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Reduced kinase function in two ultra-rare *TNNI3K* variants in families with congenital junctional ectopic tachycardia

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Abstract

Genetic missense variants in *TNNI3K*, encoding troponin-I interacting kinase, have been associated with dilated cardiomyopathy (DCM) and observed in families with supraventricular tachycardias (SVT). Previously, a family harboring the *TNNI3K*-c.1615A > G (p.Thr539Ala) variant presented with congenital junctional ectopic tachycardia (CJET), an arrhythmia that arises from the atrioventricular (AV) node and His bundle. However, this was a relatively small four-generational family with limited genetic testing ($N = 3$). We here describe a multigenerational family with CJET harboring a novel ultra-rare *TNNI3K* variant: *TNNI3K*-c.1729C > T (p.Leu577Phe). Of all 18 variant carriers, 13 individuals presented with CJET, resulting in a genetic penetrance of 72%. In addition, CJET is reported in another small family harboring

Abbreviations: AF, atrial fibrillation; AV, atrioventricular; CADD, combined annotation-dependent depletion; CCD, cardiac conduction disease; CJET, congenital junctional ectopic tachycardia; DCM, dilated cardiomyopathy; ICD, implantable cardioverter-defibrillator; SIFT, sorting intolerant from tolerant; SVT, supraventricular tachycardia; TNNI3K, troponin-I interacting kinase; VT, ventricular tachycardia.

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TNNI3K-c.2225C > T (p.Pro742Leu). Similar to the previously published CJET family, both *TNNI3K* variants demonstrate a substantial reduction of kinase activity. Our study contributes novel evidence supporting the involvement of *TNNI3K* genetic variants as significant contributors to CJET, shedding light on potential mechanisms underlying this cardiac arrhythmia.

KEYWORDS
autophosphorylation, congenital junctional ectopic tachycardia, protein kinase, rare variants, TNNI3K

1 | INTRODUCTION

Genetic variations in the *TNNI3K* gene, which encodes troponin-I interacting kinase, have been identified in families with a range of cardiac conditions, including dilated cardiomyopathy (DCM), supraventricular tachycardias (SVT), and cardiac conduction disease (CCD).[1–4] Our previous work demonstrated an increased burden of DCM and atrial fibrillation (AF) in individuals harboring TNNI3K missense variants.[1]

A rare *TNNI3K* variant (c.1615A > G; p.Thr539Ala) has previously been linked to congenital junctional ectopic tachycardia (CJET), an arrhythmia that arises from the atrioventricular (AV) node and His bundle.[4,5] This variant is located in the kinase domain of TNNI3K and has been shown to impact TNNI3K autophosphorylation negatively.[2] Unfortunately, our understanding of this variant and its link with CJET has been limited due to the small pedigree and limited genetic evidence.

In our study, we report on 13 individuals from a four-generational family who present with CJET and carried a novel ultra-rare variant in *TNNI3K* (c.1729C > T; p.Leu577Phe) associated with a significant loss-of-kinase-activity. Additionally, we have identified CJET in a family carrying *TNNI3K*-c.2225C > T (p.Pro742Leu). Collectively, our findings provide novel evidence supporting the role of genetic variants in *TNNI3K* as a contributing factor to CJET.

2 | METHODS

2.1 | Clinical study

Clinical and genetic studies were approved by the Medical Ethics Review Committee of the Amsterdam UMC or The Hospital for Sick Children's Research Ethics Board. Informed consent was obtained from all individuals of whom the clinical data are described. Patients provided general consent for DNA studies in the setting of the Amsterdam UMC Cardiogenetics biobank (BTC 2014-003#A201435) and the Hospital for Sick Children (REB #0020010161).

2.2 | Whole-exome sequencing

High quality DNA was extracted with Autopure LS (Qiagen) using the whole blood protocol and automated Puregene Chemistry at Genome Diagnostics Lab at the Department of Pediatric Laboratory Medicine, The Hospital for Sick Children, Toronto.

Genomic DNA (200 ng) was fragmented to ~200 bp using the Covaris S2. Sheared DNA was end-repaired and adenylated prior to ligation of adapters with a T-overhang. The genomic library was amplified by PCR using 10 cycles. Amplified genomic libraries were hybridized with biotinylated probes that target exonic regions including exon/intron boundaries. The enriched exome library was amplified by an additional 8 cycles of PCR and validated on a Bioanalyzer 2100. DNA size was determined with High Sensitivity Chip (Agilent Technologies) and DNA quantity was analyzed by qPCR using the Kapa Library Quantification Illumina/ABI Prism Kit protocol (KAPA Biosystems). The exome libraries were pooled and sequenced with the TruSeq sequencing chemistry V3 on the same lane of a Flowcell on a HiSeq2500 platform following Illumina's protocol. Approximately 5–7 gigabases of raw paired-end data of 100 bases were generated per exome library. Reads were trimmed for adaptor read-through and low quality using Trimomatic. Trimmed reads were aligned to the GRCh37/hg19 build human reference genome (with unplaced contigs and decoys) using BWA-MEM. PCR duplicates were marked using Picard-tools-1.112, and local re-alignment and base recalibration were performed using GATK 3.2.2. Finally, variants were identified using GATK haplotype caller 3.2.2.

2.3 | WES data analysis

Whole-exome sequencing (WES) resulted in ~94% recovery at >10× coverage. The WES data were analyzed with Golden Helix SNP and Variation Suite. Variant Call Format files were imported with quality metrics to identify shared variant (heterozygous or homozygous for autosomal dominant or autosomal recessive models, respectively) among affected individuals. All shared variants were filtered against publicly available exome sequencing databases including the Exome Aggregation Consortium database (http://exac.broadinstitute.org), NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS), and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP). Identified shared variants with a frequency less than 0.005% were validated and tested for segregation by Sanger sequencing.

2.4 | Sanger sequencing

Primers for each variant in *TNNI3K*, *CLASP2*, *CP*, *FAM83B*, *GYG1*, *HACE1*, and *RAD54L2* were designed using the PrimerQuest™ Tool from Integrated DNA Technologies (IDT) (Table S1). Primer specificity was confirmed using Primer-BLAST from the National Center for Biotechnology. PCR products were generated using a commercially available kit (Platinum SuperFi II PCR Master Mix, Invitrogen), then cleaned using a commercially available kit (PureLink™ PCR Purification Kit, Invitrogen), and subsequently submitted for Sanger sequencing using an ABI 3730 XL at The Centre for Applied Genomics (TCAG) at the SickKids Research Institute. Chromatograms were analyzed using FinchTV v.1.4.0 developed by Geospiza, Inc.

2.5 | Linkage analysis (LOD score)

A logarithm of the odds (LOD) score was calculated measuring the probability of genotype–phenotype linkage.[6] A LOD score of 3 or higher is deemed statistically significant. It indicates that the phenotype has a 1000 times greater likelihood (10^3) to result from genetic linkage of the variant to the phenotype, than chance. For this family we assumed an autosomal dominant inheritance, a penetrance of 0.7 (average penetrance of cardiovascular diseases), and a disease incidence of 0.0001.

2.6 | cDNA constructs and mutagenesis

The plasmid containing the FLAG-tagged human wild-type (WT) *TNNI3K* cDNA (NM_015978.3; cloned into a pRK5 vector) was provided by Dr. Hao Tang and Prof. Douglas Marchuk (Duke University School of Medicine, Durham, NC). Site-directed mutagenesis was performed with the Quick Change XL kit (Agilent Technologies, Santa Clara, USA) to introduce the *TNNI3K*-c.1729C > T (p.Leu577Phe) to the *TNNI3K*-WT cDNA. The mutagenesis primers are summarized in Table S2.

2.7 | Cell culture and transfection

HEK293A cells were grown in a 6-well plate in DMEM (21969–035, Gibco, USA) supplemented with 10% FBS (Biowest, France), 1% penicillin–streptomycin (Gibco, USA), and 1% L-glutamine (Gibco, USA) in a 5% CO_2 incubator at 37°C. Cells were transfected at 70% confluency with 2 µg *TNNI3K*-WT cDNA or *TNNI3K* variant cDNA using 6 µL Lipofectamine (Invitrogen, Carlsbad, USA). After 2 days, the protein was extracted with RIPA lysis buffer (0.1% SDS, 1% Triton, 50 mM Tris–HCl (pH = 8), and 150 mM NaCl, 0.5% sodium deoxycholate) supplemented with protease inhibitor cocktail (Roche, CH), and phosphatase inhibitors 4 mM phenylmethylsulfonyl fluoride and 0.5 mM sodium orthovanadate (Sigma-Aldrich, USA).

2.8 | Western blot

Western blot was performed as described previously.[1] Briefly, protein samples were heated to T = 95°C in 1x Laemmli sample buffer. 25 µg protein was loaded onto gradient Mini-PROTEAN TGX Precast gels (4%–20%, Biorad, USA). Proteins were transferred onto a PVDF membrane using a semi-dry blotting system (Biorad, USA). Blocking of the membranes was performed with either 3% milk (Protivar, Nutricia, Netherlands) or 3% BSA (A7888, Sigma, USA) in TBST for 1 h. TNNI3K protein expression was determined using an anti-FLAG mouse M2 antibody (F3165 (1:500), Sigma, USA) in 3% milk. Autophosphorylated TNNI3K protein was detected by anti-phospho-tyrosine mouse antibody PY99 (sc-7020 (1:100), Santa Cruz Biotechnology, USA) in 3% BSA. The loading control GAPDH was identified with an anti-GAPDH mouse antibody (10R-G109a (1:10000), Fitzgerald, USA) diluted in 3% milk. Primary antibodies were incubated overnight at T = 4°C. The membranes were washed three times for 15 min with TBST, then subjected to the anti-mouse horseradish peroxidase-conjugated secondary antibody (NA9310V, GE HealthCare, UK) for 1 h at room temperature. The Western blot signal was detected using Amersham ECL Prime Blotting Detection Reagent (GE HealthCare, UK). Protein expression was visualized by the LAS-4000 Lite system (Fujifilm). Analysis was performed with the ImageLab 6.0.1 software (BioRad, USA). Phospho-tyrosine and FLAG expression levels were both normalized by GAPDH. Autophosphorylation values of TNNI3K variants were normalized to TNNI3K-WT.

2.9 | Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). The data were normally distributed. Comparison of autophosphorylation levels was performed with one-way repeated measures analysis of variance (ANOVA) with Dunnett's posthoc correction. The threshold for significance was defined as $p < 0.05$.

3 | RESULTS

3.1 | Clinical data of two CJET families

3.1.1 | Family 1: TNNI3K-p.Leu577Phe

Whole-exome sequencing in three family members (I-2, III-3, and IV-2) from a four-generational family with CJET revealed a shared variant (p.Leu577Phe) in TNNI3K (Figure 1A and Table 1). Subsequently, DNA samples were collected from 23 family members to confirm and expand segregation with Sanger sequencing. We identified 16 confirmed carriers and 2 obligate carriers of *TNNI3K*-c.1729C > T (p.Leu577Phe) (NM_015978.2; rs201010209). This ultra-rare variant has been reported once in the population-based Genome Aggregation Database[7] (gnomAD; v4; ENST00000326637.8) with a minor allele frequency (MAF) of 0.000069%. In silico prediction models of variant

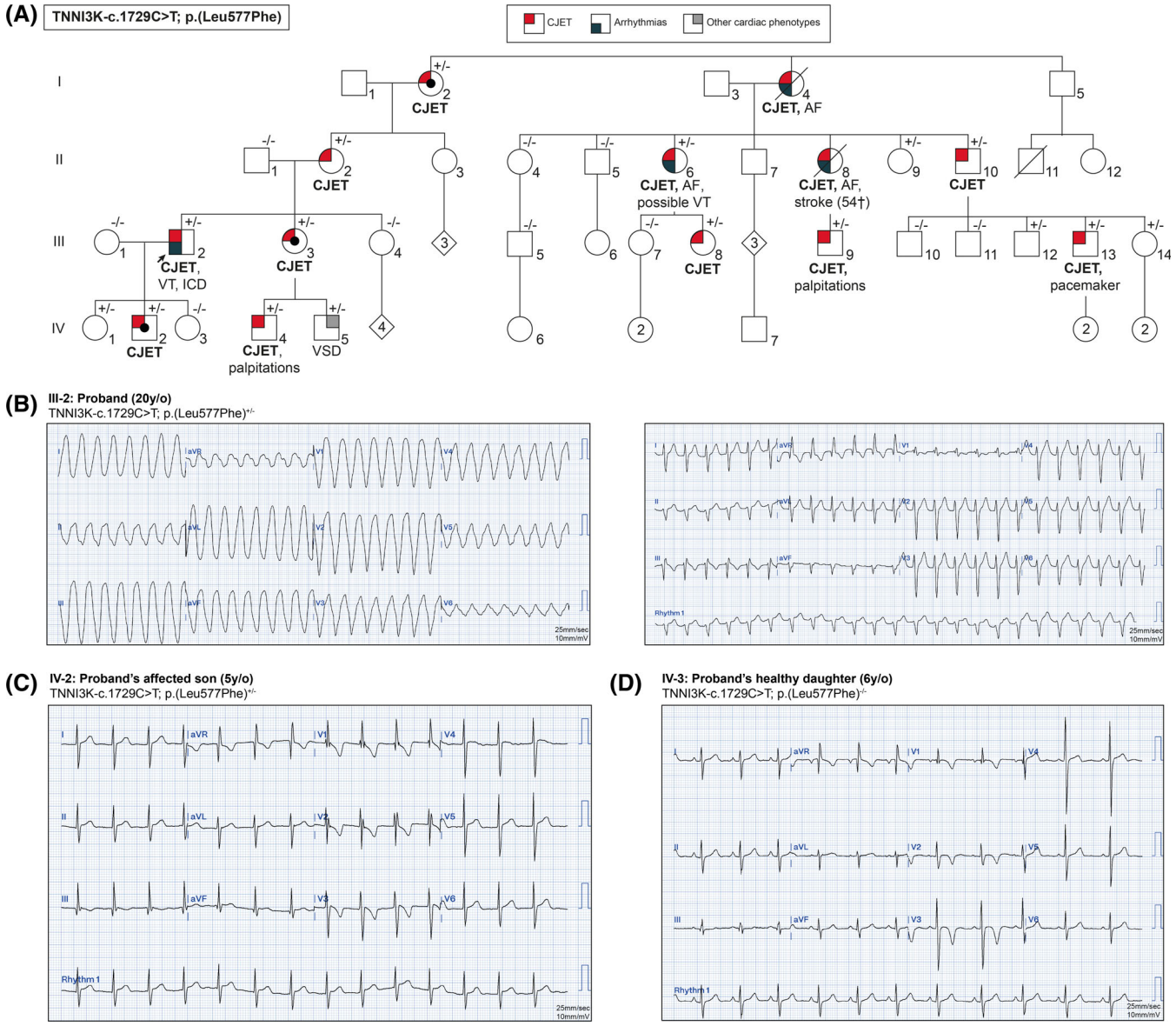

FIGURE 1 TNNI3K-p.Leu577Phe pedigree and representative ECG recordings. (A) Heterozygous TNNI3K-p.Leu577Phe variant carriers are indicated with +/−. Whole-exome sequencing was performed in the individuals marked with a closed black central circle. The proband is annotated with an arrow. (B) ECG recording of the proband (III-2) at the age of 20 years showing an episode of VT (left panel). The right panel shows an ECG recording after an ICD shock, demonstrating CJET (absent P-waves, HR = 139 bpm), RBBB (QRS = 121 ms), a slight ST elevation, and a prolonged QT interval (QTc = 480 ms). (C) First ECG of proband's affected son (IV-2) at the age of 5 years. The absence of P-waves is consistent with the CJET phenotype. (D) Normal ECG of proband's healthy daughter (IV-3) at the age of 6 years. She is a non-carrier. ECGs were reformatted with PMcardio Digitize (Powerful Medical s.r.o., Bratislavska, Slovakia). The paper speed of all depicted ECGs is 25 mm/s. †: Deceased, AF, Atrial fibrillation; CJET, congenital junctional ectopic tachycardia; ICD, implantable cardioverter-defibrillator; VSD, ventricular septal defect; VT, ventricular tachycardia. [Colour figure can be viewed at wileyonlinelibrary.com]

pathogenicity using the Combined Annotation Dependent Depletion (CADD, GRCh38-v1.6), sorting intolerant from tolerant (SIFT) and polyphen-2 models estimated *TNNI3K*-c.1729C > T (p.Leu577Phe) as a deleterious variant, with scores of 29.2, 0, and 1.0, respectively.[8]

Among the variant carriers, 13 individuals manifested CJET, which accounts for 72% of all carriers. Other observed cardiac phenotypes included atrial fibrillation (AF), right bundle branch block (RBBB), ventricular tachycardia (VT), ventricular septal defect, and palpitations. The non-carriers in this family had no known cardiac phenotypes. We computed the LOD score to evaluate the linkage between the genotype and the phenotype. Out of the 28 individuals with a genotype (26 DNA-tested and 2 obligate carriers), 23 exhibited genotype–phenotype matches, while five individuals displayed a genotype–phenotype mismatch (non-penetrant carriers). We assumed an autosomal dominant inheritance, a penetrance of 0.7, and a disease incidence of 0.0001. This yields a calculated LOD score of 3.15, signifying a statistically significant association between the observed phenotype and the variant.

TABLE 1 Individual characteristics of family members.

Fam.	ID	TNNI3K variant	Sex	Age (years)	Phenotype	Treatment/monitoring
1	I-2 (WES)	+/− (p.Leu577Phe)	F	101[a]	CJET	N/A
1	I-4	Obligate Carrier	F	[a]	CJET, AF	Pacemaker
1	II-1	−/− (p.Leu577Phe)	M	74	Healthy	
1	II-2	+/− (p.Leu577Phe)	F	74	CJET	
1	II-4	−/− (p.Leu577Phe)	F	72	Healthy	N/A
1	II-5	−/− (p.Leu577Phe)	M	62	Healthy	N/A
1	II-6	+/− (p.Leu577Phe)	F	68	CJET, pVT, AF	Ablation
1	II-8	Obligate Carrier	F	54[a]	CJET, pVT, AF, stroke	
1	II-9	+/− (p.Leu577Phe)	F	66	Healthy	
1	II-10	+/− (p.Leu577Phe)	M	70	CJET	
1	III-1	−/− (p.Leu577Phe)	F	50	Healthy	
1	III-2 (P)	+/− (p.Leu577Phe)	M	45	CJET, VT, mild AF	Ablation, ICD
1	III-3 (WES)	+/− (p.Leu577Phe)	F	52	CJET	Ablation
1	III-4	−/− (p.Leu577Phe)	F	49	Healthy	
1	III-5	−/− (p.Leu577Phe)	M	42	Healthy	
1	III-7	−/− (p.Leu577Phe)	F	43	Healthy	
1	III-8	+/− (p.Leu577Phe)	F	41	CJET	
1	III-9	+/− (p.Leu577Phe)	M	31	CJET, palpitations	
1	III-10	−/− (p.Leu577Phe)	M	41	Healthy	
1	III-11	−/− (p.Leu577Phe)	M	38	Healthy	
1	III-12	+/− (p.Leu577Phe)	M	47	Healthy	
1	III-13	+/− (p.Leu577Phe)	M	42	CJET	Pacemaker age 16
1	III-14	+/− (p.Leu577Phe)	F	45	Healthy	
1	IV-1	+/− (p.Leu577Phe)	F	17	Healthy	Holter ECG
1	IV-2 (WES)	+/− (p.Leu577Phe)	M	15	CJET	
1	IV-3	−/− (p.Leu577Phe)	F	9	Healthy	
1	IV-4	+/− (p.Leu577Phe)	M	25	CJET, palpitations	Holter ECG
1	IV-5	+/− (p.Leu577Phe)	M	22	VSD	Holter ECG
2	I-1	−/− (p.Pro742Leu)	M		Healthy	
2	I-2	+/− (p.Pro742Leu)	F	64	Healthy	
2	II-1	−/− (p.Pro742Leu)	F		Healthy	
2	II-2	+/− (p.Pro742Leu)	F	32	Healthy	
2	II-3	+/− (p.Pro742Leu)	M	34	Healthy	
2	II-4	−/− (p.Pro742Leu)	F	33	Healthy	
2	III-1 (P)	+/− (p.Pro742Leu)	M	3	CJET	

Abbreviations: AF, atrial fibrillation; CJET, congenital junctional ectopic tachycardia; ICD, implantable cardioverter-defibrillator; P, proband; pVT, possible ventricular tachycardia; VSD, ventricular septal defect; VT, ventricular tachycardia; WES, whole-exome sequencing.
[a]Deceased.

The three individuals who underwent WES exhibited additional shared missense variants of uncertain significance in the following genes: *CLASP2*, *CP*, *FAM83B*, *GYG1*, *HACE1*, and *RAD54L2* (Table S3), but testing of other affected family members demonstrated that none of these variants co-segregated with the CJET phenotype (Table S4).

The proband (III-2) is a 42-year-old male and is a carrier of the TNNI3K-p.Leu577Phe variant. At the age of 16, he was diagnosed with ventricular tachycardia (VT) and CJET. He underwent a failed ablation followed by an implantable cardioverter-defibrillator (ICD) implantation. His ECG recording at age 20 showed absent P-waves, RBBB (QRS = 121 ms), slight ST elevation, and QT prolongation (QTc = 480 ms) (Figure 1B). One episode of CJET progressed to VT. Generally, he experiences paroxysmal VT episodes and requires cardioversion approximately monthly. Two out of three of his children also carry the variant. His son (IV-2) was diagnosed with CJET at age 5 (Figure 1C), which seemed to worsen during exercise and/or fever.

An echocardiographic examination at the age of 12 did not show any cardiac abnormalities. The proband's daughters, one carrier (IV-1) and one non-carrier (IV-3, Figure 1D), show no symptoms. The proband's sister (III-3) is also a carrier and was diagnosed with CJET at the age of 25. She underwent a successful ablation. Her two children (IV-4 and IV-5) both carry the same variant in *TNNI3K*. Her son (IV-4) has been diagnosed with CJET at age 22. His brother (IV-5) manifested a ventricular septal defect, but no cardiac arrhythmias. Both the proband's father (II-2) and the grandmother (I-2) manifested CJET and tested positive for the variant.

On the other branch of the family, we again detected multiple incidences of CJET. The sister of the proband's grandmother (I-4) was reported to have had CJET and AF. Investigation of her daughter (II-6), also a carrier, revealed CJET, AF, and possible VT. She underwent a successful ablation. Her daughter (III-8) is a carrier of the *TNNI3K* variant and also manifests CJET. Her sister tested negative for the variant has no cardiac disease. Another daughter of I-4 manifested CJET and AF. She passed away at age 54 due to a stroke. Her son is a carrier and experienced cardiac palpitations and CJET. Although II-9 also tested positive for the *TNNI3K* variant, she is asymptomatic. Her brother, however, was diagnosed with CJET and carries the TNNI3K-p.Leu577Phe variant. All his five children were genetically tested for the variant in *TNNI3K*. Three siblings (III-12, III-13, and III-14) tested positive for the variant, of whom two (III-12 and III-14) were asymptomatic. The other sibling (III-18) was diagnosed with CJET and received a pacemaker at age 16.

3.1.2 | Family 2: TNNI3K-p.Pro742Leu

In addition to the TNNI3K-p.Leu577Phe variant, another variant in *TNNI3K* has been found in combination with a CJET phenotype. We here report *TNNI3K*-c.2225C > T (p.Pro742Leu) (NM_015978.2; rs1210821808) in a small family in The Netherlands (Figure 2A and Table 1). TNNI3K-c.2225C > T (p.Pro742Leu) is an ultra-rare variant that has been found in three individuals of European ancestry heterozygously in gnomAD, resulting in a MAF of 0.00019%. Pathogenicity prediction shows mixed results with deleterious CADD and SIFT scores of 26.4 and 0.01, respectively, but likely benign Polyphen-2 score of 0.444.

The proband (III-1) is a carrier of the *TNNI3K* variant and was referred with fetal tachycardia of 220/min at 36 weeks gestation. A JET with 1:1 ventriculo-atrial (VA) conduction was suspected on the M-mode fetal echocardiography (Figure 2B). The arrhythmia was controlled with sotalol prenatally and the baby was born in sinus rhythm. He then presented at 10 days of age with a narrow-complex tachycardia of 180 bpm with retrograde P waves after the QRS complex and the diagnosis of JET was confirmed with intravenous adenosine administration eliciting VA dissociation with a ventricular rate faster than the atrial rate (Figure 2C,D). Subsequent treatment with sotalol and flecainide failed, but he responded very well to ivabradine with complete conversion to sinus rhythm.

The proband additionally harbors another variant of uncertain significance in the *PRKAG2* (c.1195C > T; p.Leu399Phe) gene, encoding for protein kinase AMP-activated non-catalytic subunit gamma 2. The additional *PRKAG2* variant is absent from gnomAD (v4; ENST00000287878.9) and is predicted to be highly damaging with CADD, SIFT, and Polyphen-2 scores of 26.9, 0, and 0.98, respectively. His father (II-3), aunt (II-2), and grandmother (I-2) carry the *TNNI3K* variant but do not exhibit any cardiac abnormalities (Figure 2E–G). The proband's mother (II-4) carries the PRKAG2 variant but not the TNNI3K variant and is phenotypically unaffected (Figure 2H).

3.2 | Reduced TNNI3K protein kinase activity in variants linked to CJET

The TNNI3K protein consists of four domains: a coiled-coil domain, an ankyrin repeats domain, a kinase domain, and a serine-rich domain (Figure 3A). The TNNI3K-p.Leu577Phe missense variant is located in the kinase domain, whereas TNNI3K-p.Pro742Leu is located in the serine-rich domain. The affected residues Leu577 and Pro742 are highly conserved among various species, indicating their importance in evolution (Figure 3B). Previous studies have indicated autophosphorylation levels as an indicator of kinase function (Figure 3A).[1,2] Using the same approach, we investigated the autophosphorylation of the TNNI3K-p.Leu577Phe variant and found nearly abolished autophosphorylation levels compared to TNNI3K-WT (Figure 3C,D). In our previous work, we evaluated autophosphorylation levels of various TNNI3K variants.[1] There we found a decreased, but not abolished, kinase activity for TNNI3K-p.Pro742Leu.

4 | DISCUSSION

Genetic missense variants in *TNNI3K* have been associated with DCM, atrial fibrillation, and SVT.[1] Previously, Xi, et al. presented the first family with CJET as the main phenotype harboring the TNNI3K-p.Thr539Ala variant with reduced kinase activity.[2,4] However, this was shown in a relatively small family with limited genetic testing. Podliesna, et al. have also reported several cases of CJET in combination with other cardiac phenotypes in families carrying the TNNI3K-p.Glu768Lys variant. Nonetheless, additional evidence for TNNI3K-related CJET would be valuable.

We here present a multigenerational family with CJET harboring a novel ultra-rare *TNNI3K* variant with total loss-of-kinase-function: *TNNI3K*-c.1729C > T (p.Leu577Phe). Of all 18 variant carriers, 13 individuals presented with CJET, resulting in a genetic penetrance of 72%. The disease manifests at an early age, with the youngest patient diagnosed with CJET at the age of 5. Thus far, only high penetrant variants in *TNNI3K* have been published. However, only large families of gain-of-function *TNNI3K* variants have been described, the loss-of-kinase-function has thus far only been seen in smaller families in which detection of non-penetrance is difficult to assess.[1,2] Considering the high co-segregation rate, functional studies, population

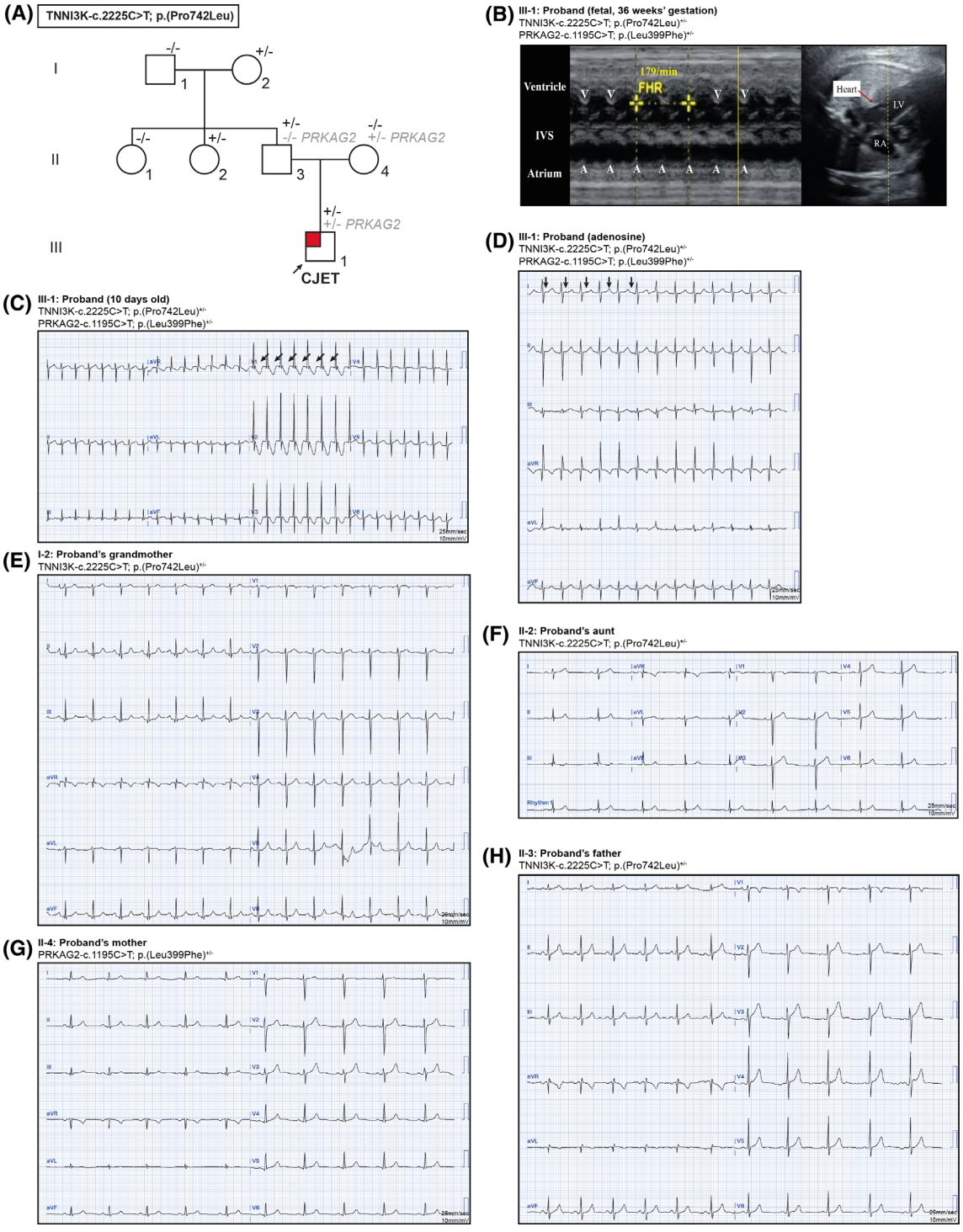

FIGURE 2 TNNI3K-p.Pro742Leu pedigree and representative ECG recordings. (A) Heterozygous TNNI3K-p.Pro742Leu and PRKAG2-p.Leu399Phe variant carriers are indicated with +/− in black and gray, respectively. The proband is annotated with an arrow. CJET: Congenital junctional ectopic tachycardia. (B) M-Mode through the left ventricle (LV) and right atrium (RA) made at presentation at 36 weeks gestation. The ventricular (V) and atrial contractions (A) are almost simultaneous (highlighted by the solid vertical yellow line). The fetal heart rate (FHR) is rather slow for a typical fetal supraventricular tachycardia and is in keeping with the diagnosis of CJET with 1:1 V-A conduction. On the right the four-chamber view of the fetal heart is seen. The dotted yellow line corresponds to the M-mode cut through the heart used in the recording. (IVS = interventricular septum). (C) ECG at age 10 days of CJET with 1:1 VA conduction showing retrograde P-waves directly after the QRS complex (black arrows in V1). (D) ECG during intravenous adenosine showing V: A dissociation with a slower rate of the P-waves (black arrows) confirming the diagnosis CJET. (E–H) ECG recordings of healthy family members. ECGs were reformatted with PMcardio Digitize (Powerful Medical s.r.o., Bratislavska, Slovakia). The paper speed of all depicted ECGs is 25 mm/s. [Colour figure can be viewed at wileyonlinelibrary.com]

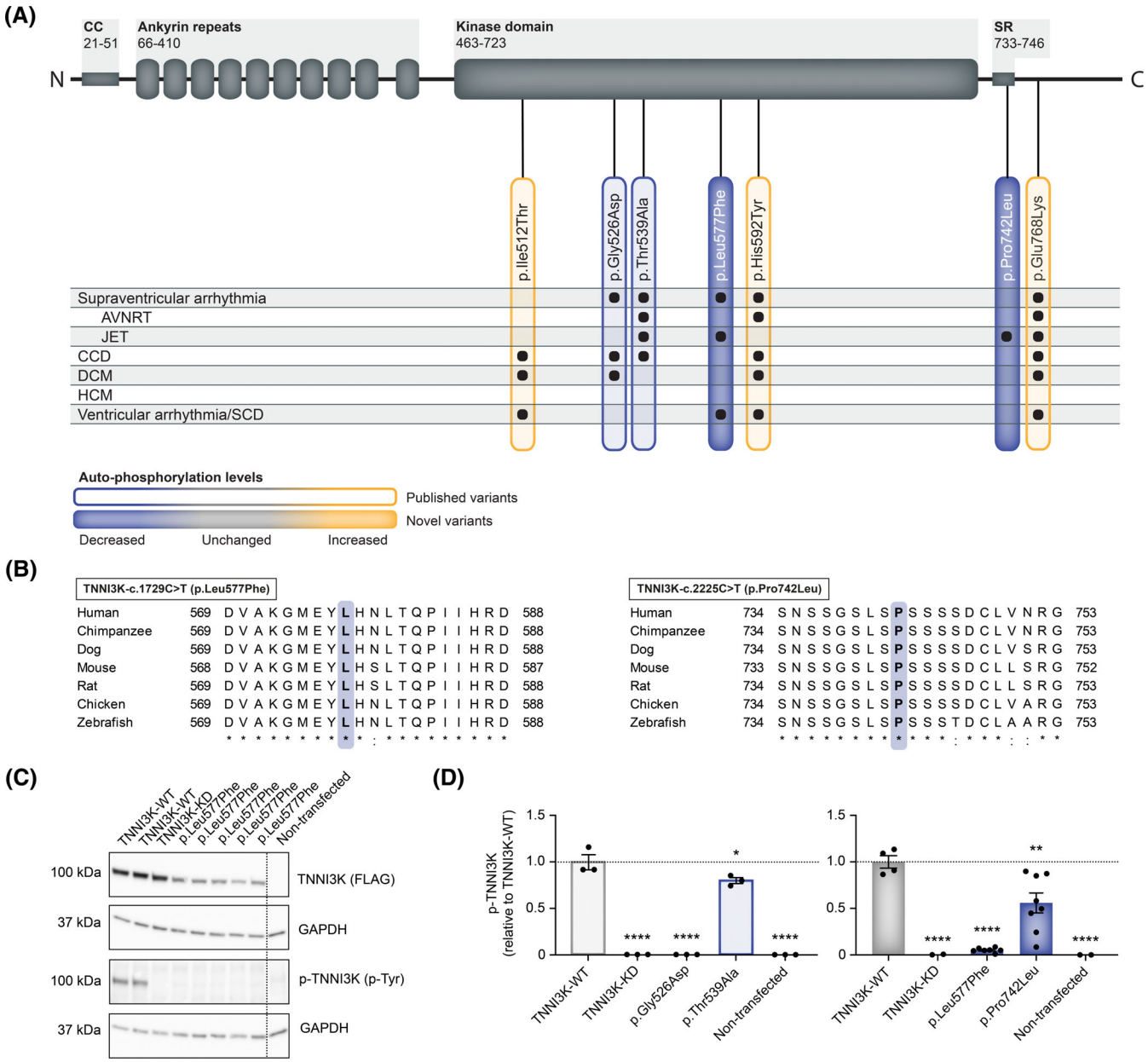

FIGURE 3 Characteristics and autophosphorylation assay of the TNNI3K-p.Leu577Phe and p.Pro742Leu variant. (A) Schematic illustration of the TNNI3K protein with the locations of TNNI3K-p.Leu577Phe and p.Pro742Leu (closed bar) and other previously published variants in TNNI3K (open bars). Phenotypes seen in variant carriers are indicated with black circles. Increased and decreased autophosphorylation levels are indicated in yellow and purple, respectively. AVNRT, atrioventricular nodal reentry tachycardia; CCD, cardiac conduction disease; CJET, congenital junctional ectopic tachycardia; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; SCD, sudden cardiac death. (B) Conservation of the L577 and P742 (highlighted in purple) residues among various species. Identical residues are indicated with *. Non-identical residues are indicated with :. (C) Representative Western blot of the TNNI3K-p.Leu577Phe variant, including TNNI3K-WT, TNNI3K-kinase-dead (KD), and non-transfected lysates. FLAG-tagged TNNI3K was detected by anti-FLAG. Phosphorylated TNNI3K was detected by a p-tyrosine antibody. GAPDH was used as a loading control. The dotted line indicates a cut in the Western blot membrane. (D) Left panel shows autophosphorylation levels of previous reported TNNI3K variants (data from Reference 2). Right panel shows autophosphorylation levels of TNNI3K-p.Leu577Phe ($n = 7$), TNNI3K-p.Pro742Leu ($n = 8$, data from Reference 1), TNNI3K-KD ($n = 2$), and non-transfected controls ($n = 2$) relative to TNNI3K-WT ($n = 4$). One-way ANOVA (Dunnett's vs. TNNI3K-WT): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. [Colour figure can be viewed at wileyonlinelibrary.com]

prevalence, and deleteriousness prediction models, *TNNI3K*-c.1729C > T can be classified as pathogenic following the American College of Medical Genetics and Genomics (ACMG) scoring system.[9] We next report CJET in one patient, diagnosed in utero, from a small family harboring an additional loss-of-kinase-function variant in *TNNI3K* (c.2225C > T; p.Pro742Leu) along with a variant in *PRKAG2* (c.1195C > T; p.Leu399Phe). Two reported cases of *TNNI3K*-c.2225C > T carriers on ClinVar yielded no additional information.

Given the absence of clear segregation in the *TNNI3K*-c.2225C > T family, this variant is classified as a variant of unknown significance based on the ACMG score. Genetic variants in *PRKAG2* have been associated with Wolff-Parkinson-White syndrome and hypertrophic cardiomyopathy,[10] but not with CJET thus far. As the inheritance in this family is not unequivocal (each variant is inherited from a phenotype-negative parent), the interaction between the variants cannot be excluded.

The direction of effect in terms of TNNI3K kinase activity is still ambiguous. Earlier reports by us and others indicate a clear pathogenicity of gain-of-function kinase variants.[1,2] The role of variants with reduced kinase function, however, has thus far remained unclear. Due to the presence of homozygous loss-of-function (i.e., truncating variants) *TNNI3K* variant carriers in the general population, haplo-insufficiency is unlikely to be pathogenic.[1,11] And yet, two previously described TNNI3K variants (p.Gly526Asp and p.Thr539Ala), and the here-reported TNNI3K-p.Leu577Phe and TNNI3K-p.Pro742Leu demonstrated a reduced kinase function. It is important to note that these variants are not predicted to lead to haplo-insufficiency. The associated phenotypes with these loss-of-kinase-function are divergent (as is the case with gain-of-kinase-function variants): TNNI3K-p.Gly526Asp variant carriers mainly demonstrate DCM without junctional arrhythmias, whereas individuals carrying TNNI3K-p.Leu577Phe are suffering from CJET without any signs of DCM at an older age. It is important to note that thus far no out-of-hospital cardiac arrests or ventricular fibrillation have been observed in the loss-of-kinase-function families in contrast to the gain-of-function families suggesting that the ventricular phenotype could be milder. The explanation of the observed mixed phenotypes remains elusive due to a lack of understanding of the TNNI3K disease mechanism, primarily due to its unknown signaling pathways. Further studies are crucial to unravel the downstream interactors of TNNI3K.

The reduced autophosphorylation level observed in TNNI3K-p.Pro742Leu is comparable to the previously documented p.Thr539Ala variant. Similarly, patient III-1 and two previously reported individuals from the p.Thr539Ala family exhibit CJET during the immediate postnatal period. In contrast to the findings of Xi et al., the arrhythmia in III-1 persisted without resolution either spontaneously or after beta-blocker administration. Instead, ivabradine was effective in resolving his arrhythmia. Moreover, it is important to highlight that Xi et al. solely tested for additional variants in *SCN5a*, yielding negative results and potentially neglecting the presence of other variants.

Altogether, we here report novel evidence for CJET as a primary consequence of TNNI3K-p.Leu577Phe, a missense variant that results in a total loss-of-kinase-function. The molecular mechanism underlying TNNI3K-driven cardiac pathologies remain to be investigated to explain the relationship between altered kinase activity and cardiac diseases.

AUTHOR CONTRIBUTIONS

Conceptualization: C.P., R.M.H., E.M.L.; Clinical data collection and interpretation: T.T.K., J.M.V., N.A.B., M.B., S-A.B.C., D.Q.C.M.B-S., R.M.H.; Experimental investigation: C.P., V.N.S., K.M., K.C.A.P.; Writing original draft: C.P., E.M.L.; All authors read, reviewed, and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/cge.14504.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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