testing of the mitochondrial genome only revealed a likely benign variant in *MT-ATP6* (m.9007A>G), which was excluded due to no segregation with the disease. The third affected fetus (IV:7) died at week 30 + 5 and was sent for autopsy.

The fourth pregnancy (IV:8) was at maternal age of 30 years. Prenatal testing of the *NEXN* variant was done through chorion villi sampling. The fetus was a heterozygous carrier of the variant. QF-PCR (chromosome 13, 18, and 21, male fetus XY) was performed and no aberrations were found. During the pregnancy, the mother developed hypertension, and later also preeclampsia. A cesarian section was performed in pregnancy week 24 + 5 due to intrauterine growth retardation (−37%) and reduced umbilical blood flow. The child (IV:8) died at age 19 days due to multiorgan failure and necrotizing enterocolitis combined with extreme prematurity. Microarray performed on post-mortem lung tissue was normal.

3.1.2 | Autopsy and microbiological investigation

The three fetuses affected by IUFD and DCM deviations (IV:5, IV:6, IV:7) underwent autopsy at Uppsala University Hospital, Sweden. They presented moderate to severe autolytic changes but no other external malformations. The first fetus (IV:5) presented cardiomegaly but with no further heart malformations. The second fetus (IV:6) had cardiomegaly, left ventricle hypertrophy with pale endocardium and dilatation of the right side of the heart. The third fetus (IV:7) presented cardiomegaly, dilatation of the right side of the heart, and biventricular apical hypertrophy was seen in both chambers. Fetus IV:7 also presented pericardial and pleural effusion and lung hypoplasia (lung weight/body weight 0.010). Pictures of the fetal hearts are presented in Figure 1b. A standard microbiological examination was performed of fetus IV:6 with no viral infection detected.

3.1.3 | Whole-exome sequencing

Exome sequencing was performed on genomic DNA from the mother and one affected fetus. The data were analyzed in a recessive mode and filtered against our in-house database, consisting of 1424 exomes. When applying the filters, only one single variant was identified, a deletion of a G in *NEXN* (NM_144573) at genomic position chr1:78401557 (hg19), NM_144573:c.1302del, leading to a frameshift and predicting a premature stop codon in exon 11 of 13, p.(Ile435Serfs*3). This variant has been reported in ClinVar as a heterozygote variant of uncertain significance in a child presenting with left ventricular noncompaction cardiomyopathy (National Center for Biotechnology Information, VCV001018253.1, <https://www.ncbi.nlm.nih.gov/clinvar/variation/VCV001018253.1>, accessed April 28, 2021) (Landrum et al., 2018). The variant has an allele frequency of 3.2e-5 in gnomAD v2.1 and interestingly, all eight heterozygous carriers are of Swedish descent (population-specific allele frequency 3.1e-4), hypothetically because of the founder effect and a common haplotype.

The *NEXN* gene is moderately depleted from heterozygous loss-of-function variants with the *loef* score of 0.78 (www.gnomad.broadinstitute.org/; obtained 2021-02-21) (Karczewski et al., 2020). To summarize, this variant has not previously been reported in a homozygous form. MutationTaster predicts the variant to have damaging effects on the protein function, resulting in nonsense-mediated mRNA decay (Schwarz et al., 2014). MetaDome predicts amino acid position 435 and onwards to be intolerant to changes (Wiel et al., 2019).

3.1.4 | Cardiomyopathy NGS gene panel

To investigate if additional variants segregated with the lethal fetal cardiomyopathy in this family, a recessive, X-linked, and dominant filtration against 121 genes associated with cardiomyopathy was performed on DNA extracted from whole blood of the heterozygote *NEXN* carrier (III:2). This revealed five potential variants for further investigations: three missense variants were located in *TTN* (rs140909116, rs72677237, and rs72646885) and one missense variant in *DSP* (rs78652302). All variants were excluded because they were classified as benign in ClinVar and/or had an allele frequency of >0.67% in gnomAD. The only variant that remained was the heterozygote variant in *NEXN* (NM_144573:c.1302del;p.(Ile435Serfs*3)).

3.1.5 | Segregation analysis

To verify the c.1302del variant in *NEXN* and analyze the segregation pattern, Sanger sequencing was performed spanning the variant in 16 available individuals. All affected fetuses (IV:5, IV:6, and IV:7) were homozygous for the mutation and their parents (III:10 and III:11), grandparents (II:5 and II:11), and other relatives (II:3, II:12, and III:2) were heterozygous. Six investigated individuals (II:1, II:2, II:4, II:6, II:9, and II:10) were homozygous for the wildtype allele (Figure 1c and Table S1). In the fourth pregnancy (IV:8), the family was offered fetal diagnostics on CVS using Sanger sequencing spanning the identified *NEXN* variant in a clinical setting. The fetus (IV:8) was heterozygote for the *NEXN* variant. To investigate whether the mutation reflects the presence of a mutational hot spot or a common founder, a haplotype analysis of the region was performed. The result revealed a homozygous haplotype block of 1.7 Mb spanning chr1:77634948–78426231 (hg19). The average recombination rate is 0.3–1 cM/Mb for this region in the human genome (based on deCODE) (Kong et al., 2002; Kong et al., 2010; Kent et al., 2002). A founder effect for this mutation is suggested by the shared 1.7 Mb haplotype on chromosome 1.

3.1.6 | Clinical examinations of dilated cardiomyopathy in heterozygote carriers

In the extended pedigree, the seven carriers were further investigated (II:3, II:5, II:11, II:12, III:2, III:10, and III:11) at the age of 32–67 years.

Two individuals presented with a DCM diagnosis at examination (III:2 age 37 and II:3 age 59), and the others presented with cardiac findings such as left bundle branch block (II:11), sinus tachycardia (III:10, III:11), borderline septal thickening (III:11), or no cardiac deviations (II:5, II:12) indicating incomplete penetrance or age-dependent expression of dilated cardiomyopathy. Variable cardiac findings of all heterozygous *NEXN* carriers are presented in Table S2.

Individual II:3 could have had DCM earlier in life. She had episodes of tachycardia and central chest pain from the fifth decade. Stenosis of the left anterior descending (LAD) coronary artery was found and treated. The additional heart condition was never suspected, which is why echocardiography was delayed until the age of 59 years. There were no signs of restenosis of the LAD.

Individual III:2 was considered to be a candidate for a heart transplant due to severe signs of heart failure (NYHA class 3A) and high NT-proBNP and ejection fraction of 15%. Individual II:3 showed great improvement after ICD and medication; furthermore, upon clinical

examination at age 67, she had NYHA class 2, normal NT-proBNP, and only minor reduction of ejection fraction.

3.2 | Functional analysis of the *NEXN* variant

3.2.1 | RNA studies

RNA sequencing of *NEXN* (NM_144573) was performed in the heterozygote parents (III:10, III:11) and controls with primers spanning exon 8–12. Three different isoforms were detected in all individuals: full-length transcript, skipping of exon 10–11, and skipping of only exon 11 (Figure 2a–c). No differences were observed in the heterozygote carriers (III:10 and III:11) compared to the healthy unrelated controls without the *NEXN* c.1302del variant, indicating detection of novel transcripts in the general population. The two novel isoforms (exon skipping of 10–11, and only 11) are in frame, and hypothetically functional. The variant could not be identified in heterozygote

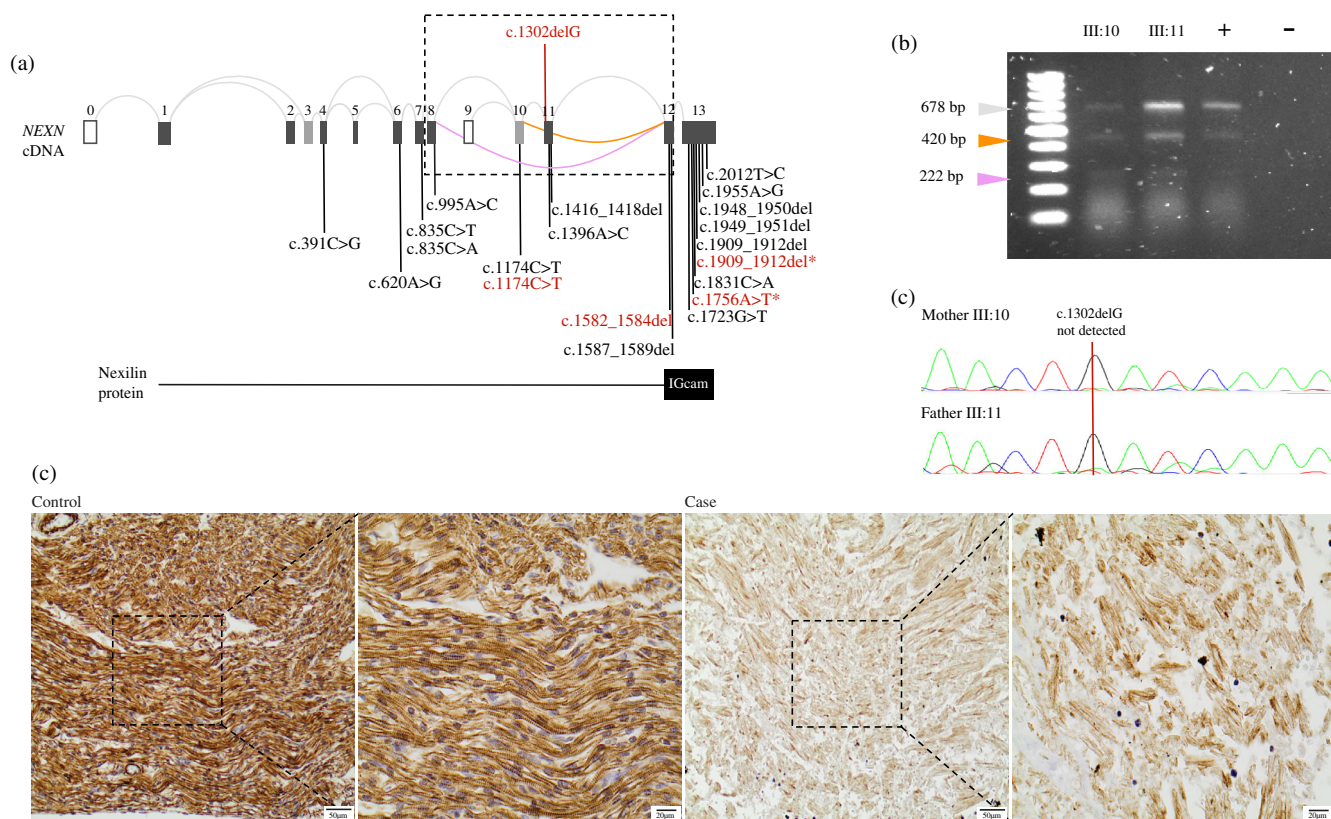


FIGURE 2 Functional analysis of the *NEXN* variant reveals reduced Nexilin expression, degraded muscle fibers, and loss of striation in the heart. (a) Schematic of the *NEXN* transcripts and the predicted protein domains of the human Nexilin protein. In gray and white are the exons with little to no expression in adult heart respectively. All previously reported cardiomyopathy patients associated with *NEXN* variants of autosomal dominant inheritance (black) and autosomal recessive inheritance (red) are included in the schematic. IGcam = immunoglobulin superfamily class domain, * = compound heterozygote variants. (a, b) RNA studies reveal two new isoforms of *NEXN* transcripts (yellow and pink), identified in both cases and controls, suggesting novel transcripts of the general population. The region spanned by the PCR are highlighted with a dotted square. (c) RNA studies of heterozygote parents could not identify the c.1302del variant (NM_144573), suggesting that the variant introduces nonsense-mediated mRNA decay. The chromatogram in the figure was performed using forward primer. (d) Immunohistochemistry of paraffin-embedded heart tissues of affected fetuses (case) and age-matched controls using the *NEXN* antibody (NEXN-TA590405) indicated loss of Nexilin function by reduced Nexilin expression, degraded muscle fibers, and loss of cross striations in the heart

parents, confirming that nonsense-mediated mRNA decay removes the mutated transcripts (Figure 2c).

3.2.2 | Immunohistochemistry

Sections of paraffin-embedded hearts tissue from the affected fetuses (IV:5, IV:6, and IV:7) and two age-matched controls were stained with a polyclonal antibody, NEXN-TA590405. Immunohistochemistry staining of cases revealed degraded muscle fibers with a loss of cross striations. Furthermore, the affected fetuses have reduced normal NEXN antibody staining of the myofibrils and unspecific staining in clumps (Figure 2d, Figure S1). This observation could not be seen in the age-matched IUFD controls.

3.2.3 | Histological studies

Microscopic analyses were performed on sections of the heart from the same individuals as above. H&E staining of the heart sections revealed a thickening of the endocardium and degraded muscle fibers with a loss of striation. MAT staining revealed an increased deposition of collagen fibers (stained in blue) in all affected fetuses compared to the controls. Increased deposition of elastin deposits was detected with SR (stained in red) and VVG (stained in dark purple) in cases compared to controls (Figure 3a, Figure S2). Quantification of elastin and collagen revealed a great increase in the cases compared to the controls (Figure 3b,c, Table 1, Table S3).

4 | DISCUSSION

Here, we describe the first natural course of a lethal fetal form of DCM (lfDCM) caused by a biallelic variant in *NEXN* (NM_144573: c.1302del;p.(Ile435Serfs*3)). The variant segregated with a recessive inheritance pattern in a large pedigree of four generations (Figure 1, Table S1). All three fetuses affected by lfDCM presented with a homozygous genotype, while heterozygous carriers presented with DCM of incomplete penetrance or age-dependent expression (cardiac deviations of carriers described in Table S2). We propose that the c.1302del variant in *NEXN* possibly is a founder mutation, due to the long homozygous haplotype spanning 1.7 Mb in the affected fetus. This haplotype spans seven genes, but the age of the mutation cannot be estimated due to the limited numbers of individuals analyzed. The lfDCM characteristics included an uneventful pregnancy until week 23–24 and intra uterine fetal death (IUFD) at week 24–30. IUFD and severe DCM caused by biallelic loss-of-function variants in *NEXN* variants were confirmed by two previous reports (Rinaldi et al., 2020; Bruyndonckx et al., 2021) and studies on *Nexn* global and cardiomyocyte-specific knock-out (–/–) mice (Aherrahrou et al., 2016; Liu et al., 2019). Fetal echocardiography in affected fetuses of our study was compatible with DCM, but the DCM characteristics could not be confirmed with micro- and macroscopic

examination due to autolytic changes. The affected fetuses (IV:5–7) had thickening of the ventricular endocardium, defined as endocardial fibroelastosis (EFE). The EFE diagnosis was confirmed with histology staining, revealing a distinct increase of connective tissue and elastic fibers in all three fetuses' hearts (IV:5–7) (Figure 3, Table 1, Figure S1, Table S3). EFE is a common finding reported in 25% of pediatric DCM patients (Seki et al., 2013), and has only been reported in this lfDCM subtype once before (Bruyndonckx et al., 2021). The EFE phenotype is further confirmed by a mice study, where *Nexn* –/– mice present with DCM progression and EFE (Aherrahrou et al., 2016).

The genetic diagnosis in the current study is strengthened by screening for variants in other known cardiomyopathy genes and ruling out other known potential disease-causing factors such as diabetes, infection, and autoimmunity. In addition, a ClinVar report, variant predictions using MutationTaster, MetaDome, gnomAD, and functional studies using RNA sequencing, histology staining, and immunohistochemistry strengthen the diagnosis. ClinVar has reported this variant as a heterozygote variant of unknown significance in a child with cardiomyopathy, confirming the variant's association with DCM in heterozygote form (Landrum et al., 2018). MutationTaster (Schwarz et al., 2014) and MetaDome (Wiel et al., 2019) predict the variant to be damaging on the protein function (Figure S2), resulting in nonsense-mediated mRNA decay. In addition, gnomAD is moderately depleted from loss-of-function variants in *NEXN* (l'oeuf = 0.78) with no homozygous variants (besides one late homozygous variant likely escaping nonsense-mediated mRNA decay because of last exon rule), as expected by lethal conditions caused by recessive loss (Karczewski et al., 2020). RNA sequencing spanning the variant in cDNA synthesized from RNA extracted from blood of heterozygote individuals showed only expression of the wildtype allele (Figure 2c). This suggests that the mutated allele was targeted and removed by nonsense-mediated mRNA decay, indicating a damaging effect by haploinsufficiency in blood. Furthermore, the RNA analysis revealed two novel *NEXN* transcripts present in the general population that was not affected by the variant (Figure 2a), suggesting that these transcripts, if present in relevant tissues, are not enough for compensation. Functional analysis of the identified variant using immunohistochemistry in fetal hearts showed a reduced normal NEXN antibody staining of the myofibrils and unspecific staining in clumps and striation in the heart (Figure 2d, Figure S1), supporting the genetic diagnosis and loss of Nexilin function. Complete loss of NEXN antibody staining in affected fetuses was not observed, which is consistent with previous findings using a different antibody (Bruyndonckx et al., 2021). This could possibly be explained by the novel transcript isoforms identified in this study, which are not affected by the investigated c.1302del and c.1174C > T variants (Bruyndonckx et al., 2021) (Figure 2a–c), unspecific antibodies, and/or a truncated Nexilin protein in the heart. In addition, samples from the affected fetuses had autolytic changes that could possibly have affected the histology findings; however, no staining reduction was seen in the age-matched IUFD controls. The variant loss-of-function effect was supported by the phenotypic overlap (including lethality) with *NEXN* knock-out

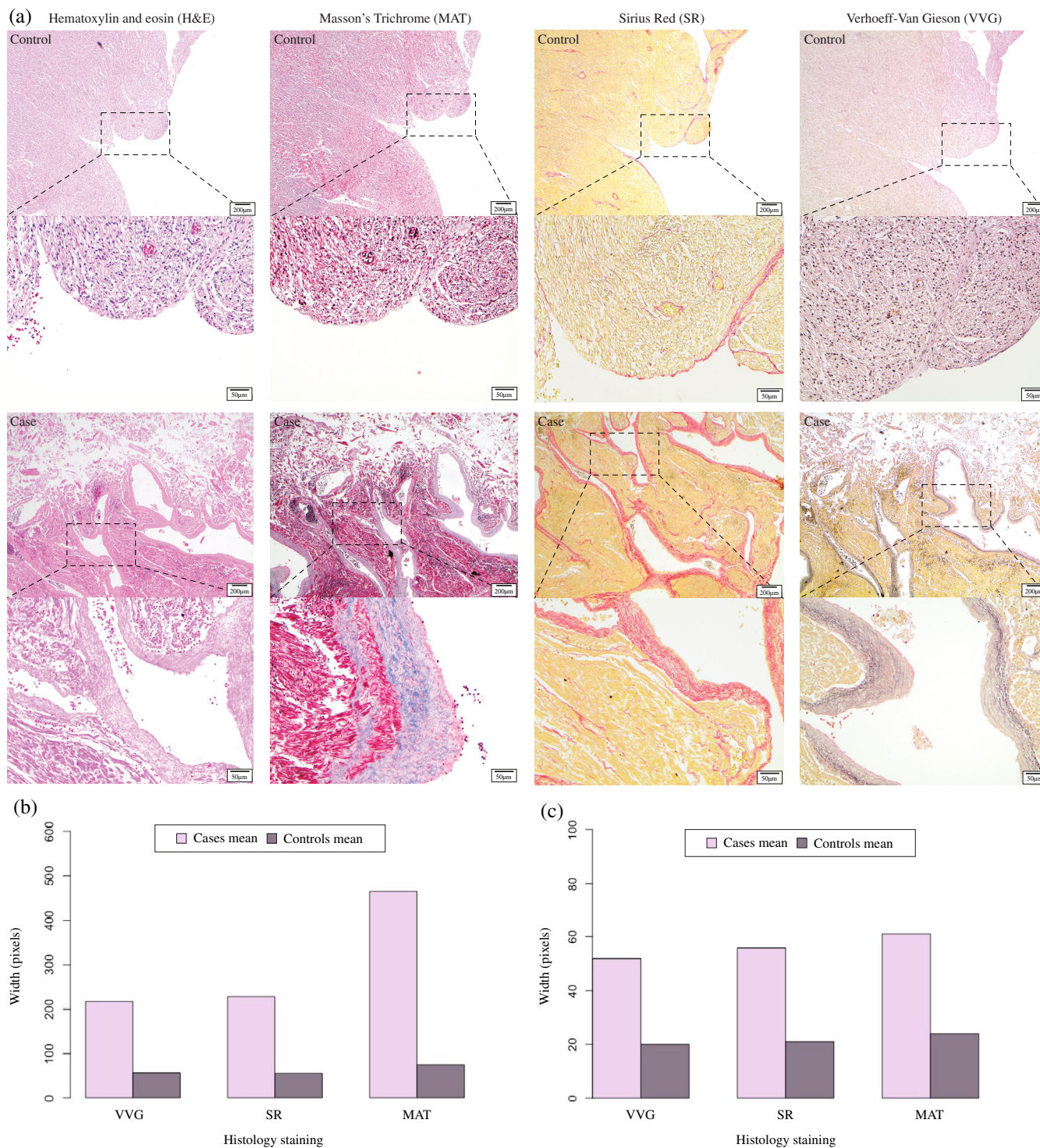


FIGURE 3 Endocardial fibroelastosis (EFE) as a characteristic for lethal fetal dilated cardiomyopathy (IfDCM) caused by a homozygous variant in *NEXN*. (a) Histology staining of heart sections from three affected fetuses (cases) and two age-matched controls revealed increased deposition of collagen and elastin in cases compared to controls. Staining was performed with Hematoxylin & Eosin (H&E), Masson's trichrome (MAT), Sirius red (SR), and Verhoeff-Van Gieson (VVG). The scale bars in the top images are 200 μm , and 50 μm in the zoomed in images below. (b) Barplots visualizing the increase in width of connective tissue and elastic fibers in the above heart sections of the same individuals. The values are mean values of the width (pixels) from 10 representative measurements per individual in histology-stained slides with 20 \times of magnification (C) and 4 \times of magnification

(--/--) mice (Aherrahrou et al., 2016; Liu et al., 2019) and the two previously described IfDCM patients with biallelic LoF variants in *NEXN* (Rinaldi et al., 2020; Bruyndonckx et al., 2021).

Heterozygous variants in *NEXN* have previously been described in 31 patients with DCM or HCM (Table S4) with various ages of onset, ranging from infantile to adult (Andersen et al., 2019;

TABLE 1 Quantification of elastin and collagen in the heart sections from three affected fetuses (cases) and two age-matched controls

Histology	IV:5 mean	IV:6 mean	IV:7 mean	Control I mean	Control II mean	Cases mean	Control mean
VVG (×4)	45	75	36	29	10	52	20
VVG (×20)	227	250	176	124	16	217	70
SR (×4)	47	91	29	30	12	56	21
SR (×20)	166	401	116	82	27	228	55
MAT (×4)	53	89	39	33	15	61	24
MAT (×20)	436	812	143	132	18	464	75

Histology staining was performed with Hematoxylin & Eosin (H&E), Masson's trichrome (MAT), Sirius red (SR), and Verhoeff-Van Gieson (VVG). The values are mean values of the width (pixels) from each individual (10 measurements) and group (cases or controls) in histology-stained slides with 20× of magnitude and 4× of magnitude.

Bruyndonckx et al., 2021; Gigli et al., 2019; Hassel et al., 2009; Kean et al., 2019; Klauke et al., 2017; Richard et al., 2019; Verdonschot et al., 2018; Waldmüller et al., 2015; Wang et al., 2010; Zhang et al., 2020). The carriers in this family with a DCM diagnosis (II:3 and III:2) show consistent characteristics with the previously described heterozygous DCM individuals of the *NEXN* gene (Bruyndonckx et al., 2021; Gigli et al., 2019; Hassel et al., 2009; Kean et al., 2019; Klauke et al., 2017; Verdonschot et al., 2018; Zhang et al., 2020). DCM and cardiac deviations caused by heterozygote variants in *NEXN* are also supported by a mice study where loss of *NEXN* in adult mice cardiomyocytes led to cardiac stress and DCM, indicating an important role for cardiac function in adults (Spinozzi et al., 2020). Age-dependent expression or incomplete penetrance of DCM in carriers of this family (II:5, II:11, II:12, III:10, and III:11) was observed. Age-dependent expression or incomplete penetrance has been described in *NEXN* carriers previously (Bruyndonckx et al., 2021) and is a well-known phenomenon that is present in most other familial DCM families previously described, with DCM expression ranging from asymptomatic to cardiac death (Mahon et al., 2005; Baig et al., 1998; McKenna & Judge, 2021). Furthermore, heterozygous *Nexn* $-/-$ mice displayed mild DCM at postnatal day 4–6 but not at 3 months of age, indicating a possible window of importance for *NEXN* expression, age-dependent expression, or an incomplete penetrance effect (Aherrahrou et al., 2016). In the fourth pregnancy, the mother of this family (III:10) presented with preeclampsia. Heterozygous missense variants in *NEXN* have previously been associated with preeclampsia, a condition that has been shown to increase the risk of cardiovascular-related mortality later in life (Gammill et al., 2018). Except that biallelic loss-of-function variants result in a severe lethal DCM phenotype, there seems to be no clear genotype–phenotype correlation in heterozygote affected individuals, considering variant subtypes, variant effects, transcripts, or protein domains affected (Figure 2a and Table S4) (Howe et al., 2020).

Familial lethal fetal cardiomyopathies are rare, and recessively inherited DCM in *NEXN* has only been described in three families previously. In the first report, two infants presented with a homozygous non-frameshift deletion in *NEXN* (NM_144573:c.1582_1584del;p.Glu529del) and severe, but not lethal, DCM (Al-Hassnan et al., 2013). However, this variant has low evidence for pathogenicity due to

limited segregation analysis and no functional studies. In the next two reports, compound heterozygote variants (nonsense NM_144573:c.1756A>T;p.(Lys586*), frameshift NM_144573:c.1909_1912del;p.(Tyr637Alafs*684)) and a homozygous variant (stop gain NM_144573:c.1174C>T;p.[R392*]) were identified in *NEXN* in fetuses of terminated pregnancy or emergency caesarian delivery. The emergency caesarian delivery was followed by an attempt to get the fetus to survive with treatment, but without success, resulting in a lethal outcome after 2 weeks. The previously described fetuses presented with cardiomyopathy including cardiomegaly, EFE, low contractility, and fibroelastosis (Bruyndonckx et al., 2021; Rinaldi et al., 2020). These reports support the genotype–phenotype correlation in the current study. But the previously described pregnancies were not full-term, and the studies have a limited segregation analysis, and restricted evidence supporting the pathogenic variant effect, highlighting the novelty of this study.

To summarize, we present the first natural course of a recessively inherited lethal form of human fetal cardiomyopathy caused by loss of Nexilin function. The pregnancies were uneventful until week 23–24, followed by sudden fetal death at week 24–30, characterized by cardiomegaly and endocardial fibroelastosis.

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DISCLOSURES

Do not have any financial interests or relationships to disclose.

AUTHORS CONTRIBUTIONS

Josefin Johansson, Katharina Ericson, Amanda Lindberg, Nancy Mulaiase, Sarah Lidéus, Adam Ameer, and Sanna Gudmundsson, performed the experiments. Carina Frykholm, Katharina Ericson, Kalliopi Kazamia, Geir Falck, and Per-Erik Gustafsson performed the clinical investigations. Maria Wilbe, Marie-Louise Bondeson, and Josefin Johansson designed the experiments and analyzed the data. Maria Wilbe and Marie-Louise Bondeson supervised research. Josefin

Johansson and Carina Frykholm prepared the figures and the manuscript with input and approval from all authors.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ORCID

Josefin Johansson  <https://orcid.org/0000-0002-5152-4096>

Adam Ameer  <https://orcid.org/0000-0001-6085-6749>

Maria Wilbe  <https://orcid.org/0000-0003-3000-0696>

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