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# CLINICAL AND GENETIC ANALYSIS OF RARE ION CHANNEL DISEASES

PhD Thesis

**János Borbás, M.D.**

Supervisor:

Róbert Sepp, MD, PhD, DSc

Department of Internal Medicine - Cardiology Center
Faculty of Medicine
University of Szeged

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# LIST OF PUBLICATIONS

**Publications directly related to the thesis:**

1. Sepp R, Hategan L, Bácsi A, Cseklye J, Környei L, **Borbás J**, Széll M, Forster T, Nagy I, Hegedűs Z. Timothy syndrome 1 genotype without syndactyly and major extracardiac manifestations. *Am J Med Genet A* 2017; 173(3):784-789. doi: 10.1002/ajmg.a.38084. (Q2, IF: 2.264)

2. **Borbás J**, Vámos M, Hategan L, Hanák L, Farkas N, Szakács Zs, Csupor D, Tél B, Kupó P, Csányi B, Nagy V, Komócsi A, Habon T, Hegyi P, Sepp R. Geno- and phenotypic characteristics and clinical outcomes of *CACNA1C* gene mutation associated Timothy syndrome, "cardiac only" Timothy syndrome and isolated long QT syndrome 8: A systematic review. *Front Cardiovasc Med* 2022; 9:1021009. doi: 10.3389/fcvm.2022.1021009. (Q1, IF: 3,6)

3. Déri Sz,* **Borbás J**,* Hartai T, Hategan L, Csányi B, Visnyovszki Á, Madácsy T, Maléth J, Hegedűs Z, Nagy I, Arora R, Labro AJ, Környei L, Varró A, Sepp R, Ördög B. Impaired cytoplasmic domain interactions cause co-assembly defect and loss of function in the p.Glu293Lys *KNCJ2* variant isolated from an Andersen-Tawil Syndrome patient. *Cardiovasc Res* 2021; 117:1923–1934. doi:10.1093/cvr/cvaa249 (D1, IF: 13,081).*Shared first authorship.

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1. **Borbás J**, Hategan L, Vámos M, Hanák L, Szakács Zs, Hegyi P, Sepp R. *CACNA1C* -génmutáció asszociált kórképek klinikai jellemzői és lefolyása. Clinical characteristics and progression of *CACNA1C* gene mutation associated syndromes. *Cardiologia Hungarica* 2020; 50: Suppl. D pp. D151-D151.

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# LIST OF ABBREVIATIONS

ABPM: Ambulatory Blood Pressure Monitor, usually 24 Hours

ACA: aborted cardiac arrest

ACMG/AMP: American College of Medical Genetics and Genomics and the Association for Molecular Pathology

AED: Automated External Defibrillator

*ANK2:* Ankyrin 2

AP: action potential

ATS: Andersen-Tawil syndrome

AV: Antrio-ventricular

B: Benign

BS: Brugada syndrome

*CACNA1C*: calcium voltage-gated channel subunit alpha1 C

*CASQ2:* Calsequestrin 2

CD-I: cytoplasmic domain interface

CDI: calcium-dependent inactivation

CK: creatine-phosphokinase, CK-MB: creatine-phosphokinase MB isoenzyme

COTS: cardiac only Timothy syndrome

CPVT: catecholaminergic polymorphic ventricular tachycardia

DI: domain I

DNA: Deoxyribonucleic acid

dbSNP: Single Nucleotide Polymorphism Database

ECG: electrocardiogram

ERS: early repolarization syndromes

ES: extrasystole

HGNC: HUGO Gene Nomenclature Committee

ICD: implantable cardioverter defibrillator

$I_{K1:}$ cardiac inwardly rectifying potassium current

$I_{Na:}$ inward sodium current

JLNS: Jervell and Lange-Nielsen Syndrome

*KCNJ2*: potassium inwardly rectifying channel subfamily J member 2

*KCNQ1*: Potassium Voltage-Gated Channel Subfamily Q Member 1

LB: likely benign

LP: likely pathogenic
LQTS: long QT syndrome
MACE: major adverse cardiac events
M-CHAT: Modified Checklist for Autism in Toddlers
MRI: magnetic resonance imaging
NanoBiT: NanoLucVR Binary Technology
NSVT: non-sustained ventricular tachycardia
NY: New York
PIP2: phosphatidilinositol-4,5-bisphosphate
RyR2: ryanodine receptor 2
P: pathogenic
PM: pacemaker
PVB: premature ventricular beats
PVC: premature ventricular contractions
RNA: ribonucleic acid
QTc: corrected QT interval of the ECG
QUc: corrected QU interval of the ECG
*SCN4A*: Sodium Voltage-Gated Channel Alpha Subunit 4
SCD: sudden cardiac death
SSI: steady-state inactivation
SQTS: short QT syndrome
SZTE: University of Szeged
TS: Timothy syndrome, TS1: type 1, TS2: type 2
TdP: torsades de pointe arrhythmia
UK: United Kingdom
USA: United States of America
VDI: voltage-dependent inactivation
VES: ventricular extrasystole
VF: ventricular fibrillation
VT: ventricular tachycardia
VUS: variant of unknown significance
WT: wild-type

# 1. INTRODUCTION

## 1.1 Channelopathies

Channelopathies constitute a diverse group of disorders resulting from the malfunction of ion channel subunits or their associated proteins. These disorders can have genetic origins or be acquired through other medical conditions, pharmacological agents, or environmental toxins. The predominant cause of channelopathies is mutations in genes encoding ion channels, leading to impaired channel function.(1) There are over 400 genes that encode ion channels, which are present in all human cell types and play crucial roles in virtually every physiological process.(2) Each ion channel type is a multimeric complex composed of multiple subunits, each encoded by different genes. The location of the mutation within these genes can influence various aspects of the channel, including its gating mechanisms, conductance properties, ion selectivity, or signal transduction pathways. As a result, the functional impact of channelopathies is highly variable, depending on the expression location of ion channels, specific mutation and its effect on the ion channel's performance and regulation.

Cardiac myocytes are specialized excitable cells capable of generating and propagating an action potential (AP), an electrical signal that culminates in myocardial contraction. The genesis of a cardiac AP is driven by orchestrated ion fluxes across the cell membrane, transitioning the cell from a quiescent state to an activated state (depolarization) and subsequently returning it to the resting membrane potential (repolarization). Each phase of this process is the result of a finely tuned interplay of multiple voltage-gated ion channels. In contractile cardiomyocytes, the initiation of the AP is characterized by a rapid influx of sodium ions ($Na^+$), producing an inward current ($I_{Na}$) that elevates the membrane potential from its resting level (−90 mV) to a peak depolarization (+20 mV). This depolarization phase is succeeded by the efflux of potassium ions ($K^+$) through an outward current designated Ito, which marks the commencement of cellular repolarization.(3)

Cardiac channelopathies arise from mutations in genes (either loss-of-function or gain-of-function) related to various cardiac membrane channels (such as in long QT syndrome [LQTS], short QT syndrome [SQTS], and Brugada syndrome [BS]) or cellular structures impacting $Ca^{2+}$ availability (as seen in certain forms of catecholaminergic polymorphic ventricular tachycardia [CPVT]). Most of these channelopathies follow an autosomal dominant inheritance pattern with variable expressivity, though one form of LQTS, known as Jervell and Lange-Nielsen

Syndrome (JLNS), is autosomal recessive. Different syndromes are typically triggered by distinct factors, although this can vary within the same syndrome depending on the specific mutation, as observed in LQTS. Early symptoms often include palpitations, syncope, near-syncope, seizures, and sudden cardiac death (SCD). While many channelopathies present characteristic electrocardiogram (ECG) findings, these indicators may not always be consistently present.

While symptoms of some ion channel diseases, like LQTS, SQTS, BS or CPVT, are mainly confined to the heart, there are several channelopathies, like Andersen-Tawil syndrome (ATS) or Timothy syndrome (TS), in which symptoms may affect multiple organs and the underlying mutation may lead to a multisystemic disorder. Andersen-Tawil syndrome, caused by mutations in the *KCNJ2* gene and Timothy syndrome, caused by mutations in the *CACNA1C* gene, are such a rare conditions affecting several organ systems, with cardiac phenotypic manifestations including QT prolongation and ventricular arrhythmias.

## 1.2. Andersen-Tawil syndrome

Andersen-Tawil syndrome (ATS) is an autosomal dominant multisystem disorder characterized by periodic paralysis, ventricular arrhythmias, and distinctive facial and skeletal deformities. Additionally, hypoplastic kidneys and valvular diseases have been described in association with ATS. The disorder is notable for its significant intrafamilial variability and incomplete penetrance.(4)

Physical abnormalities associated with ATS prominently affect the head, face, and lower limbs. These features often include disproportionally small mandible (micrognathia), low-set ears, and finger curvature abnormalities known as clinodactyly. (Figure 1)

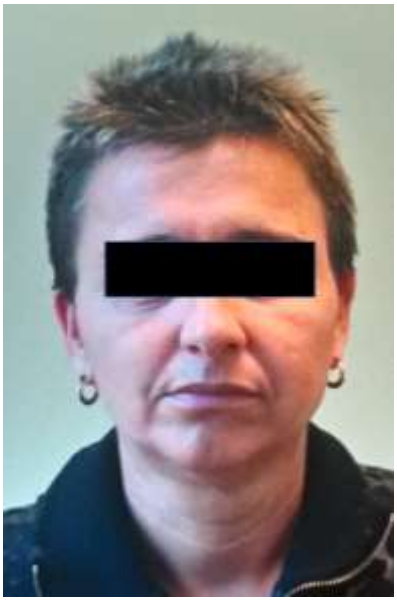

Figure 1: Craniofacial abnormalities of Andersen-Tawil syndrome which include low-set ears, widely spaced eyes, hypertelorism, broad forehead, broad nasal bridge, micrognathia, and short stature.

Among the cardiac manifestations of ATS, the most common are the electrocardiogram (ECG) abnormalities. Some patients exhibit mild QTc prolongation; however, the most characteristic features are the significant prolongation of the QU interval and the presence of pronounced U waves. These U waves are typically broad and tall, most prominently seen in the precordial V2-3 leads. The arrhythmias observed in ATS include frequent ventricular extrasystoles, bidirectional couplets, and polymorphic ventricular tachycardias (VT), which can be either sustained or non-sustained (Figure 2 and Figure 9).

In Andersen-Tawil syndrome type 1, the condition is linked to a loss-of-function mutation in the *KCNJ2* gene, which encodes the Kir2.1 inward rectifier potassium channel. The flow of potassium ions through this channel is critical for maintaining the normal membrane potential in both cardiac and skeletal muscles. Andersen-Tawil syndrome type 2 presents with identical clinical symptoms to ATS1, but the genetic defect remains unidentified. Consequently, ATS2 encompasses all ATS cases where genetic testing does not detect a mutation in *KCNJ2*. The loss-of-function mutations in *KCNJ2* in ATS1 affect the excitability of both skeletal and cardiac muscle, leading to the cardiac arrhythmias and periodic paralysis characteristic of the syndrome.(5)

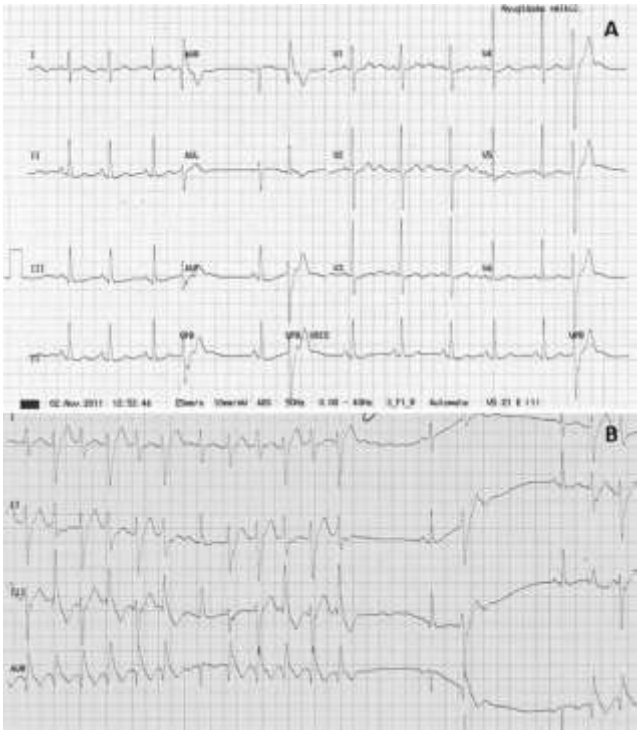


Figure 2: ECG abnormalities in Andersen-Tawil syndrome. Panel A: Mildly prolonged QT interval (QTc: 451 ms), prominent U wave in V1-3 leads. 1-1 monomorphic isolated PVCs. Panel B: Prolonged QT interval (QTc: 492 ms), bidirectional non-sustained ventricular tachycardia.

In ATS type 1 patients, gene-specific T-U wave abnormalities arise due to the reduced $I_{K1}$ current, resulting from mutations in the *KCNJ2* gene. The normal QTc, abnormal ECG and other clinical features help distinguish ATS from other long QT syndromes. Hence, ATS1 is often referred to as LQT7. (https://pubmed.ncbi.nlm.nih.gov/12163457/) The clinical diagnosis of ATS is made on the presence of periodic paralysis, symptomatic arrhythmias or evidence of prominent U waves, and characteristic facial and dental anomalies (Table 1).(6)

| | **Symptoms** |
|---|---|
| **Case A** (presence of TWO of the following three clinical criteria) | • Periodic paralysis<br>• Symptomatic arrhythmias or evidence of prominent U waves, ventricular ectopy, prolonged QTc or QUc interval<br>• Characteristic facial and dental anomalies, along with small hand and foot size, plus at least two additional features:<br>• Low-set ears<br>• Widely spaced eyes<br>• Small mandible<br>• Clinodactyly of the fifth finger<br>• Syndactyly of the 2nd and 3rd toes |
| **Case B** | The presence of ONE of the above three clinical criteria if there is a first-degree relative who meets TWO of the clinical criteria listed above. |

Table 1: Clinical diagnosis of Andersen-Tawil syndrome based on phenotypic and clinical characteristics

Mutations in the *KCNJ2* gene generally disrupt the structure and function of the potassium channel or prevent the channel from correctly positioning in the cell membrane. Many mutations impair the binding of the molecule phosphatidylinositol-4,5-bisphosphate (PIP2) to the channel, which is crucial for regulating channel activity. Currently, 103 mutations are known in the literature, a significant increase from just a few years ago. Most cases involve missense or nonsense mutations, with only a few small deletions observed.

The general structure of the Kir2.1 protein, like all Kir2.x proteins, includes two α-helices forming the transmembrane region, connected by a pore-forming loop (Figure 8). Both the amino and carboxyl termini are intracellular, with a sliding helix near the N-terminus. Four Kir2.x subunits can form the functional tetrameric channel through heteromerization or homomerization.

A critical phenomenon is that mutated channels exhibit altered sensitivity to the secondary messenger PIP2 within the membrane, an essential activator for most inward-rectifier potassium channels. About half of the mutations in *KCNJ2* affect parts of the channel vital for interaction with PIP2, highlighting the importance of the PIP2-channel interaction in the pathogenesis of ATS.

Mutations in the pore-forming loop of Kir2.1 are also significant. This loop is crucial for the channel's functionality and is well-conserved across the entire Kir family. Protein maturation and folding are common targets for mutations, leading to faulty protein structures that hinder transport and incorporation into the membrane - reducing the number of expressed channels on the cell surface. Additionally, even correctly positioned channels in the membrane may lose their activity.

The $I_{K1}$ current, produced by the Kir2.x family, is the most significant determinant of the resting membrane potential. A reduction in $I_{K1}$ current leads to sustained ventricular activity and QT prolongation. Typically, a reduced $I_{K1}$ current would cause a less negative membrane potential, leading to opposing processes. However, depolarization alters the activity of most sodium channels encoded by *SCN4A*, resulting in decreased membrane excitability, explaining the muscle weakness and periodic paralysis characteristic of ATS1. The persistent sodium window current increases sodium-potassium pump activity, leading to substantial potassium influx and thus explaining the hypokalemia observed in patients. Osteoclast dysfunction (ion imbalance) results in bone and cranial deformities.

Genetic testing for ATS is limited due to high variance in penetrance and expressivity. Generally mild cardiac involvement carries a relatively low risk of fatal arrhythmias, aiding in prognosis. *KCNJ2* mutations are usually more benign compared to mutations in CPVT genes (*RYR2, CASQ2*).

As a multisystem disorder, ATS stands out in the family of channelopathies. The symptoms range widely, including ventricular arrhythmias, physical deformities, and recurrent paralysis. Most patients suffer from mutations in the *KCNJ2* gene, affecting the inward-rectifier potassium current through modifications to the Kir2.1 protein. The $I_{K1}$ current causes repolarization at the end of the repolarization phase of muscle cells and is a primary controller of the resting membrane potential current. Therefore, ATS is a disease of cardiac repolarization.(7) There is limited evidence suggesting that *KCNJ2* may cause isolated long QT syndrome (LQTS). (8)

## 1.3. Timothy syndrome

Timothy syndrome (TS) is a multisystem disorder characterized by syndactyly and cardiac arrhythmias, which can ultimately lead to sudden cardiac death. TS is often associated with cognitive, neurological, and craniofacial abnormalities, as well as immunodeficiency. It is a distinct, severe, and rare form of long QT syndrome (LQTS), referred to as LQT8, because it involves the *CACNA1C* gene associated with LQTS. This gene encodes the L-type calcium channel, Cav1.2. The clinical manifestations of Timothy syndrome affect multiple organ systems, including the skin, heart, immune system, brain, eyes, and teeth. Patients with TS exhibit prolonged QT intervals on ECG and experience cardiac arrhythmias such as bradycardia, atrioventricular block, torsades de pointes ventricular tachycardia, and ventricular fibrillation.(9)

Timothy syndrome is inherited in an autosomal dominant manner. In rare cases, a "new" (de novo) mutation may occur in the gene. The *CACNA1C* gene is located on chromosome 12 and has 36 transcripts. The primary transcript comprises 13,433 base pairs and 47 exons. The protein-based channel encoded by this gene consists of 2138 amino acids (http://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000151067;r=12:1970786-2697950)

In cardiac myocytes, inwardly rectifying $Ca^{2+}$ current passes through the L-type calcium channel, the Cav1.2 voltage- gated calcium channel, thereby initiating calcium release through activation of ryanodine receptor 2 (RyR2) from the sarcoplasmic reticulum. The channel has several subunits, including the α1C-subunit, responsible for voltage sensing, for conduction pore formation, and for the gating mechanism. The fully functional channel complex is comprised of an additional intracellular β subunit and an extracellular α2δ subunit. The α1C subunit has 4 domains (I–IV), each with six transmembrane-spanning segments (S1–S6). S1–S4 are the voltage-sensitive subunits, whereas S5–S6 are the selectivity filter. When the membrane is depolarized, the S4 domain approaches the cytoplasmic ends of the S5 and S6 helices and the conduction pore opens. Changes in L-type calcium channel function (via phosphorylation, mutations, drugs, etc.) can affect cardiac myocyte contractility and arrhythmogenicity. The channel is expressed in many tissues of the body.(10)(11)(12)

The α1C subunit of the channel is encoded by the *CACNA1C* gene. Mutations affecting the *CACNA1C* gene may exhibit a variety of clinical manifestations. These manifestations include the typical Timothy syndrome (Timothy syndrome 1, TS1), which is characterized by QTc prolongation, AV-block, congenital heart defects, facial dysmorphisms, episodic hypoglycemia and neurologic symptoms including developmental delays, possible autism, seizures, and intellectual disability. The most distinctive morphological hallmark of TS1 is syndactyly, in contrast to atypical Timothy syndrome (Timothy syndrome 2, TS2) patients, who have no syndactyly but carry many of the other multisystemic manifestations of the disease (recently reviewed by Bauer et al.)(13). Further clinical manifestation of *CACNA1C* gene mutations include “cardiac only” Timothy syndrome (COTS)(14), which is characterized by QTc prolongation and congenital heart defects without extracardiac manifestations. In contrast to the above-mentioned phenotypes, some *CACNA1C* gene mutations are associated with isolated QTc prolongation (isolated long QT syndrome 8, LQT8), exhibiting QTc prolongation only without additional cardiac or extra-cardiac manifestations.(15)(16)(17) Because the *CACNA1C* gene was the eighth gene proved to cause QTc prolongation, it was historically called LQT8, but today a clear distinction exists between multi-organ Timothy syndrome and isolated LQT8.(18) These phenotypes are associated with a variety of *CACNA1C* gene mutations, affecting different regions of the *CACNA1C* gene. The predominant genetic cause of TS1, identified in 2004, is a recurrent, canonical “de novo” heterozygous missense mutation, p.Gly406Arg, in the alternatively spliced exon 8A.(9) In cases with TS2, additional mutations, originally p.Gly406Arg and p.Gly402Ser, were identified in exon 8 of the gene.(8) With the

clinical description of “cardiac only” TS and isolated LQT8, an increasing number of *CACNA1C* mutations have been described in the literature. The accumulated data indicated a great amount of genetic and phenotypic heterogeneity of *CACNA1C* mutations associated phenotypes, i.e., “typical” TS mutations showing variant phenotypes(19) and “non-typical” TS mutations associated with typical TS phenotype (Figure 3).(20)(21)(22)(23)

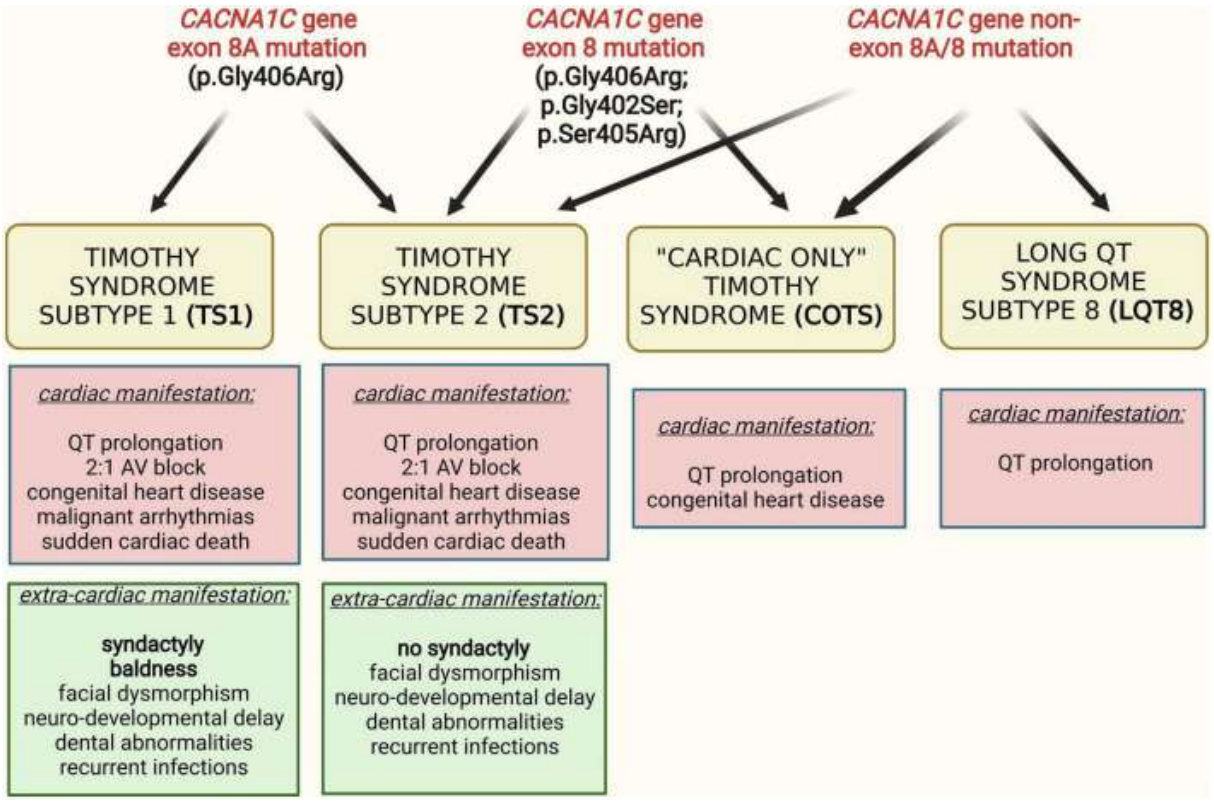


Figure 3: Interrelation of different clinical phenotypes and genotypes in *CACNA1C* gene mutation associated diseases.

# 2. AIMS

In my PhD work I aimed:

1. To screen for mutations causing Timothy syndrome among Hungarian patients with ion channel diseases;

2. To assess geno- and phenotypic characteristics and clinical outcomes of *CACNA1C* gene mutation associated Timothy syndrome, “cardiac only” Timothy syndrome and isolated long QT syndrome 8 by conducting a systematic literature search;

3. To identify novel and known *KCNJ2* gene mutations in Hungarian patients with Andersen-Tawil syndrome;

4. To analyze qualitative and quantitative ECG characteristics in patients with Andersen–Tawil syndrome.

# 3. PATIENTS AND METHODS

## 3.1. Patients

Patients for the different study projects reported in this PhD thesis have been selected from the in-house database of the Cardiology Center, University of Szeged, which records clinical data of patients with ion channel diseases. The details of the database (named CardioAdmin) have been published previously.(24) In brief, the registry is a single-center, observational database which collects data of patients with ion channel diseases who are diagnosed, treated and followed up in the in-patient or out-patient departments of the Cardiology Center, University of Szeged. The registry also includes patients who are referred to the Center for specialist evaluation or for treatment. Ion channel diseases, registered in the database, include long QT syndrome (LQTS), short QT syndrome (SQTS), Brugada syndrome (BS), catecholaminergic polymorphic tachycardia (CPVT), sudden cardiac death (SCD), progressive conduction diseases and uncharacterized ion channel diseases. Diseases are diagnosed according to published international guidelines. Follow-up patient visits take place during time intervals recommended by national and international protocols (usually every six months to one year). The diagnosis and treatment of patients (including medication and device treatments) is always based on the decision of the treating physician. The results of routine cardiological examinations and treatments obtained during the examination, treatment and follow-up of the patient are entered into the registry. It is also possible to record events and complaints listed as medical history by date. Individual diagnoses can also be made on a family basis. Measured and quantified variables of basic cardiological examinations (ECG, Holter ECG, ABPM, ergometry, etc.), imaging and invasive examinations (echocardiography, cardiac MRI, cardiac catheterization, etc.) are recorded in every case. The findings of special examinations (electromyography, myocardial biopsy, etc.) are also available in many cases. Accurate records are also kept for the patients' drug and invasive device treatments (ICD and pace-maker implants). Special or extra examinations and treatments related to the registry are not carried out. The ion channel patient registry operates under the ethical license of the Regional Committee on Human Biomedical Research Ethics (approval numbers: 166/2016 SZTE, 165/2016 SZTE). After detailed information, patients to be included in the registry confirm their intention to participate by signing a consent form.

### 3.1.1. Screening for mutations in the *CACNA1C* gene in a patient with suspected Timothy syndrome 1

A variant case of long QT syndrome with 2:1 AV block was referred to the Cardiology Center, University of Szeged, Hungary through a nation-wide collaboration.

The male patient was born as a second child to a healthy 31-year-old woman and 31-year-old man, both of Caucasian descent. There was no report of consanguinity, and the family histories were non-contributory. Especially, family history was negative for premature sudden cardiac death, arrhythmic disorders, syndactyly, facial dysmorphism, or autism. The pregnancy was uncomplicated.

Intermittent fetal bradycardia (heart rate of 72 bpm) was noted at 37 weeks gestation. The baby was born via normal uncomplicated vaginal delivery at 38 weeks gestation. Apgar scores were 9 and 10 at 1 and 5 min, respectively. The baby's birth weight was 2,950 g (18th centile), length was 54 cm (98th centile), and head circumference measured 34 cm (35th centile). On the second day of life cardiac evaluation revealed bradycardia due to 2:1 atrioventricular (AV) conduction and marked QTc-interval prolongation of 600 ms (Figure 4, Panel A). On medical therapy (propranolol and mexiletine) QTc duration stabilized in the range of 470–580 ms; 2:1 AV conduction resolved, and there was consistent 1:1 AV conduction with T-wave alternans (Figure 4, Panel B). Initial imaging with echocardiography demonstrated a patent foramen ovale, with a diameter of 2.5 mm with left-to-right shunt, and a mildly dilated right ventricle. The ductus arteriosus was closed and there were no signs of ventricular septal defect, cardiomyopathy or ventricular dysfunction. Laboratory findings did not reveal hypoglycemia or hypocalcaemia. The patient did not have facial dysmorphisms (hypertelorism, depressed nasal bridge, prominent forehead, etc.) and was not bald. He did not have joint hypermobility. Most importantly no syndactyly, either on the hands or on the feet, were present (Figure 4, Panel C and D). No hypoplastic teeth and decay was noted. During his first 3 years of life he has made steady developmental progress. He sat unassisted at age 7.5 months, crawled at age 8 months, walked with support at age 8.5 months, and walked independently at age 12 months. First words were at the age of 14 months. At age 2 years an M-CHAT (Modified Checklist for Autism in Toddlers) test was done which did not indicate autism or autism spectrum disorder. He does not have frequent or serious infections. As the patient's father is a board-certified cardiologist, he is under continuous bed-side ECG monitoring during nights with frequent traditional Holter monitoring performed every 3–6 months. Only on one occasion a 2:1 AV

conduction was detected for 12 hours, without symptoms, but no sustained or non-sustained ventricular tachyarrhythmias were ever encountered. At age 2 years he was hospitalized because of an episode of severe hypoglycemia (blood glucose level 1.4 mmol/L) associated with convulsions. As head trauma could not be excluded, a cranial CT was performed under general anesthesia. No VT or VF was observed under medication with propofol, midazolam, fentanyl, and muscle relaxants. The only observed rhythm disturbance was an intermittent 2:1 AV block which returned to normal conduction after the correction of serum glucose levels. The episode was resolved without any further consequence. Glucose monitoring with tissue glucose sensor did not reveal any further episode of hypoglycemia in the subsequent days. At present, at age 3 years, his mental and physical development seems to be normal. He is in good general health and attends kindergarten without assistance. He is able to express feelings, understands jokes, and sadness.

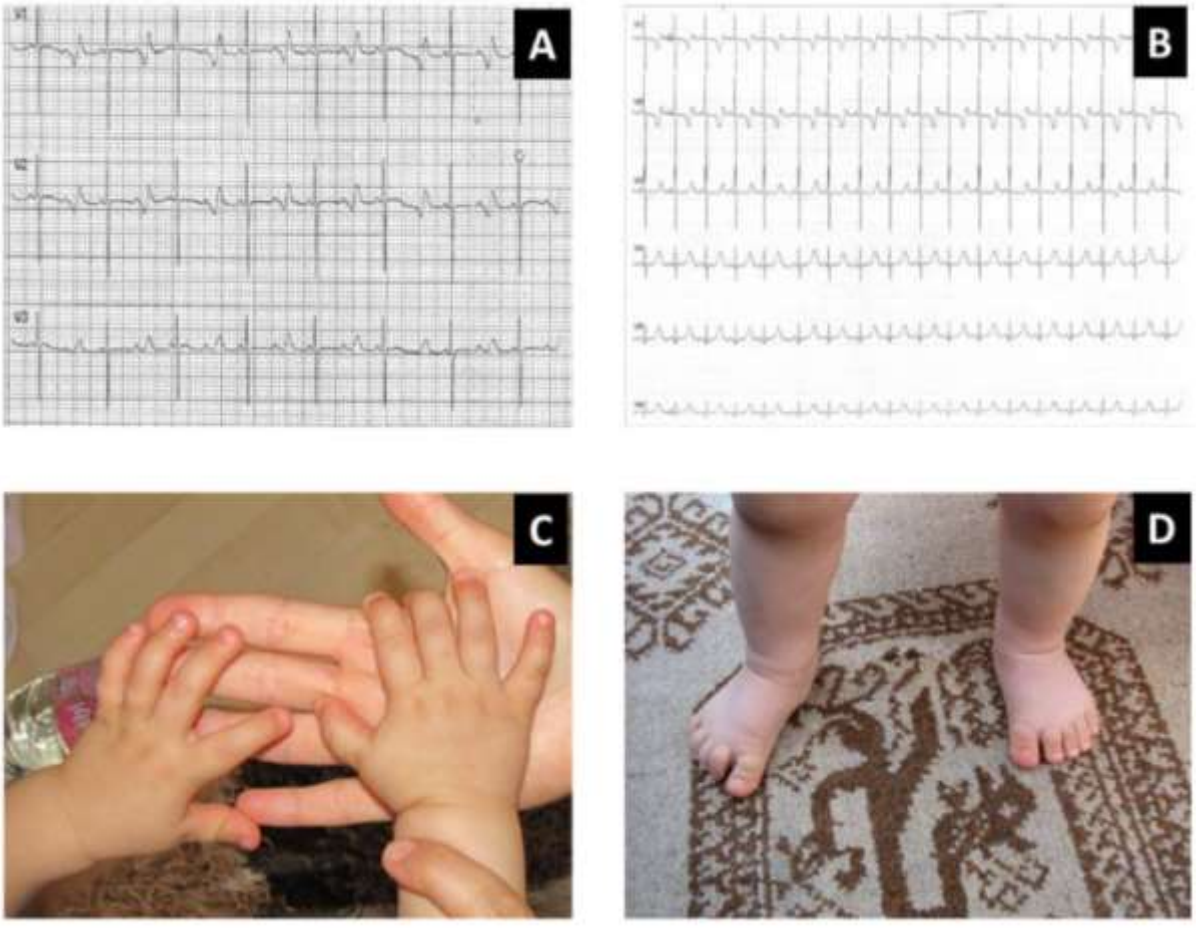


Figure 4: 12-lead ECG of the proband illustrating 2:1 AV block and marked QTc-interval prolongation of 600 ms at a heart rate of 72 bpm (Panel A). On medical therapy (propranolol and mexiletine) 2:1AV conduction resolved, and there is a consistent 1:1 AV conduction with QTc prolongation of 516 ms with T-wave alternans at a heart rate of 123 bpm (Panel B). Paper speed 25 mm/s, calibration 10 mm/mV. Panel C and D: Photograph of the proband's hands and feet, at age 12 months, demonstrating no syndactyly of the fingers or toes.

### 3.1.2. Assessing geno- and phenotypic characteristics and clinical outcomes of *CACNA1C* gene mutation associated Timothy syndrome, 'cardiac only' Timothy syndrome and isolated long QT syndrome 8 through a systematic review

A comprehensive search was conducted in MEDLINE (via PubMed), Embase, Web of Science, and Scopus databases from 2004 through 2019 focusing on full-text papers published reporting data on patients with Timothy syndrome or isolated long QT syndrome 8 (LQT8) affected by mutations of the *CACNA1C* gene. Excluding reports on mosaic patients,(25)(26)(27)(28)(29) a total of 134 patients were identified (Table 2). Most of the publications reported data of one case, whereas 16 studies summarized genotypic and clinical data of more patients/pedigrees.

As there was a considerable overlap between genotypes and phenotypes, comparator groups have been defined both based on the genotype and based on the phenotype. Accordingly, patients were divided into the following groups, defined on the genotype:

1) Carriers of exon 8A p.Gly406Arg *CACNA1C* mutations [formerly categorized as Timothy syndrome subtype 1 (TS1)].(13)

2) Carriers of exon 8 p.Gly406Arg *CACNA1C* mutations [formerly categorized as Timothy syndrome subtype 2 (TS2) in the strictest sense].(13)

3) Carriers of exon 8 p.Gly406Arg and pGly402Ser *CACNA1C* mutations [formerly categorized as Timothy syndrome subtype 2 (TS2), according to the original report].(8)

4) Carriers of all exon 8 *CACNA1C* mutations [including mutations p.Gly406Arg, p.Gly402Ser, p.Ser405Arg, p.Gly402Arg, and p.Pro381Ser mutations, formerly categorized as Timothy syndrome subtype 2 (TS2) in the broader sense].

5) Carriers of non-exon 8/8A *CACNA1C* mutations.

According to reported phenotypic manifestations patients were divided into the following three groups:

1) TS (Timothy syndrome, either typical or atypical, defined as patients with characteristic cardiac, and extra-cardiac manifestations). Two subgroups, i.e., typical TS (defined as patients with syndactyly) and atypical TS (defined as patients without syndactyly) were also analyzed separately.

2) Cardiac only TS (COTS, defined as patients with cardiac manifestations only).

3) Isolated long-QT syndrome 8 (LQT8, defined as patients with QTc prolongation only). Proven mosaic TS cases, reported as such, present in very small numbers in the reported publications, were not considered, taking the very specific genetic constellation of these cases into consideration. For all the comparator groups two sets of comparisons were made: (i) including index patients only (i.e., one affected person per family) (ii) including all affected patients (i.e., including index patients and all affected family members).

Out of the 134 patients, there were 85 index patients and 49 additional family members. Out of the 85 index patients 59 suffered from TS, 6 from COTS and 20 from isolated LQT8, respectively. In the entire patient population (index patients and relatives), there were 60 patients with TS, 15 patients with COTS and 59 patients with isolated LQT8 (Table 2).

| | Genotype | | | | | Phenotype | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *CACNA1C* exon 8A p.Gly406Arg mutation carrier[†‡] | *CACNA1C* exon 8 p.Gly406Arg mutation carrier[†] | *CACNA1C* exon 8 p.Gly406Arg/ p.Gly402Ser mutation carrier[†] | *CACNA1C* exon 8 mutation carrier[†] | *CACNA1C* non-exon 8/8A mutation carrier | Timothy syndrome (TS) | | | COTS | LQT8 | |
| | | | | | | TS with syndactyly | TS without syndactyly | TS total | | | |
| index patients, n | 31 | 6 | 12 | 16 | 33 | 45 | 14 | 59 | 6 | 20 | 85 |
| all patients*, n | 31 | 6 | 13 | 17 | 81 | 46 | 14 | 60 | 15 | 59 | 134 |

COTS, "cardiac only" Timothy syndrome; LQT8, isolated long QT syndrome, subtype 8. *Including family members. †In 5 patients (all carriers of the p.Gly406Arg mutation) it was not reported whether the patients carried the p.Gly406Arg mutation in exon 8 or in exon 8A. ‡Exon 8A mutations carriers all carried the p.Gly406Arg mutation.

Table 2. Comparator groups and number of patients in the comparator groups of the study. COTS: „cardiac only" Timothy syndrome. LQT8: long QT syndrome, subtype 8. *including family members. †In 5 patients (all carriers of the p.Gly406Arg mutation) it was not reported whether the patients carried the p.Gly406Arg mutation in exon 8 or in exon 8A.

### 3.1.3. Screening for mutations in the *KCNJ2* gene in patients with suspected Andersen-Tawil syndrome

Patients with suspected Andersen-Tawil syndrome were selected from our ion channel patient database based on suggested clinical criteria published in the literature (see Table 1). Seven patients met the pre-specified criteria whose clinical details are summarized in Table 3 (patients L5.0, L49.0, L84.0, L111.0, L114.0, L131.0, L154.0). The age at diagnosis was 12±7 years. There were 6 females and one male among the probands. Typical periodic paralysis was observed in three patients, while muscle weakness in one additional patient. Dysmorphic features (micrognathia, hypertelorism, low set ears, short stature) were noted in five cases. There were no patients with syndactyly or clinodactyly. Frequent premature ventricular beats

(PVBs) were observed in every case, while non-sustained ventricular tachycardia (NSVT) was recorded in four cases, all in bidirectional form. There were three aborted cardiac death among the probands at age 24, 38 and 20 years, which indicated implantable cardioverter defibrillator (ICD) implantation. Medical therapy included beta-blockers in six and flecainide in three cases.

Case histories of two ATS patients merit special attention: patient L84.0 with the *KCNJ2* p.Val302del mutation, as it is a novel variant; and patient L114.0 with the *KCNJ2* p.Glu293Lys mutation, as the cellular electrophysiological analysis of the mutation provided a novel pathophysiological mechanism.

#### 3.1.3.1 Case history of the patient with the *KCNJ2* p.Val302del mutation (L 84.0)

The female patient (born in 1996) was first hospitalized because of muscle weakness leading to walking difficulties at age of 5 years, although the term "neonatal hemiplegia" appeared on a medical certificate issued at age of 1 year, without further details. Laboratory findings revealed normal potassium levels and an elevated creatine-phosphokinase (CK) level of 596 U/L (upper limit of normal: 195 U/L), which returned to normal in 6 days. No special investigations other than orthopedic consultation were initiated, and the patient's symptoms ceased without consequences. In later years, she was admitted to different hospitals several times with symptoms of painless muscle weakness and proximal, asymmetric hemiplegia with sudden onset and variable duration. Serum potassium levels were in normal range in the majority of cases, except for one occasion when a below-normal potassium level (3.1 mmol/L) was noted. During episodes of muscle weakness, elevated CK levels were registered in the range of 507–629 U/L, with a normal CK-MB fraction, normalizing within days. Neurological examinations including lumbar puncture and liquor chemistry, cerebral and cervical magnetic resonance imaging, electromyography, electroencephalography, and electroneuronography were normal. Symptoms usually ceased within days with potassium and magnesium supplementation, and the patient was discharged in good condition every time.

Cardiac arrhythmia was noted first at age of 9 years, which consisted of frequent multifocal premature ventricular beats (PVB) or short runs of non-sustained ventricular tachycardia on the resting ECG. The arrhythmia did not lead to symptoms (palpitation, dizziness, or light-headedness) and no syncope ever occurred. Echocardiography proved normal cardiac structure and function. Twenty-four-hour Holter monitoring revealed 13% PVB, 1% couplets, and numerous short runs of ventricular tachycardia; the longest consisting of 11 beats with a

frequency of 148 beats/min. Propranolol and magnesium supplementation was started, which had no effect, then amiodarone treatment was initiated. Amiodarone decreased the number of PVBs but did not lead to a complete elimination of arrhythmia; furthermore, it induced thyrotoxicosis, and therefore had to be stopped. Sotalol treatment was started, which showed some effect. Based on the available clinical data and the characteristic appearance of the proband (short stature, microcephaly, facial dysmorphic features including low set ears, micrognathia), clinical diagnosis of Andersen–Tawil syndrome was raised at age of 15 years. On the last follow-up at age of 18 years, the patient was alive with unchanged clinical status. As the patient was abandoned by her parents and was brought up by her maternal aunt, no family members were available for clinical or genetic screening, apart from the maternal aunt who exhibited normal ECG and echocardiography.

| Family | Subject | Affected | Age at diagnosis | Sex | Neuro-muscular symptoms | | | Dysmorphic Features | | | | ECG characteristics | | | | | Fatal arrhythmias | | | Therapy | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | periodic paralysis | documented hypokalemia | muscle weakness | micro-gnathia | hyper-telorism | low set ears | short stature | Enlarged U wave | Frequent PVCs | NSVT | VT | Bidirec-tional VT | Syncope | ACA/ SCD | Age at ACA/SCD | Beta-Blocker | Fleca-inide | ICD |
| L 5 | 5.0 | yes | 24 | female | no | no | yes | yes | yes | yes | yes | yes | yes | yes | no | yes | yes | yes | 24 | metoprolol | no | yes |
| | 5.1 | yes | 10 | female | no | no | yes | yes | yes | yes | yes | yes | yes | yes | no | yes | yes | yes | 17 | bisoprolol | no | yes |
| | 5.4 | no | – | male | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| | 5.8 | yes | 0 | male | no | no | no | no | no | no | no | no | yes | no | no | no | no | no | – | metoprolol | no | no |
| | 5.10 | yes | 0 | male | no | no | no | no | no | no | no | no | yes | no | no | no | no | no | – | no | no | no |
| L 49 | 49.0 | yes | 11 | female | no | yes | no | yes | yes | yes | yes | yes | yes | no | no | no | yes | yes | 38 | metoprolol | yes | yes |
| | 49.1 | yes | 29 | female | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | no | no | no | no | – | metoprolol | no | no |
| | 49.2 | yes | 20 | female | no | no | yes | yes | yes | yes | yes | yes | yes | no | no | no | no | no | – | metoprolol | no | no |
| | 49.3 | yes | 22 | female | yes | no | yes | yes | yes | yes | yes | yes | no | yes | no | no | no | no | – | metoprolol | no | no |
| | 49.9 | no | – | male | – | – | – | – | – | – | – | – | – | – | – | – | no | no | – | metoprolol | no | no |
| L 84 | 84.0 | yes | 15 | female | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | no | yes | no | no | – | no | yes | no |
| L 111 | 111.0 | yes | 18 | male | no | yes | yes | yes | yes | yes | no | yes | yes | no | no | no | yes | yes | 20 | nadolol | yes | yes |
| | 111.2 | no | – | male | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| | 111.4 | no | – | female | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| L 114 | 114.0 | yes | 4 | female | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | no | yes | no | no | – | propranolol | no | no |
| L 131 | 131.0 | yes | 3 | male | yes | yes | yes | no | no | no | no | yes | yes | yes | yes | yes | no | no | – | propranolol | no | no |
| L 154 | 154.0 | yes | 13 | female | no | no | no | no | no | no | yes | no | yes | no | no | no | yes | no | – | metoprolol | no | no |

Table 3. Demographic, clinical, ECG and outcome characteristics of index patients and family members carrying *KCNJ2* gene mutations identified in the study. Non-carrier family members are not shown in the table. PVC: premature ventricular complex, NSVT: non-sustained ventricular tachycardia, VT: ventricular tachycardia, ACA: aborted cardiac arrest, SCD: sudden cardiac death, ICD: implantable cardioverter defibrillator

### 3.1.3.2. Case history of the patient with the *KCNJ2* p.Glu293Lys mutation (L 114.0)

The female index patient was diagnosed with ATS1 at age of 7 years. She was born from an uneventful pregnancy at the end of 40 gestational weeks, with 2900 g birth weight. Dysmorphic features were evident at the time of diagnosis and included hypertelorism, low-set ears, mandibular hypoplasia, broad nose, and broad forehead. Symptomless premature ventricular beats (PVBs), which were diagnosed as multifocal (with two different PVB morphology), sometimes bigeminal PVBs, with short runs of non-sustained ventricular tachycardia (NSVT) were detected at age of 4 years. On Holter examination, PVBs made up 38% of all recorded beats. Corrected QT interval was increased and was in the range of 456–485 ms. Echocardiography did not reveal any significant structural heart disease. Due to regular NSVT, flecainide therapy was initiated at the age of 11 years. Ventricular PVBs ceased promptly after the initiation of iv. flecainide and remained so on po. therapy (2x100 mg). Beta-blocker therapy (propranolol 320 mg) was continued. Control Holter revealed 0% PVB of all recorded beats. Skeletal muscle symptoms, apart from general fatigue and occasional numbness of the feet, started to occur as periodical paralysis at age of 8 years. The latter was characterized by inability to raise and walk, lasting for 3 days, and was unrelated to serum potassium levels (3.86–4.6 mmol/L). Acetazolamide did not significantly improve symptoms. The episodes of periodical paralysis became more severe, and the patient is wheelchair-bound at age of 10 years. Flecainide treatment, which was shown to be particularly effective in reducing ventricular arrhythmic activity after 1-year follow-up, seemed to have no effect on paralytic symptoms. Paralysis often affected all four extremities. The patient's mental development and ability to cooperate is according to age.

At the time of the last visit, the patient was 12 years old. She complains of retrosternal chest pain, which does not occur at night. Shortness of breath also occurs. Respiratory function is in normal range in lying position, while a moderate restrictive respiratory dysfunction was demonstrated in upright position, characteristic for muscle weakness, possibly indicating intercostal muscle weakness. Rarely, abdominal pain develops. Abdominal ultrasound did not confirm any abnormality in the localization corresponding to the complaints.

### 3.1.4. Analysis of ECG characteristic of patients with genetically confirmed Andersen-Tawil syndrome

Seven patients (6 females, 1 male, avg. age 25,4±11 years; patients No. L 5.0, L 49.0, L 49.1, L 49.3, L 84.0, L 114.0, L 131.0, see Table 3) with genetically confirmed ATS (three patients with p.Arg218His, one patient each with p.Arg312Glu, p.del302Val, p.Glu293Lys and p.Met307Ile *KCNJ2* mutations) were examined. Patients with ATS were compared to an age- and sex-matched cohort of patients with long QT syndrome.

## 3.2 Methods

### 3.2.1. Screening for mutations in the *CACNA1C* gene in a patient with suspected Timothy syndrome 1

#### 3.2.1.1. Sequencing

Coding sequences and exon-intron boundaries of all 13 causative long QT genes were analyzed by next-generation sequencing using Agilent's SureSelect technology with custom-designed 120-mer RNA baits specific to target region (Agilent Technologies, Santa Clara, CA, United States). Targeted resequencing was carried out on a SOLiD 5500xl System (Life Technologies, Grand Island, NY, United States). Variants, identified by targeted resequencing, were validated by standard capillary sequencing using custom-designed primers. Exon 37 of *ANK2* gene, exon 8A of *CACNA1C* gene, and exon 1 of *KCNQ1* gene were direct sequenced on a ABI Prism 310 Genetic Analyzer (Applied Biosystems by Life Technologies, Grand Island, NY, United States). Genetic mosaicism was assessed in the index patient and both parents using DNA extracted from buccal cells and from sperm, in the case of the father.

#### 3.2.1.2. Bioinformatic analysis

Mapping of the SOLiD reads were accomplished by Genomic Workbench ver 7.0.3 (CLC Bio) using the human genome assembly hg19 as reference sequence. Variant calling and variant annotation were performed by the same software. The functional impact of amino acid changes caused by missense mutations was predicted by SIFT and PROVEAN programs. Nucleotide and amino acid changes are reported according to the Ensembl database (release: 85), based on reference sequences ENST00000155840.9 for *KCNQ1*, ENST00000399591.5 for *CACNA1C* and ENST00000264366.10 for *ANK2*.

#### 3.2.1.3. Interpretation of variants

Identified variants were evaluated according to the standards for the interpretation of sequence variants issued by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) in 2015, and were classified as benign (B), likely benign (LB), variant of unknown significance (VUS), likely pathogenic (LP), and pathogenic (P). Variants were interpreted using CardioClassifier, an automated and interactive

web tool that supports disease specific interpretation of genetic variants in genes associated with inherited cardiac conditions and assessing ClinVar variants entries (https://www.ncbi.nlm.nih.gov/clinvar/). In case of discrepancy between CardioClassifier and ClinVar interpretations, a final verdict was reached by assessing clinical evidence for disease causation (especially data on the number of affected individuals with the condition and evidence for co-segregation). In case of novel variants with no ClinVar entry, and not covered by CardioClassifier, the Varsome (https://varsome.com/) or Franklin on-line interpretation programs were used.

### 3.2.2. Assessing geno- and phenotypic characteristics and clinical outcomes of *CACNA1C* gene mutation associated Timothy syndrome, 'cardiac only' Timothy syndrome and isolated long QT syndrome 8 through a systematic review

This systematic review was reported in accordance with the PRISMA Statement for reporting systematic reviews and meta-analyses. Our predefined review protocol was published in the PROSPERO database under the registration number of CRD42020184737. There was no deviation from the predefined and published protocol during the study.

A comprehensive search was conducted in MEDLINE (via PubMed), Embase, Web of Science, and Scopus databases from 2004 through 2019 focusing on full-text papers published reporting data on patients with Timothy syndrome or isolated long QT syndrome 8 (LQT8) affected by mutations of the *CACNA1C* gene. Conference abstracts were included when same data could be extracted from full-text papers. Studies eligible for inclusion were identified by using the following search query as full text search: "Timothy syndrome" OR ("LQT8 OR CACNA1C").

#### 3.2.2.1. Eligibility criteria and selection

The eligibility criteria for this systematic review were as follows:

1) Reporting data on patients and/or relatives with documented mutation of the *CACNA1C* gene in the English language;

2) Describing detailed geno- and/or phenotypic features of the case;

3) Reporting data on clinical outcomes. Reports on *CACNA1C* gene mutation associated with Brugada syndrome (BS3), short QT syndrome (SQT4) or early repolarization syndromes (ERS), exhibiting fundamentally different pathomechanisms, were excluded. Duplicate cases

were identified, and the less informative ones were excluded. Two review authors independently evaluated all potentially relevant articles for eligibility. Any disagreement was subsequently resolved by consensus.

Excluding reports on mosaic patients, a total of 34 publications comprising data of 134 patients were identified. (Appendix Figure 1, Appendix Table 1)

#### 3.2.2.2. Data extraction

From the eligible reports, patient-level data were extracted. Beyond the detailed description of the mutation affecting the *CACNA1C* gene, the following sets of data has been extracted from the source reports: (1) demographic data; (2) manifestations of the disease (categorized as cardiac or extracardiac manifestations); (3) utilized medical and device therapy; and (4) outcome of the disease (categorized as death or cardiac events). Major adverse cardiac events (MACE) were defined as death, aborted cardiac arrest (ACA), sudden cardiac death (SCD), or appropriate ICD discharge. Arrhythmia type and circumstances at ACA/SCD/ICD discharge were also recorded if available.

#### 3.2.2.3. Re-evaluation of the interpretation of different *CACNA1C* mutations

As many of the considered publications were published before the standards for the interpretation of sequence variants were issued by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) in 2015,(30) we reassessed the interpretations of all extracted *CACNA1C* mutations. All the mutations were re-evaluated in ClinVar and Varsome.

### 3.2.3. Molecular genetic analysis of the *KCNJ2* gene

Genetic analysis of the *KCNJ2* gene was performed as detailed under 3.2.1. Nucleotide and amino acid changes are reported according to the Ensembl database (release: 85), based on the reference RefSeqGene LRG_328 sequence for *KCNJ2.*

### 3.2.4. Analysis of ECG characteristics of genetically confirmed patients with Andersen-Tawil syndrome

In patients with ATS and LQTS, standard resting 12-lead ECG recordings were obtained during their clinical evaluation. The ECG recordings were analyzed both qualitatively and quantitatively. Among the qualitative abnormalities, the presence of ventricular extrasystoles

(VES)/bigeminy, as well as couplets and NSVT (non-sustained ventricular tachycardia), were assessed. NSVT was defined as a rhythm disturbance consisting of three or more beats, with a frequency >100/min and lasting <30 seconds. Bidirectional VT was characterized by a 180-degree shift in the QRS axis from beat to beat. Quantitative ECG parameters included the corrected QT (QTc) and QU (QUc) intervals, the duration of the U-wave, and the amplitude of the U-wave, measured in lead V2 or V3. These parameters were determined based on manual measurements. Heart rate correction was performed using the Bazett's formula. Data were compared to that of age- and sex-matched control patients with LQTS.

# 4. RESULTS

## 4.1. Identification of mutations causing Timothy syndrome among Hungarian patients with ion channel diseases

### 4.1.1. Mutation data

Three different heterozygous genetic variants in three genes have been detected using targeted resequencing by next-generation sequencing: p.Gly406Arg (c.1216G>A, rs79891110) in exon 8A of the *CACNA1C* gene (Figure 5); p.Tyr94Cys (c.281A>G, rs781717051) in exon 1 of the *KCNQ1* gene; and p.Ile3252Thr (c.9755T>C, rs36210417) in exon 37 of the *ANK2* gene. All three variants were previously reported. The *KCNQ1* p.Tyr94Cys variant was predicted to be deleterious by PROVEAN and to be tolerant by SIFT, not reported in ClinVar and was classified as a VUS by CardioClassifier. The *ANK2* p.Ile3252Thr variant was predicted to be neutral/tolerant by both prediction methods and is annotated as benign/likely benign in ClinVar.

However, the identified *CACNA1C* gene exon 8A p.Gly406Arg variant, classified as pathogenic by ClinVar, is the canonical Timothy syndrome 1 causing mutation and therefore was regarded pathogenic. Although sequence homology is high between exons 8 and 8A of the *CACNA1C* gene, the presence of several "exon specific" nucleotides made it possible to distinguish between the two. Sequence comparison with all available published sequences of *CACNA1C* exon 8 and 8A in previous reports proved that our patient indeed carried the p.Gly406Arg variant affecting exon 8A of the *CACNA1C* gene (Figure 5). The *CACNA1C* p.Gly406Arg variant was also present in the DNA sample of the index patient extracted from buccal, uroepithelial, and hair follicular cells. The height of the mutant nucleotide peak was similar in the different samples and were equal to the normal nucleotide peak.

### 4.1.2. Genetic screening of family members

First degree family members of the index patient (mother, father, and sister) underwent genetic screening for all three variants. The proband's father was proved to be the carrier of the *KCNQ1* p. Tyr94Cys variant, while mother and sister were non-carriers for both the *KCNQ1* and *ANK2*

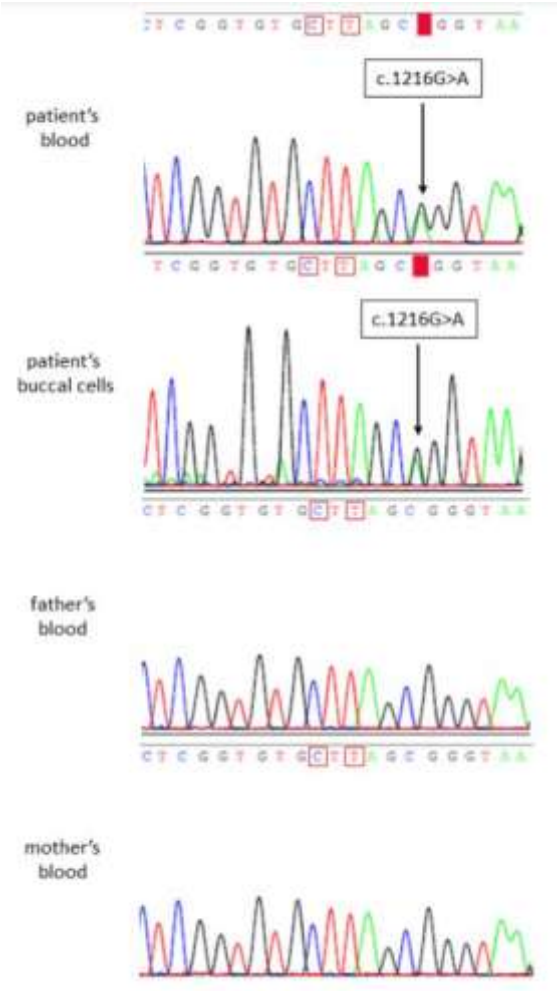


Figure 5: Sequence analysis of exon 8A of the *CACNA1C* gene illustrating the c.1216G>A mutation. The mutation changes the GGA triplet at codon406, encoding for glycine, to AGA, encoding for arginine (p.Gly406Arg). Sequencing result is shown from DNA isolated from blood (patient, father, mother) and from buccal cells (patient). Testing of parents suggested that the mutation is 'de novo'. Nucleotides framed in red are unique for exon 8Awhen compared to homologous exon 8.

variants. Neither the parents nor the sister of the index patient carried the *CACNA1C* p.Gly406Arg variant (Figure 5), therefore, this variant must have arisen "de novo" (paternity was proven). The *KCNQ1* p.Tyr94Cys variant-carrying father has no signs or symptoms of long QT syndrome and has a completely normal ECG and echocardiogram (data not shown), suggesting that the *KCNQ1* p.Tyr94Cys variant has no major influence on the disease phenotype. In order to assess whether the *CACNA1C* p.Gly406Arg mutation has indeed arisen "de novo" in the family, or one of the parents has mosaicism for the mutation, we performed genetic analysis of the DNA extracted from buccal cells (in case of both parents) and sperm (in case of father). The *CACNA1C* p.Gly406Arg variant was not present in any sample extracted from different tissues, therefore no proof for genetic mosaicism was found (Figure 5).

## 4.2. Assessment of geno- and phenotypic characteristics and clinical outcomes of *CACNA1C* gene mutation associated Timothy syndrome, "cardiac only" Timothy syndrome and isolated long QT syndrome 8

### 4.2.1. Re-evaluation of the interpretation of different *CACNA1C* mutations

Altogether, 33 *CACNA1C* mutations were extracted from the reports (Figure 6 and Table 4). Twenty-eight mutations (85%) had an interpretation of pathogenic (P), likely pathogenic (LP), or variant of unknown significance (VUS) favoring P/LP, either according to ClinVar or Varsome. In general, the number of ClinVar submissions were low (1–4 submissions). Only five mutations (p.Ala28Thr, p.Met456Ile, p.Gly1783Cys, p.Arg1906Gln, p.Gly1911Arg) had a verdict of benign (B), likely benign (LB), or VUS. However, in the case of p.Ala28Thr, reported familial segregation of the mutation in affected family members and functional studies providing evidence for deleterious effect(15) were not considered in the Varsome algorithm which would activate an additional PP1 and a PS3 criteria shifting the interpretation of the variant as VUS favoring LP. Also, for the p.Gly1911Arg variant, reported to be associated with TS, significant differences in the functional analysis and "de novo" occurrence of the variant have been demonstrated,(21) activating a PS2 and PS3 criteria and shifting the interpretation of the variant as VUS favoring P. In the case of the p.Met456Ile, Gly1783Cys and Arg1906Gln variants, all reported to be associated with isolated LQT8, no significant additional features (e.g., differences in the functional analysis, familial segregation, etc.) were demonstrated to alter the Varsome verdict. However, for the sake of data integrity all the mutations with the reported phenotypes were considered as they were reported.

### 4.2.2. Comparison of patient groups defining different subgroups of Timothy syndrome

#### 4.2.2.1. Comparison of different subgroups of Timothy syndrome, defined on genotype (patients with exon 8A p.Gly406Arg vs. exon 8 p.Gly406Arg mutations; vs. exon 8 p.Gly406Arg/p.Gly402Ser mutations, vs. all exon 8 mutations)

There were 31 index pts. with exon 8A mutations (all carriers of canonical p.Gly406Arg mutation), 6 pts. with exon 8 p.Gly406Arg mutations, 12 pts. with exon 8

p.Gly406Arg/p.Gly402Ser mutations and 16 pts. with exon 8 mutations (six p.Gly406Arg, six p.Gly402Ser, two p.Ser405Arg, one p.Gly402Arg and one p.Pro381Ser mutations). In further five patients (all carriers of the p.Gly406Arg mutation) it was not unequivocally reported and was impossible to determine whether the patients carried the p.Gly406Arg mutation in exon8 or in exon 8A.(31)(32)(33)(34) (Table 4 and Figure 6).

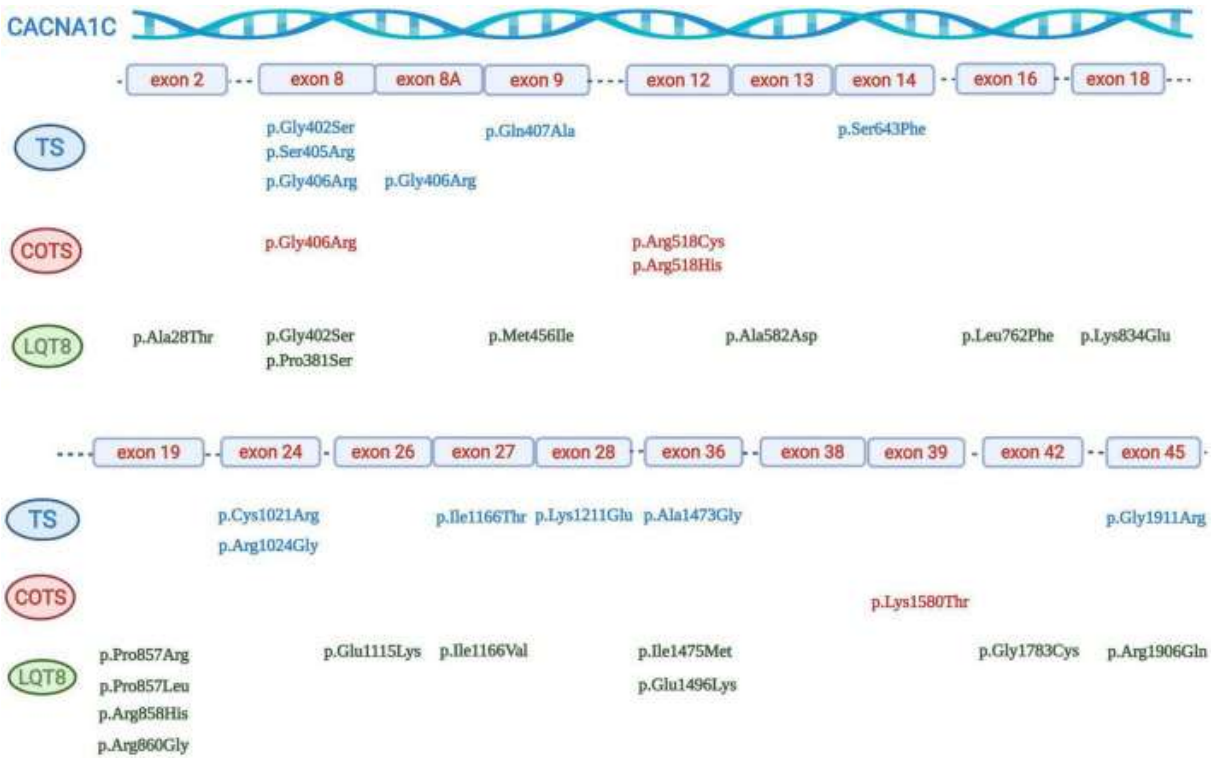


Figure 6: Graphical representation of the location of different *CACNA1C* mutations. Mutations associated with Timothy syndrome are in blue, mutations associated with "cardiac only" Timothy syndrome are in red, and mutations associated with isolated LQT8 are in green.

The detailed comparison of the groups is presented in Table 5. Comparing data on demographics, clinical and ECG manifestations, and outcome, it was only syndactyly which was significantly more frequent in pts. with exon 8A p.Gly406Arg mutations in all comparisons, and baldness which was again more frequent in pts. with exon 8A p.Gly406Arg mutations in comparison with pts. carrying exon 8 p.Gly406Arg/Gly402Ser mutations or all exon 8 mutations. The presence of AV block was also more frequent and the age at MACE was lower in pts. with exon 8A p.Gly406Arg mutations in comparison with pts. with all exon 8 mutations. In addition, patients with exon 8A p.Gly406Arg mutations were significantly

younger at the time of diagnosis (median 0 vs. 32 months; p=0.019) and more pts. were diagnosed in the first year of life (89 vs. 44%; p=0.009). Marked QTc prolongation (>500 ms) was present in all the patients, except two patients with exon 8 mutation. The degree of QTc prolongation (maximum QTc) was similar in the groups (median ≥600 ms in all groups). There was no difference in the utilization of pacemaker/ICD implantation or of left cervical sympathectomy. MACE rate was high (67–83%) but was not different in the groups.

#### 4.2.2.2. Comparison of different subgroups of Timothy syndrome, defined on phenotype (Timothy syndromes with or without syndactyly)

The detailed comparison of the groups is presented in Table 5. There were 45 TS pts. with syndactyly and 14 pts. without syndactyly. Only baldness was more frequent and the age at MACE was lower in TS pts. with syndactyly. The degree of QTc prolongation was marked (median ≥600 ms in both groups) and MACE rate was high (68–71%) but showed no statistical difference.

#### 4.2.2.3. Analysis of all patients, including family members

There was one additional family member with exon 8 mutation. His inclusion did not alter any of the statistical comparisons.

### 4.2.3. Comparison of patient groups defining different forms of *CACNA1C* gene associated diseases

#### 4.2.3.1. Comparison of different forms of *CACNA1C* gene associated diseases, defined on genotype (patients with exon 8/8A *CACNA1C* mutations vs. non-exon 8/8A *CACNA1C* mutations)

There were 52 index patients with exon 8/8A and 33 index patients with non-exon 8/8A mutations. Exon 8/8A mutations almost all clustered at codon 402 (7 cases), 405 (2 cases), and 406 (42 cases), while the most frequently affected codons in non-exon 8/8A mutation carriers were codon 518 (three cases with p.Arg518Cys mutations and one case with p.Arg518His mutation), codon 857 (one case with p.Pro857Leu and p.Pro857Arg mutations each), codon 858 (four cases with p.Arg858His mutation), codon 1166 (two cases with p.Ile1166Thr, and one case with Ile1166Val mutation) and codon 1473 (two cases with p.Ala1473Gly mutation) (Table 4 and Figure 6).

| Exon | Codon | Mutation | ClinVar | | | | Varsome | | TS ($n$ = 59) | | COTS ($n$ = 6) | | LQT8 ($n$ = 20) | | Total ($n$ = 85) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Verdict | Max | Score | Submiss. | Verdict | Items | $n$ | % | $n$ | % | $n$ | % | $n$ | % |
| 2 | 28 | Ala28Thr | VUS | 3 | 3 | 1 | Likely benign | BS2 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| 8 | 381 | Pro381Ser | VUS | 3 | 3 | 1 | VUS-favoring LP | PM2, PP3, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| | 402 | Gly402Arg | VUS | 3 | 3 | 1 | VUS-favoring P | PM2, PP3, PP5, BP1 | 1 | 2% | 0 | 0% | 0 | 0% | 1 | 1% |
| | 402 | Gly402Ser | P | 5 | 4.6 | 3 | Likely pathogenic | PP5, PM2, P3, BP1 | 5 | 8% | 0 | 0% | 1 | 5% | 6 | 7% |
| | 405 | Ser405Arg | | | ND | | Likely pathogenic | PP3, PM2, BP1 | 2 | 3% | 0 | 0% | 0 | 0% | 2 | 2% |
| | 406 | Gly406Arg | P | 5 | 5 | 3 | Pathogenic | PS1, PP3, PP5, PM2, BP1 | 6 | 10% | 0 | 0% | 0 | 0% | 6 | 7% |
| 8A | 406 | Gly406Arg | P | 5 | 5 | 3 | Pathogenic | PS1, PP3, PP5, PM2, BP1 | 31 | 53% | 0 | 0% | 0 | 0% | 31 | 36% |
| 8/8A? | 406 | Gly406Arg | P | 5 | 5 | 3 | Pathogenic | PS1, PP3, PP5, PM2, BP1 | 4 | 7% | 1 | 17% | 0 | 0% | 5 | 6% |
| 9 | 407 | Glu407Ala | | | ND | | VUS-favoring LP | PM2, PP3, BP1 | 1 | 2% | 0 | 0% | 0 | 0% | 1 | 1% |
| | 456 | Met456Ile | | | ND | | VUS | PM2, BP4 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| 12 | 518 | Arg518Cys | P | 5 | 5 | 2 | Likely pathogenic | PP5, PM2, PM5, PP3, BP1 | 0 | 0% | 3 | 50% | 0 | 0% | 3 | 4% |
| | 518 | Arg518His | P | 5 | 4.7 | 3 | Likely pathogenic | PP5, PM2, PM5, PP3, BP1 | 0 | 0% | 1 | 17% | 0 | 0% | 1 | 1% |
| 13 | 582 | Ala582Asp | P | 5 | 5 | 1 | VUS-favoring LP | PM2, PP3, PP5, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| 14 | 643 | Ser643Phe | | | ND | | VUS-favoring LP | PM2, PP3, BP1 | 1 | 2% | 0 | 0% | 0 | 0% | 1 | 1% |
| 16 | 762 | Leu762Phe | LP | 4 | 4 | 1 | VUS-favoring LP | PM2, PP3, PP5, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| 18 | 834 | Lys834Glu | P | 5 | 5 | 1 | Likely pathogenic | PS3, Pm2, PP3, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| 19 | 857 | Pro857Arg | P | 5 | 5 | 1 | Likely pathogenic | PS3, PM2, PP3, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| | 857 | Pro857Leu | LP | 5 | 4 | 3 | VUS-favoring P | PM2, PM5, PP3, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| | 858 | Arg858His | P | 5 | 4.7 | 4 | Likely pathogenic | PP5, PM2, PP3, BP1 | 0 | 0% | 0 | 0% | 4 | 20% | 4 | 5% |
| | 860 | Arg860Gly | P | 5 | 5 | 1 | VUS-favoring LP | PM2, PP3, PP5, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| 24 | 1021 | Cys1021Arg | VUS | 3 | 3 | 1 | VUS-favoring LP | PM2, PP3, BP1 | 1 | 2% | 0 | 0% | 0 | 0% | 1 | 1% |
| | 1024 | Arg1024Gly | | | ND | | VUS-favoring LP | PM2, PP3, BP1 | 1 | 2% | 0 | 0% | 0 | 0% | 1 | 1% |
| 26 | 1115 | Glu1115Lys | VUS | 3 | 3 | 1 | VUS-favoring LP | PM2, PP3, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| 27 | 1166 | Ile1166Thr | P | 5 | 5 | 1 | VUS-favoring LP | PM2, PP3, PP5, BP1 | 2 | 3% | 0 | 0% | 0 | 0% | 2 | 2% |
| | 1166 | Ile1166Val | | | ND | | VUS-favoring LP | PM2, PP3, PP5, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| 28 | 1211 | Lys1211Glu | VUS | 3 | 3 | 1 | VUS-favoring LP | PM2, PP3, PP5, BP1 | 1 | 2% | 0 | 0% | 0 | 0% | 1 | 1% |
| 36 | 1475 | Ile1475Met | P | 5 | 5 | 1 | VUS-favoring LP | PM2, PP3, PP5, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| | 1496 | Glu1496Lys | | | ND | | VUS-favoring LP | PM2, PP3, BP1 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| 38 | 1473 | Ala1473Gly | LP | 5 | 4.5 | 2 | VUS-favoring LP | PM2, PP3, PP5, BP1 | 2 | 3% | 0 | 0% | 0 | 0% | 2 | 2% |
| 39 | 1580 | Lys1580Thr | | | ND | | VUS-favoring LP | PM2, PP3, BP1 | 0 | 0% | 1 | 17% | 0 | 0% | 1 | 1% |
| 42 | 1783 | Gly1783Cys | VUS | 3 | 3 | 1 | Likely benign | PM2, BP1, BP4 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| 45 | 1906 | Arg1906Gln | VUS | 3 | 3 | 8 | Benign | BS1, BS2, PP3 | 0 | 0% | 0 | 0% | 1 | 5% | 1 | 1% |
| | 1911 | Gly1911Arg | VUS | 3 | 3 | 3 | Benign | BS1, BS2 | 1 | 2% | 0 | 0% | 0 | 0% | 1 | 1% |

Table 4: Distribution among phenotypes and interpretation of the different *CACNA1C* mutations according to ClinVar and Varsome.

The detailed comparison of the two groups is presented in Appendix Table 2. Patients with exon 8/8A mutations were significantly younger at the time of diagnosis, and a higher percentage of the patients were diagnosed at birth or in the first year of life. The predominant phenotype associated with exon 8/8A mutations was TS in 49 patients (96%), COTS in 1 patient (2%) and isolated LQT8 in 2 patient (2%), while with non-exon 8/8A mutations it was TS in 10 patients (29%), COTS in 5 patients (15%) and isolated LQT8 in 18 patients (56%) ($p<0.001$). Extracardiac manifestations (94 vs. 32%; $p < 0.001$) were significantly more frequent in patients with exon 8/8A mutations. As TS was the overwhelmingly prevalent phenotype in patients with exon 8/8A mutations, the major phenotypic characteristics of TS were all significantly more frequent in patients with exon 8/8A mutations. QTc prolongation was present in all the 52 patients with exon 8/8A mutations, while it was seen in only in 79% of the patients with non-exon 8/8A mutations ($p=0.0025$). The degree of QTc prolongation (maximum QTc) was much more pronounced in patients with exon 8/8A mutations (median 606 vs. 498 ms.; $p<0.0001$) and the rate of pts. with >500 ms QTc prolongation was much higher (92 vs. 36%; $p<0.001$). AV block was also observed in significantly more cases in patients with exon 8/8A mutations (74 vs. 33%; $p=0.002$). There was no difference in the utilization of pacemaker/ICD implantation or of left cervical sympathectomy. There was a marked difference in terms of outcome, as much higher number of pts. with exon 8/8A mutations died (33 vs, 9%; $p=0.017$) or experienced MACE (71 vs. 34%; $p=0.001$).

#### 4.2.3.2. Comparison of different forms of *CACNA1C* gene associated diseases, defined on phenotype (patients with Timothy syndromes vs. "cardiac only" Timothy syndrome vs. isolated LQT8)

Out of the 85 index patients there were 59 (69%) with TS, 6 (7%) with COTS and 20 (24%) with the isolated LQT8 phenotype.

As detailed previously, exon 8 or 8A mutations, affecting codons 402, 405 and 406 made up the majority of the genotypes in patients with TS (49/59 patients, 83%). Most mutations leading to COTS affected codon 518 in 4/6 patients (67%). Mutations causing isolated LQT8 scattered through the gene with only one codon having affected in more than one patient (codon 858 in 4/20 patients, 20%) (Table 4 and Figure 6)

The detailed comparison of the three groups is presented in Appendix Table 2. Patients with TS were significantly much younger at the time of diagnosis than patients with COTS or isolated LQT8 (median 1 month vs. 180 months vs. 174 months, respectively; $p<0.001$). In addition,

significantly much more pts. with TS were diagnosed at birth or in the first year of life. The degree of QTc prolongation was much more prominent in patients with TS than in patients with COTS or with isolated LQT8 (median 603 vs. 490 vs. 480 ms, respectively; $p<0.001$) and the number of pts. with >500 ms QTc prolongation was much higher (94 vs. 17% vs. 20%, respectively; $p<0.001$). There was no significant difference regarding PM/ICD/AED implantation or utilization of left cervical sympathectomy among the groups. There was a marked difference in terms of outcome, as a much higher number of pts. with TS died, as compared with COTS, or isolated LQT8 (32 vs. 17% vs. 0%, respectively; $p=0.006$) or experienced MACE (71 vs. 33% vs. 30%, respectively; $p=0.004$).

#### 4.2.3.3. Analysis of all patients, including family members

There was one additional family member with exon 8/8A mutations (total of 53 patients) and additional 48 family members with non-exon 8/8A mutations (total of 81 patients), and there was one additional family member with TS (total of 60 cases), 9 family members with COTS (total of 15 cases) and 39 family members with isolated LQT8 (total of 59 cases).

As family members in every group usually exhibited a milder phenotype, all the statistical differences, observed between the index patient groups regarding disease and outcome parameters, remained statistically different, even at a higher significance level. Furthermore, the difference in age at death or at the time of MACE became statistically different, as patient with exon 8/8A mutations or patients with TS died at an earlier age and had MACE at an earlier age.

| | *CACNA1C* exon 8A Gly406Arg mutation carrier *n* = 31 | *CACNA1C* exon 8 Gly406Arg mutation carrier *n* = 6 | *p* | *CACNA1C* exon 8 Gly406Arg/Gly402Ser mutation carrier *n* = 12 | *p* | *CACNA1C* all exon 8 mutation carrier *n* = 16 | *p* | TS with syndactyly *n* = 45 | TS w/o syndactyly *n* = 14 | *p* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | vs. exon 8A Gly406Arg mutation carrier | | | | | | |
| ***Demographics*** | | | | | | | | | | |
| Age at diagnosis, months [median (IQR)] | 0 (0–11) | 0 (0–0) | 0.378 | 5 (0–51) | 0.189 | **32 (0–63)** | **0.019** | 6 (0–24) | 0 (0–41) | 0.721 |
| Diagnosis at birth, *n* (%) | 10/18 (55.6) | 5/6 (83.3) | 0.351 | 5/12 (41.7) | 0.710 | 5/16 (31.2) | 0.185 | 14/32 (43.7) | 9/14 (64.3) | 0.205 |
| Diagnosis in first year of life, n (%) | 16/18 (88.9) | 5/6 (83.3) | 1.000 | 7/12 (58.3) | 0.084 | **7/16 (43.7)** | **0.009** | 23/32 (71.9) | 9/14 (64.3) | 0.611 |
| Sex (M/F) | 15/9 | 2/4 | 0.360 | 4/6 | 0.276 | 4/7 | 0.156 | 16/18 | 8/3 | 0.143 |
| ***Disease manifestation*** | | | | | | | | | | |
| Extra cardiac manifestation, *n* (%) | | NA | | NA | | 14/16 (93.3) | 0.111 | | NA | |
| Syndactyly, n (%) | **29/31 (93.5)** | **1/6 (16.7)** | **0.000** | **4/12 (33.3)** | **0.000** | **6/16 (37.5)** | **0.000** | | NA | |
| Baldness, *n* (%) | 18/23 (78.3) | 1/2 (50%) | 0.430 | **1/5 (20)** | **0.026** | **1/8 (12.5)** | **0.002** | **21/27 (77.8)** | **2/7 (20.6)** | **0.024** |
| Facial abnormality, *n* (%) | 7/13 (53.8) | 3/6 (50) | 1.000 | 6/12 (50) | 1.000 | 6/16 (37.5) | 0.467 | 14/23 (60.9) | 8/14 (57.1) | 1.000 |
| Seizures, *n* (%) | 3/6 (50) | 2/4 (50) | 1.000 | 2/7 (28.6) | 0.592 | 2/8 (25.0) | 0.580 | 5/9 (55.6) | 5/9 (55.6) | 1.000 |
| Neuro developmental delay, *n* (%) | 7/11 (63.6) | 5/5 (100) | 0.245 | 8/11 (72.7) | 1.000 | 10/15 (66.7) | 1.000 | 15/21 (63.6) | 9/12 (75.0) | 1.000 |
| Autism/ASD, *n* (%) | 0 | 0 | – | 0 | – | 2/2 (100) | 1.000 | 0/1 (0.0) | 1/3 (33.3) | 1.000 |
| Recurrent infections, *n* (%) | 3/4 (75) | 1/2 (50) | 1.000 | 2/5 (40) | 0.524 | 2/6 (33.3) | 0.524 | 4/6 (66.7) | 2/4 (50.0) | 1.000 |
| Dental abnormalities, *n* (%) | 14/15 (93.3) | 1/1 (100) | 1.000 | 1/2 (50) | 0.228 | 1/3 (33.3) | 0.056 | 14/14 (100) | 3/5 (60.0) | 0.058 |
| Hypocalcemia, *n* (%) | 0/1 (0.0) | 1/1 (100) | 1.000 | 1/1 (100) | 1.000 | 1/2 (100.0) | 1.000 | 1/1 (100) | 1/2 (50.0) | 1.000 |
| Hypoglycemia, *n* (%) | 8/14 (57.1) | 2/2 (100) | 0.500 | 2/5 (40) | 0.628 | 2/9 (22.2) | 0.197 | 9/20 (45.0) | 4/7 (57.1) | 0.678 |
| Orthopedic disorder, *n* (%) | 0/1 (0.0) | 3/3 (100) | 0.250 | 4/4 (100) | 0200 | 4/5 (80.0) | 0.333 | 3/3 (100) | 5/6 (83.3) | 1.000 |
| ***ECG and arrhythmia manifestations*** | | | | | | | | | | |
| Max. QTc, ms [median (IQR)] | 600 (570–650) | 666 (555–702) | 0.639 | 610 (555–699) | 0.655 | 603 (555–681) | 0.500 | 603 (570–650) | 610 (554–702) | 0.991 |
| QTc > 500 ms, *n* (%) | 17/18 (94.4) | 6/6 (100) | 1.000 | 11/12 (91.7) | 1.000 | 14/16 (87.5) | 0.484 | 30/32 (93.7) | 13/14 (92.9) | 0.911 |

| | *CACNA1C* exon 8A Gly406Arg mutation carrier *n* = 31 | *CACNA1C* exon 8 Gly406Arg mutation carrier *n* = 6 | *p* | *CACNA1C* exon 8 Gly406Arg/Gly402Ser mutation carrier *n* = 12 | *p* | *CACNA1C* all exon 8 mutation carrier *n* = 16 | *p* | TS with syndactyly *n* = 45 | TS w/o syndactyly *n* = 14 | *p* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | vs. exon 8A Gly406Arg mutation carrier | | | | | | |
| AV block, *n* (%) | 27/31 (87.1) | 6/6 (100) | 1.000 | 7/11 (63.6) | 0.174 | **7/15 (46.7)** | **0.009** | 34/43 (79.1) | 9/13 (69.2) | 0.472 |
| Syncope, *n* (%) | 1/3 (33.3) | 2/2 (100) | 0.400 | 4/4 (100) | 0.143 | 4/4 (100.0) | 0.143 | 3/4 (75.0) | 4/5 (80.0) | 1.000 |
| T wave alternans, *n* (%) | 9/14 (64.3) | 5/6 (83.3) | 0.613 | 7/12 (58.3) | 1.000 | 9/15 (60.0) | 1.000 | 16/21 (76.2) | 6/12 (50.0) | 0.149 |
| Documented major arrhythmia NOT leading to ACA/SCD/ICDD, *n* (%) | 11/21 (52.4) | 4/6 (66.7) | 0.662 | 5/9 (55.6) | 1.000 | 5/9 (55.6) | 0.875 | 13/30 (43.39 | 7/10 (70.0) | 0.169 |
| ***Devices and interventions*** | | | | | | | | | | |
| PM, *n* (%) | 5/11 (45.5) | 4/5 (80.0) | 0.308 | 6/9 (66.7) | 0.406 | 6/9 (66.7) | 0.406 | 9/18 (50.0) | 7/10 (70.0) | 0.434 |
| ICD/AED, *n* (%) | 11/18 (61.1) | 3/6 (50.0) | 0.665 | 9/12 (75) | 0.694 | 11/15 (73.3) | 0.712 | 19/29 (65.5) | 9/14 (64.3) | 1.000 |
| LCSD, *n* (%) | 3/18 (16.7) | 1/6 (16.7) | 1.000 | 1/12 (8.3) | 0.632 | 1/15 (6.7) | 0.607 | 3/28 (10.7) | 2/14 (14.3) | 1.000 |
| ***Outcome*** | | | | | | | | | | |
| Death, *n* (%) | 13/31 (41.9) | 2/6 (33.3) | 1.000 | 2/12 (16.7) | 0.164 | 2/16 (12.5) | 0.052 | 16/45 (35.6) | 3/14 (21.4) | 0.514 |
| Age at death, months [median (IQR)] | 3 (1–25) | 37 (–) | 0.434 | 37 (–) | 0.434 | 37 | 0.434 | 2 (1–18) | 44 | 0.102 |
| Major adverse cardiac event (death/ACA/SCD/ICDD), *n* (%) | 22/31 (71.0) | 4/6 (66.7) | 1.000 | 10/12 (83.3) | 0.698 | 11/16 (68.7) | 0.876 | 30/44 (68.2) | 10/14 (71.4) | 1.000 |
| Age at MACE, months [median (IQR)] | 24 (2–29) | 45 (16–66) | 0.151 | 54 (16–72) | 0.062 | **54 (16–72)** | **0.034** | **17 (2–30)** | **60 (46–72)** | **0.003** |

Table 5: Comparison of clinical characteristics and outcome data in the index patients in Timothy syndrome subtypes.

## 4.3. Identification of known and novel *KCNJ2* mutations in Hungarian patients with Andersen-Tawil syndrome

### 4.3.1. Mutation data

Seven mutations in seven patients in the *KCNJ2* gene were identified. All the mutations were private, occurring only in one family each. The characteristics of the mutations are summarized in Table 6. The relative position of the mutations in the *KCNJ2* gene and the relative topological location of the mutations in the Kir2.1 channel protein is illustrated in Figure 7 and 8.

| Family | dbSNP ID | nucleotide change | protein change | ClinVar report | ClinVar classification | Franklin classification | novel | comment |
|---|---|---|---|---|---|---|---|---|
| L5 | rs786205820 | NM_000891.3:c.935G>A | NP_000882.1:p.Arg312His | yes | pathogenic/likely pathogenic | pathogenic | no | |
| L49 | rs199473384 | NM_000891.3:c.653G>A | NP_000882.1:p.Arg218Gln | yes | pathogenic | pathogenic | no | |
| L84 | – | NM_000891.3:c.905_907del | NP_000882.1:p.Val302del | no | – | likely pathogenic | yes | p.Val302Met is reported as pathogenic in ClinVar |
| L111 | rs199473653 | NM_000891.3:c.245G>A | NP_000882.1:p.Arg82Gln | yes | pathogenic/likely pathogenic | pathogenic | no | |
| L114 | – | NM_000891.3: c.877G>A | NP_000882.1:p.Glu293Lys | no | – | likely pathogenic | yes | p.Glu293Gln is reported as VUS in ClinVar |
| L131 | rs199473658 | NM_000891.3:c.921G>A | NP_000882.1:p.Met307Ile | yes | VUS | likely pathogenic | no | |
| L154 | – | NM_000891.3:c.161G>A | NP_000882.1:p.Cys54Tyr | no | – | likely pathogenic | yes | p.Cys54Phe is reported as pathogenic in ClinVar |

Table 6. Characteristics of the *KCNJ2* mutations identified in the cohort of patients with Andersen-Tawil syndrome.

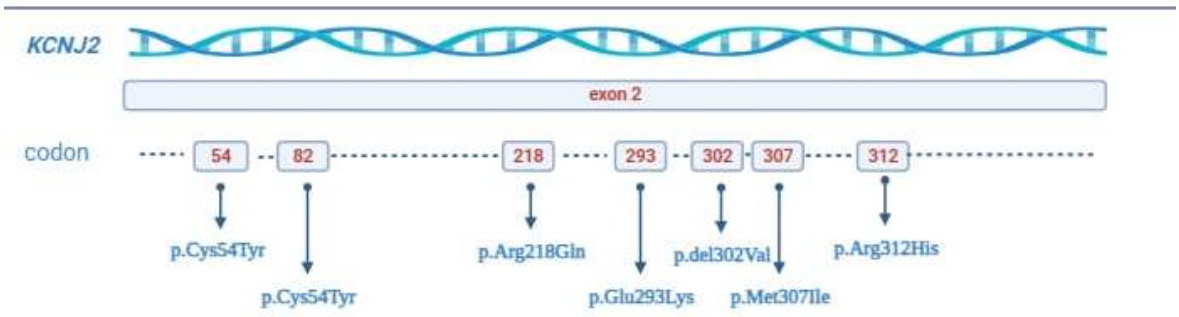


Figure 7. Localization of the identified *KCNJ2* mutations in the schematic structure of the *KCNJ2* gene.

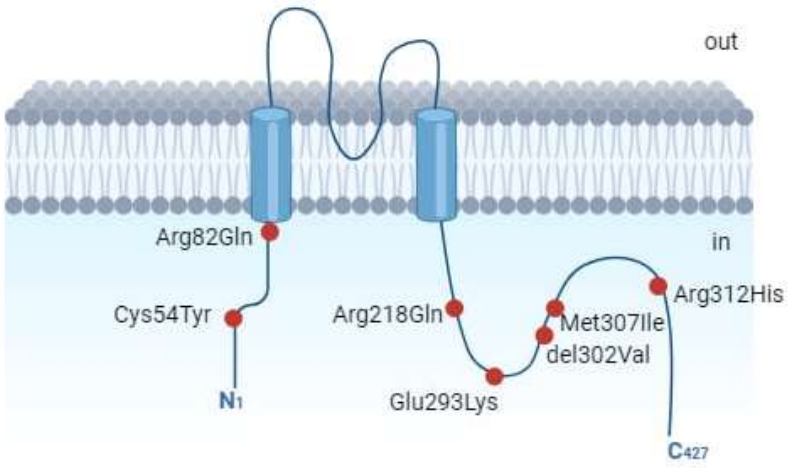


Figure 8. Topological localization of the identified *KCNJ2* mutations in the schematic structure of the Kir2.1 ion channel.

Out of the seven mutations, six were missense mutations and one was a single amino-acid deletion (NM_000891.3:c.905_907del, NP_000882.1:p.Val302del). The patient carrying the latter mutation was heterozygous for the mutant allele, as indicated by the superimposed normal and the shifted mutant sequences (Appendix Figure 2). The mutation appeared to affect the last two nucleotides of codon 302 (TG) and the first nucleotide of codon 303 (G). As the remaining first nucleotide of codon 302 (G) and the last two nucleotides of codon 303 (AA) leave the same coding sequence (GAA) as the original codon 303, the consequence of the deletion is the complete in-frame loss of codon 302, encoding for Valine (reference sequence: ENSP00000441848, www.ensembl.org), while the rest of the original reading frame is preserved (Appendix Figure 2 Panel C).

Two mutations affected the N-terminal and 5 mutations affected the C-terminal part of the protein (Figure 8). Three of the mutations (p.Val302del, p.Glu293Lys, p.Cys54Tyr) were novel, while the others were previously reported mutations. All but one variant with a ClinVar entry were classified as pathogenic/likely pathogenic, the one variant (p.Met307Ile) was classified as VUS by ClinVar, based on just one entry, judged as insufficient information. However, all the variants were classified as pathogenic/likely pathogenic by Franklin. Based on the typical phenotype associated with the p.Met307Ile and the Franklin classification we considered the pMet307Ile variant as likely pathogenic.

### 4.3.2. Family screening

Altogether 30 family members were available for family screening. Out of them, 10 family members proved to be carriers of any of the *KCNJ2* mutations (Table 3). Among the mutation carriers 6 family members proved to be clinically affected (see family trees in Appendix Figure 4). Two mutation carrier newborns were diagnosed based on the presence of the frequent PVBs on the ECG, while the other four adult mutation carriers showed typical manifestations of the disease including dysmorphic features and ECG characteristics (Table 1). The other family members did not show any signs and symptoms of the disease, neither cardiac nor extra-cardiac, even at an advanced age.

### 4.3.3. Functional characterization of the identified novel *KCNJ2* gene mutations, p.Val302del and p.Glu293Lys

Functional characterization of the identified novel *KCNJ2* gene mutations, p.Val302del and p.Glu293Lys was performed at the Department of Pharamacology and Pharamacotherapy, University of Szeged. The data of functional characterization is not part of this PhD thesis, but for the sake of completeness it is covered briefly here.

As for the p.Val302del mutation, heterologously expressed wild-type (WT) and p.Val302del mutant alleles showed similar subcellular distribution of the Kir2.1 protein with high intensity labelling from the membrane region, indicating that membrane transportation of Kir2.1 is not affected by the p.Val302del mutation. Cells transfected with the WT allele displayed a robust current with strong inward rectification, while no current above background was detected in cells expressing the p.Val302del Kir2.1 subunit. Co-transfection of CHO cells with the WT and the p.Val302del Kir2.1 revealed a dose-dependent inhibitory effect of the p.Val302del Kir2.1 mutant subunit on WT Kir2.1 currents. These observations indicated that the wild type and the p.Val302del mutant subunits co-assemble in the cell membrane and that the mutation affects potassium conductivity and/or gating of the WT/Val302del heteromeric Kir2.1 channels. (referencia)

As for the p.Glu293Lys variant, the variant produced no current in homomeric form and showed dominant-negative effect over WT subunits. Immunocytochemical labelling showed the p.Glu293Lys subunits to distribute in the subsarcolemmal space. Salt bridge prediction indicated the presence of an inter-subunit salt bridge network at the CD-I of Kir2.1, with the

involvement of Glu293. Subunit interactions were studied by the NanoLucVR Binary Technology (NanoBiT) split reporter assay. Reporter constructs carrying NanoBiT tags on the intracellular termini produced no bioluminescent signal above background with the p.Glu293Lys variant in homomeric configuration and significantly reduced signals in cells co-expressing WT and p.Glu293Lys subunits simultaneously. Extracellularly presented reporter tags, however, generated comparable bioluminescent signals with heteromeric WT and p.Glu293Lys subunits and with homomeric WT channels. Loss of function and dominant-negative effect confirmed the causative role of p.Glu293Lys in ATS1. Co-assembly of Kir2.1 subunits is impaired in homomeric channels consisting of p.Glu293Lys subunits and is partially rescued in heteromeric complexes of WT and p.Glu293Lys Kir2.1 variants. These data point to an important role of Glu293 in mediating subunit assembly, as well as in gating of Kir2.1 channels.

## 4.4. Qualitative and quantitative assessment of ECG characteristics in patients with Andersen-Tawil syndrome

There was no significant difference between the ATS and LQTS patient groups in terms of age (25.4±11.6 vs. 26.0±9.3 years; p=0.9249) and gender distribution (6 females/1 male vs. 6 females/1 male; p=1.000), matched for age and gender (Table 7).

### 4.4.1. Qualitative evaluation of ECG recordings in ATS patients

Frequent ventricular extrasystoles (or bigeminy) were present in 71% (5/7) of cases with ATS (Figure 9, Panel A). Typical bidirectional extrasystoles (ES) or NSVT were observed in 57% (4/7) of cases with ATS (Figure 9, Panel B). These ECG characteristics were not observed in any of the LQTS patients.

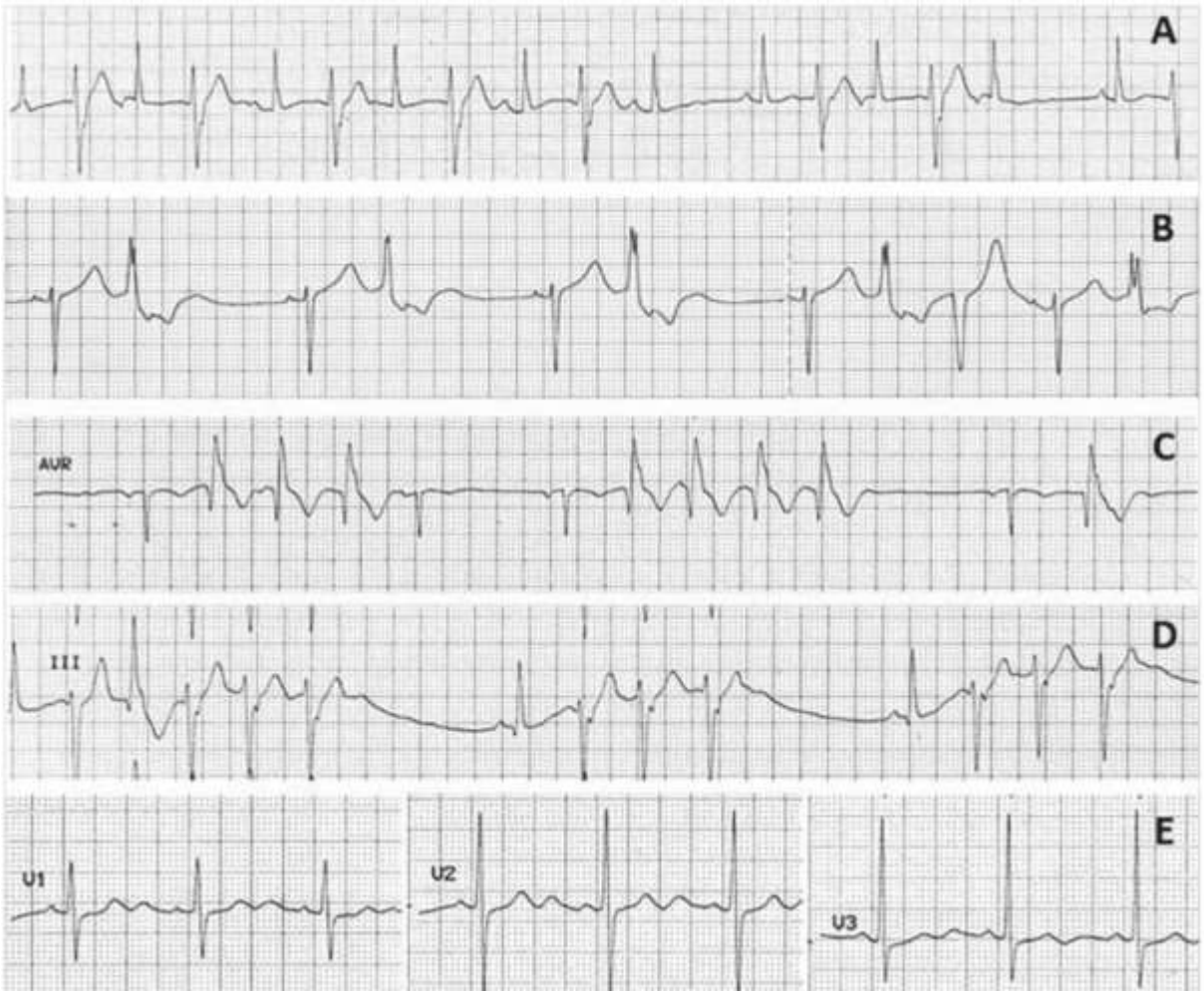


Figure 9: Characteristic ECG abnormalities in Andersen-Tawil syndrome. A-panel: frequent premature ventricular contractions (PVCs), bigeminy; B-panel: frequent PVCs, bigeminy, couplet, with bidirectional morphology; C-panel: non-sustained ventricular tachycardia; D-panel: non-sustained ventricular tachycardia; with bidirectional morphology; E-panel: prominent U wave in leads V1-3.

### 4.4.2. Quantitative analysis of ECG parameters in ATS and LQTS patients

The QTc, QUc, U-wave width, and U-wave height parameters for ATS and LQTS patients are presented in Table 7. Regarding quantitative ECG parameters, the corrected QT interval was significantly shorter in ATS patients compared to LQTS patients (451.2 vs. 518.4 ms, $p<0.04$), while the corrected QU interval did not differ significantly (575.7 vs. 583.8 ms, $p<0.84$). The QTc was within the normal range (<460 ms) in 57% (4/7) of ATS cases. The U-waves were significantly longer (U-wave duration: 143.0 vs. 78.5 ms, $p<0.004$) and higher (U-wave amplitude: 0.139 vs. 0.064 mV, $p<0.0002$) in ATS patients (Figure 9, Panel C).

| Subject | Diagnosis | Sex | Age | QTc (ms) | Quc (ms) | Uwidth (ms) | Uhight (mV) |
|---|---|---|---|---|---|---|---|
| L 5.0 | ATS | female | 20 | 411 | 649,5 | 190 | 0,16 |
| L 49.0 | ATS | female | 44 | 483 | 599 | 150 | 0,1 |
| L 49.1 | ATS | female | 37 | 420 | 510 | 130 | 0,15 |
| L 49.3 | ATS | female | 25 | 401 | 482 | 150 | 0,1 |
| L 114.0 | ATS | female | 9 | 522 | 639,5 | 110 | 0,23 |
| L 131.0 | ATS | male | 23 | 477 | 500 | 120 | 0,1 |
| L 84.0 | ATS | female | 20 | 444,25 | 649,88 | 151,25 | 0,13 |
| | | | **25,4±11,6** | **451,2±44,4** | **575,7±75,7** | **143±26** | **0,139±0,05** |
| L 60.0 | LQTS | female | 20 | 488,43 | 533,14 | 97,14 | 0,07 |
| L 71.0 | LQTS | female | 44 | 454,5 | 573 | 100 | 0,05 |
| L 76.0 | LQTS | female | 20 | 626 | 730 | 70 | 0,06 |
| L 1.0 | LQTS | female | 28 | 525 | 622,2 | 52 | 0,07 |
| L 112.0 | LQTS | female | 10 | 456,5 | 529 | 60 | 0,1 |
| L 50.0 | LQTS | male | 23 | 590 | 504 | 90 | 0,05 |
| L 92.0 | LQTS | female | 21 | 488 | 595 | 80 | 0,05 |
| | | | **26,0±9,3** | **518,4±66,4** | **583,8±76,4** | **78,5±19** | **0,064±0,02** |
| **p** | | **NS** | **NS** | **p<0,04** | **p<0,84** | **p<0,004** | **p<0,0002** |

Table 7: Comparison of quantitative ECG parameters between Andersen-Tawil syndrome (ATS) and long QT syndrome (LQTS) patients. QTc: corrected QT interval, QUc: corrected QU interval, U width: width of U wave, U height: height of U wave.

# 5. DISCUSSION

## 5.1. Identification of mutations causing Timothy syndrome among Hungarian patients with ion channel diseases

In our work we reported a variant case of Timothy syndrome, caused by a TS1-specific *CACNA1C* exon 8A p.Gly406Arg mutation. The clinical phenotype was characterized by neonatal 2:1 AV block and marked QT prolongation. However, the patient lacked many of the other hallmarks of TS1, most notably syndactyly, and no other major extracardiac manifestations were present.

Syndactyly is reported in 100% of patients with *CACNA1C* exon 8A p.Gly406Arg mutation. Moreover, syndactyly was also present in patients proved to be somatic or germline mosaic carriers of the mutation.(26)(25) In one of these cases the direct presence of the *CACNA1C* exon 8A p.Gly406Arg mutation was demonstrated in left/right arm skin biopsy samples with an estimated percent mosaicism of 6.5%.(25) As Cav1.2 was highly expressed in apical ectodermal ridge cells of developing digits, syndactyly is thought to be caused in TS by $Ca^{2+}$-induced cell death in the apical ectodermal ridge.(9)

There are several possible explanations for the lack of syndactyly and major extracardiac manifestations in our case. One of the possible reasons for the variant phenotype might be the variable expression of exons 8A and 8 of the *CACNA1C* gene in different tissues. Exons 8 or 8A are mutually exclusive exons, which encode the transmembrane segment 6 of domain I (DI/S6). Exon 8A, although, expressed in multiple adult and fetal tissues, including heart, brain, gastrointestinal system, lungs, immune system, smooth muscle, and testis, represents the less dominant cardiac and brain isoform of *CACNA1C*, as its relative expression was consistently shown to be significantly much lower than exon 8 in heart and brain (23% vs. 77%).(9) The mutant exon 8A isoform might be present in even less amount in non-affected tissues and the milder dysfunction in the Cav1.2 ion channel could possibly be compensated by the non-mutant exon 8 isoform or by other mechanisms. In addition, the role of modifier genes, known to alter the disease phenotype in many diseases,(35)(36) cannot be ruled out either. Another explanation for the variant phenotype is the possible presence of somatic mosaicism in our patient, as reports on mosaic patients with TS1 describe a partial phenotype of the disease, similar to ours.(25)(26) The possible presence of somatic mosaicism was also reported with

regard to TS2.(8)(37) According to this scenario, the mutant Cav1.2 channel must be absent from unaffected organs (limbs, brain) and might be present in low levels in probably affected organs (heart, pancreas) in our patient. However, as we had four sources of tissue (lymphocytes, buccal cells, uroepithelial cells, hair follicles), and all were shown to harbor the mutation, direct evidence for the above explanation is lacking. Despite this, mosaicism cannot be ruled out completely, as samples were not available from tissues characteristically affected in TS (i.e., brain and digits). In conclusion, we described a Timothy syndrome 1 genotype without syndactyly and major extracardiac manifestations. The case highlights further phenotypic variability in Timothy syndrome. Most importantly, it underlines that the lack of syndactyly doesn't exclude the presence of Timothy syndrome 1 genotype.

## 5.2. Assessment of geno- and phenotypic characteristics and clinical outcomes of *CACNA1C* gene mutation associated Timothy syndrome, "cardiac only" Timothy syndrome and isolated long QT syndrome 8

In our systemic review we examined *CACNA1C* gene mutation associated Timothy syndrome, "cardiac only" Timothy syndrome, isolated long QT syndrome 8 and provided data that these disease forms exhibit major differences regarding clinical manifestations and outcome. These differences can be defined either based on the genotype or the phenotype. In the literature, there is a high degree of controversy regarding the classification of Timothy syndrome. Since the original reports on TS1 and TS2, several proposals have been made to identify TS subtypes. One proposal suggested that all TS phenotypes resulting from mutations in *CACNA1C* should be classified as TS1, and when the next TS disease gene is discovered, it can be classified as TS2.(25) Another proposal recommended that TS1 and TS2 should include exclusively only patients with the p.Gly406Arg mutation in exon 8A (TS1) or in exon 8 (TS2), while the remaining alleles be called atypical TS.(13) It has been also argued that there is no clinical utility to distinguishing TS1 from TS2.(38)

However, these proposals were based on small case series and mostly lacked evidence for the most profound question as to whether there is any major difference in the clinical manifestation or, more importantly, the outcome of TS subtypes. Here we provide data, that apart of syndactyly or baldness, there is no major differences regarding clinical manifestations or

outcome measures between TS1 and TS2, either defining TS subtypes on the genotype or on the phenotype. Both subtypes are characterized by an extreme degree of QTc prolongation (median ≥600 ms) which is reflected in the similarly high MACE rate. All the above probably makes the distinction between TS1 and TS2 obsolete, at least with regard to clinical outcome, and may make the use of the descriptive terms “classical TS” (with syndactyly) and “non-classical TS” (without syndactyly) more appropriate, as it emphasizes that the two are similar diseases with comparable clinical outcomes.

On the other hand, there are important differences between TS, COTS and isolated LQT8. Based on the phenotype, Timothy syndrome is characterized by a much earlier disease onset, much more pronounced QTc prolongation and much higher mortality than “cardiac only” Timothy syndrome or isolated LQT8. Although phenotypic differences are the basis for the categorization of these disease forms, this is the first time that differences in ECG parameters or in the clinical outcome have been demonstrated. The degree of QTc prolongation is often extreme in TS (>600 ms), and it is >500 ms in >90% of TS patients, while it is only mildly prolonged (<500 ms) in COTS or LQTS. This might be the primary reason for the higher rate of clinical complications, either death or MACE. Similar observations were made by Landstrom et al. based on 28 independent probands.(33) Based on the above findings, the established categorization of these disease groups (TS, COTS and isolated LQT8) seems to be justified also on the grounds of clinical outcome.

Differences, like those outlined above, can be encountered if comparisons are made based on the genotype, i.e., between carriers of exon 8/8A mutations vs. non-exon 8/8A mutations.

As exon 8/8A mutations are responsible for 83% of TS cases and only 17% of COTS and 10% of isolated LQT8, similar differences can be observed that was seen among TS, COTS and isolated LQT8. These include that disease onset is at much a younger age, phenotypic characteristics of TS are more prevalent, QTc prolongation is much more pronounced and clinical outcome is much more severe in patients with exon 8/8A mutations.

Additionally, the results of our systemic review demonstrated that TS is a rather homogenous disease genetically, as the p.Gly406Arg mutation either in exon 8 or exon 8A alone is responsible for 70% of the cases, and mutations affecting codons 402–407 is responsible for 85% of TS cases. The strongest relationship was seen between mutation p.Gly406Arg in exon 8A and “classical” TS, which is present in 93.5% of the patients. COTS and isolated LQT8 are

more heterogenous in this regard having their causative mutations more scattered through the gene.

The cellular electrophysiological alterations caused by the different *CACNA1C* gene mutations alone are not sufficient to explain the above differences in phenotypic expression. *CACNA1C* mutations may result in channel dysfunction in different ways [recently reviewed in detail by Bauer et al.](13), but the general mechanism is that these mutations lead to gain-of-function alleles of the gene, thereby prolonging the cardiac action potential (AP) and consequently the QT interval.(8)(9)(21) The classical mechanism of channel dysfunction, demonstrated for TS mutations (exon 8A and 8 p.Gly406Arg, and exon 8 p.Gly402Ser variants) in different expression systems, is a loss of voltage-dependent inactivation (VDI) of the channel, which leads to prolonged opening of the channel and subsequently causes an increase in the maximum flow of Ca2+ through the channel (peak current density). (8)(9)(21) Additional mechanisms, affecting calcium-dependent inactivation (CDI)(39) and steady-state inactivation (SSI) of the channel, significantly increasing the window current, has also been demonstrated (in case of the p.Gly1911Arg mutation).(39)(40) Alternative channel dysfunctional mechanisms are characterized by a reduction in peak current density which is associated with a negative shift in V1/2 of activation, the degree of depolarization necessary for activation of the channel (p.Ile1166Thr, p.Arg518His, p.Arg518Cys, p.Ser643Phe, p.Gly419Arg variants). (14)(15)(23)(41) (42) This would also result in a net gain-of-function effect on Cav1.2 channels.

For explaining the wide variations in phenotypic expression for TS, COTS and isolated LQT8, many different possibilities have been proposed [recently reviewed in detail by Bauer et al.](13). One possible mechanism is the presence of parental or individual mosaicism, which has been reported in several cases with TS.(26)(28) In the scenario of parental mosaicism, a “de novo” mutation may arise during gametogenesis of a parent (who is mosaic for the variant, since only his/her gamete is affected) but the descendant is fully heterozygous for the variant. In the case of individual mosaicism, mosaicism would occur in the developing embryo due to a “de novo” mutation which may arise post-zygotically. The variant may be present or absent in the cells and organs of the affected mosaic individual, depending on the timing and location of the mutation happened during embryogenesis.(13)

Another explanation could involve the many different isoforms of the *CACNA1C* protein. The transcript profile of the *CACNA1C* gene was reported to be substantially more complex than appreciated by Clark et al., as they identified 38 novel exons and 241 novel transcripts.(43)

Importantly, many of the novel variants were abundant, and predicted to encode channels with altered function. It has been also demonstrated by Dick et al., based on studies of the p.Gly406Arg mutation in adult guinea pig ventricular myocytes, that cells could tolerate a certain proportion of mutant *CACNA1C* channels.(39) A low level of mutant channels caused only a slight AP prolongation; however, above a certain threshold (about 12% for Gly406Arg and about 40% for Gly402Ser), APs became unstable, and cells became arrhythmogenic. This may be further affected by the "repolarization reserve," as congenital or acquired impairment of one type of transmembrane ion channel does not necessarily result in excessive repolarization changes because other repolarizing currents can take over and compensate.(44)

## 5.3. Identification of known and novel *KCNJ2* mutations in Hungarian patients with Andersen-Tawil syndrome

In our study involving a Hungarian cohort of patients with Andersen-Tawil syndrome, we identified a total of seven distinct *KCNJ2* mutations. These mutations included p.Arg218His, p.Arg312Glu, p.del302Val, p.Glu293Lys, p.Met307Ile, p.Cys54Tyr, and p.Arg82Gln.

Four of these mutations have been documented in the literature with various clinical manifestations.

The p.Arg312Glu mutation was reported to be associated with hypokalemic periodic paralysis, characterized by two to three episodes per year. Major triggers included exercise, post-exercise, and stress. Additionally, patients experienced episodic pain and sustained cramps in the lower legs, particularly in the calves, and in the hands. Physical examination revealed subtle dysmorphisms, such as short palpebral fissures, thin upper lips, a high arched palate, mild facial asymmetry, and a long nose. Electrocardiogram analysis typically showed the presence of U waves.(45)

The p.Arg218His mutation presented with dysmorphic facial features, including hypertelorism and micrognathia. Clinical manifestations included episodes of weakness, hypokalemia, premature ventricular contractions, and ventricular tachycardia during sleep.(46)

The p.Arg82Gln mutation was linked to premature ventricular contractions. Patients exhibited characteristic dysmorphic features such as low-set ears, hypertelorism, a relatively broad forehead, a thin upper lip, micrognathia, small hands, and clinodactyly of the fifth digits.(47)

The p.Met307Ile mutation manifested as intermittent quadriparesis and shares common dysmorphic features, including hypertelorism, a broad-based nose, low-set ears, and micrognathia. Clinodactyly was also observed. Unlike the other mutations, patients with p.Met307Ile mutation exhibited normokalaemia and asymptomatic premature ventricular and atrial contractions.(46)

The remaining three mutations were novel, and all were private, occurring only in one family each.

By functional analysis we were able to demonstrate that p.Glu293Lys causes loss of function and exerts a dominant-negative effect on Kir2.1 currents, as indicated by the patch-clamp experiments. Importantly, the dominant-negative effect was detected over a physiologically relevant membrane potential range between -70 and -10 mV and it was comparable to that of a well-known ATS1 variant p.Arg218Gln. Furthermore, Glu293 plays an important role in mediating subunit interactions within the Kir2.1 ion channel complex, possibly maintaining a salt bridge network at the CD-I. The charge reversal in the p.Glu293Lys variant leads to impaired subunit co-assembly in homomeric channels consisting of p.Glu293Lys subunits and to gating abnormalities in heteromeric complexes of WT and p.Glu293Lys subunits. These data indicate that p.Glu293Lys is a novel causative *KNCJ2* variant in ATS1, exerting dominant-negative effect on the WT allele on a heterozygous genetic background. These findings demonstrate the causative role of the p.Glu293Lys mutation in ATS1.

The second novel *KCNJ2* mutation, p.Val302del, was associated with the full clinical spectrum of ATS including periodic paralysis, ventricular arrhythmias, and dysmorphic features. Cardiac symptoms included mild QT prolongation, prominent U-waves, frequent premature ventricular beats, and bidirectional ventricular tachycardia. Functional analysis of the mutation using heterologously expressed wild type and p.Val302del mutant alleles demonstrated normal membrane trafficking of the p.Val302del Kir2.1 variant. However, co-expression of the WT and the p.Val302del Kir2.1 revealed a dose-dependent inhibitory effect of the p.Val302del Kir2.1 mutant subunit on WT Kir2.1 currents. These observations indicate that the WT and the p.Val302del mutant subunits co-assemble in the cell membrane and that the mutation affects potassium conductivity and (or) gating of the WT/Val302del heteromeric Kir2.1 channels, strongly indicating a causative role of the p.Val302del mutant.

Results of the functional analysis of the third novel *KCNJ2* variant, p.Cys54Tyr, is not available yet. However, annotation programs classified the variant as likely pathogenic, and another

variant, affecting the same amino acid, p.Cys54Phe was reported as pathogenic in ClinVar. Therefore we classified the *KCNJ2* p.Cys54Tyr variant as likely pathogenic.

## 5.4. Qualitative and quantitative assessment of ECG characteristics in patients with Andersen-Tawil syndrome

In our study, we conducted a qualitative and quantitative analysis of ECG abnormalities in Hungarian patients with Andersen-Tawil syndrome. Our results indicate that patients with ATS exhibit characteristic ECG changes, such as frequent ventricular extrasystoles, bidirectional bigeminy, or runs. The corrected QT interval is often shorter and can even fall within the normal range. The U-wave is more prominent, longer, and higher in these patients.

It is well known and clinically significant that certain surface ECG abnormalities and the shape of the T wave can differ and be characteristic of different LQT subgroups. In the LQT1 subgroup, an early appearing, broad-based, prolonged T wave is typical, while the LQT2 subgroup is characterized by a significant decrease in T-wave amplitude, bifid, split, confluent U-wave-like T waves with multiple wave components, primarily observed in the chest leads. The LQT3 subgroup's typical ECG change is the prolonged ST segment, meaning the QT prolongation is due to a late-appearing T wave of normal duration and amplitude.

It appears that such distinct ECG characteristics exist in Andersen-Tawil syndrome, too. Zhang et al. were the first to investigate the ECG characteristics observed in ATS.(6) In the studied population (96 ATS patients), the degree of QTc prolongation was not significant (20 ms), and the median QTc value (440 ms) was within the normal range. Only 17% of patients had a QTc value exceeding 460 ms. In 91% of patients, the T-U-wave morphology, particularly the enlarged U wave, was characteristic. In some patients, the descending limb of the T wave was prolonged, and the U wave was biphasic.

Kukla and colleagues attempted to standardize the ECG characteristics of Andersen-Tawil syndrome patients by describing diagnostic features during ECG analysis of ATS patients:(48) (a) in tachycardic ATS patients, when the P wave and U wave "merge", a P-pulmonale morphology develops, and the P wave height can exceed 3 mm; (b) considering the presence of the U wave and excluding it from the calculations, the patients' QTc duration falls within the normal range; (c) in the first cardiac cycle following a VES, the T and U waves can fuse, resulting in a pseudo-LQTS morphology; (d) in leads V2-3, the U wave may be present, and its

amplitude may be further increased by tachycardia, unlike in healthy controls; (e) in the adrenaline provocation test, the U/T wave ratio changes from <1 to >1.

Among the rhythm disturbances observed in ATS, frequent ventricular extrasystoles, possible bigeminy, and bidirectional polymorphic ventricular tachycardia (VT) are most characteristic. Typically, VT is non-sustained and relatively slow, with a frequency of ≤150/min. Although VT is not rapid and is generally well-tolerated, the number of ES and VT episodes can lead to tachycardia-induced cardiomyopathy. It is crucial to distinguish bidirectional VT observed in ATS from that seen in digitalis toxicity(49) and catecholaminergic polymorphic ventricular tachycardia (CPVT),(50) as the prognosis and treatment of ATS and CPVT differ. While the number of ES and VT episodes is typically high in ATS, the transition to life-threatening ventricular arrhythmias is not characteristic, unlike in CPVT patients.(6) Physical exertion does not induce arrhythmias in ATS patients, unlike in CPVT patients, and according to some reports, exertion can even suppress arrhythmias. The two diseases can be differentiated through genetic testing, with most CPVT cases and approximately 60% of ATS cases being diagnosable using genetic methods.

# 6. SUMMARY AND ORIGINAL FINDINGS

**1. We identified a *CACNA1C* gene exon 8A p.Gly406Arg mutation, specific to Timothy syndrome type 1, in a patient with a variant phenotype of Timothy syndrome characterized by lack of syndactyly and of substantial extracardiac manifestations.**

Our case draws attention to phenotype variants of Timothy syndrome. It should be emphasized that the absence of syndactyly does not exclude the presence of Timothy syndrome type 1 genotype.

**2. Assessing geno- and phenotypic characteristics and clinical outcomes of *CACNA1C* gene mutation associated Timothy syndrome, "cardiac only" Timothy syndrome and isolated long QT syndrome 8, we observed that clinical phenotypes associated with mutations in the *CACNA1C* gene show important clinical differences.**

Timothy syndrome is associated with the most severe clinical phenotype and with the highest risk of morbidity and mortality. However, distinguishing TS subtypes, in any form, are not supported by our data.

**3. We identified new and known *KCNJ2* gene mutations in Hungarian patients with Andersen-Tawil syndrome.**

In a Hungarian patient cohort of ion channel patients we identified 7 *KCNJ2* mutations (p.Arg218His, p.Arg312Glu, p.del302Val, p.Glu293Lys, p.Met307Ile, p.Cys54Tyr, p.Arg82Gln). Three of the mutations were novel. The mutations were identified in 17 patients, belonging to 7 families.

**4. By analyzing qualitative and quantitative ECG characteristics in patients with Andersen–Tawil syndrome we found that patients with ATS usually exhibit typical ECG changes representing frequent ventricular ES, bigeminy or NSVT.**

Prolongation of the corrected QT interval is not typical and QTc may be in the normal range. U waves are more prominent, being longer and taller in these patients.

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# Appendix and supplementary material

## 2. Assessment of geno- and phenotypic characteristics and clinical outcomes of *CACNA1C* gene mutation associated Timothy syndrome, "cardiac only" Timothy syndrome and isolated long QT syndrome 8

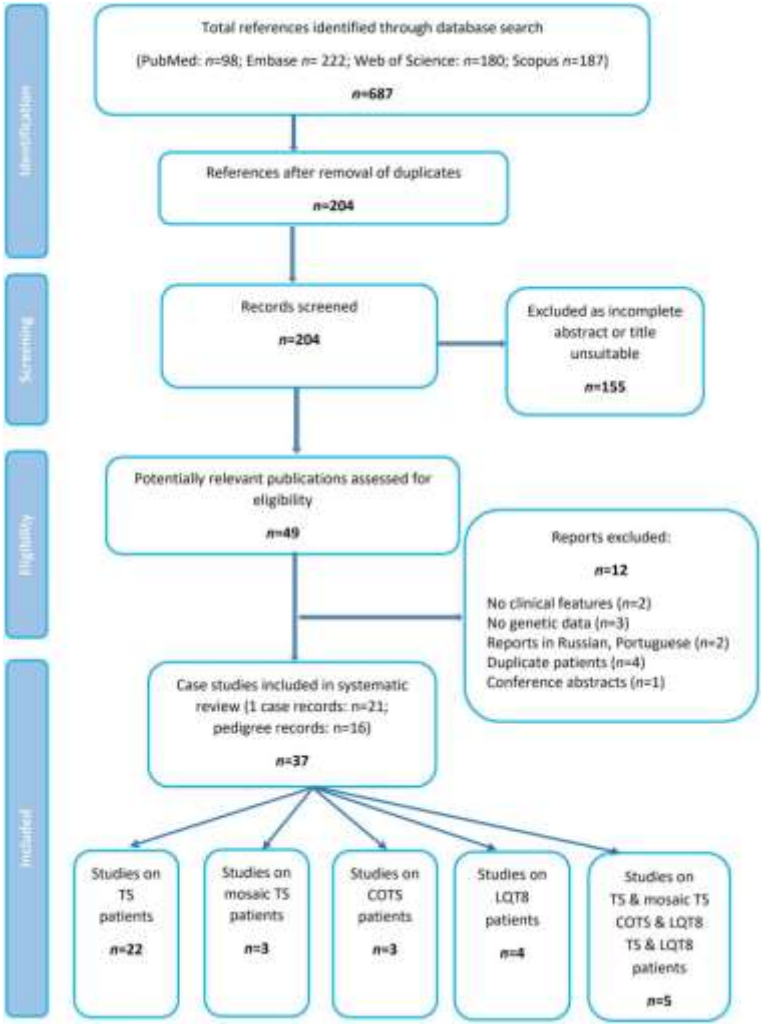



Figure 1: Flow chart of study selection

| First author of report | Year of report | Origin of report | Reported number of index patients | Reported disease phenotype | Reported CACNA1C_mutation | Reference |
|---|---|---|---|---|---|---|
| Splawski et al. 2004 | 2004 | USA | 13 | TS | Gly406Arg | Splawski I et al. Cell. 2004;119:19-31. |
| Lo-A-Njoe et al. 2005 | 2005 | Netherlands | 2 | TS | Gly406Arg | Lo-A-Njoe SM et al. Heart Rhythm 2005;2:1365-8. |
| Splawski et al. 2005 | 2005 | USA | 2 | TS | Gly406Arg, Gly402Ser | Splawski et al. Proc Natl Acad Sci USA 2005;102:8089-96 |
| Jacobs et al. 2006 | 2006 | USA | 1 | TS | Gly402Ser | Jacobs et al. Heart Rhythm. 2006;3:967-70. |
| Krause et al. 2011 | 2011 | Germany | 1 | TS | Gly406Arg | Krause et al. Clin Res Cardiol. 2011;100:1123-7. |
| Gillis et al. 2012 | 2012 | Canada | 1 | TS | Ala1473Gly | Gillis et al. Am J Med Genet A. 2012;158A:182-7. |
| An et al. 2013 | 2013 | South-Korea | 1 | TS | Gly406Arg | An et al. J Korean Med Sci. 2013 May;28(5):788-91. |
| Boczek et al. 2013 | 2013 | USA | 4 | LQT8 | Pro857Arg, Lys834Glu, Pro857Leu, Arg1906Gln | Boczek et al. Circ Cardiovasc Genet. 2013 Jun;6:279-89. |
| Gao et al. 2013 | 2013 | China | 1 | TS | Gly406Arg | Gao et al. Circ Arrhythm Electrophysiol. 2013;6:614-22. |
| Fröhler et al. 2014 | 2014 | Lebanon | 1 | TS | Gly402Ser | Fröhler et al. BMC Med Genet. 2014;15:48. |
| Fukuyama et al. 2014 | 2014 | Japan | 7 | LQT8 | P381S, M456I, A582D, R858H, G1783C | Fukuyama et al. Europace 2014;16:1828-37. |
| Hennessey et al. 2014 | 2014 | Fillipines | 1 | TS | G1911R | Hennessey et al. PLoS One. 2014;9:e106982. |
| Boczek et al. 2015 | 2015 | USA | 4 | COTS:3, TS:1 | Arg518Cys, Arg518His, Ile1166Thr | Boczek et al. Heart Rhythm. 2015;12:211-9. |
| Corona-Rivera 2015 | 2015 | Mexico | 1 | TS | Gly406Arg | Corona-Rivera et al. Eur J Med Genet. 2015;58:332-5. |
| Diep et al. 2015 | 2015 | Hawaii | 1 | TS | Gly406Arg | Diep et al. Am J Med Genet A. 2015;167A:2780-5. |
| Ergül et al. 2015 | 2015 | Turkey | 1 | TS | Gly406Arg | Ergül et al. Anatol J Cardiol. 2015;15:672-4. |
| Hiippala et al. 2015 | 2015 | Finland | 2 | COTS:1, LQT8:1 | Gly406Arg, Gly402Ser | Hiippala et al. Am J Med Genet A. 2015;167A:629-34. |
| Wemhöner et al. 2015 | 2015 | Germany | 6 | TS:1, LQT8:5 | Ala28 Thr, Ile1166Val, Ile1475Met, Arg860Gly, Ile1166Thr, Glu1496Lys | Wemhöner et al. J Mol Cell Cardiol. 2015;80:186-95. |
| Gunay et al. 2016 | 2016 | Turkey | 1 | TS | Ala1473Gly | Gunay et al. Cardiology in the Young. 2016;26:S72-S73. |
| Kawaida et al. 2016 | 2016 | Japan | 1 | TS | Gly406Arg | Kawaida et al. Pathol Int. 2016;66:587-592. |
| Landstrom et al. 2016 | 2016 | hispanic | 1 | LQT8 | Leu762Phe | Landstrom et al. Int J Cardiol. 2016;220:290-8. |
| Landstrom et al. 2016 | 2016 | hispanic | 1 | TS | Gly406Arg | Landstrom et al. Int J Cardiol. 2016;220:290-8. |
| Philipp et al. 2016 | 2016 | USA | 1 | TS | Gly406Arg | Philipp et al. Proc (Bayl Univ Med Cent). 2016;29:160-2. |
| Sepp et al. 2017 | 2017 | Hungary | 1 | TS | Gly406Arg | Sepp et al. Am J Med Genet A. 2017;173:784-9. |
| Dufendach et al. 2018 | 2018 | USA | 16 | TS | Gly402Ser, Gly406Arg, Ser405Arg, Gly402Arg, Lys1211Glu,Cys1021Arg | Dufendach et al. JACC Clin Electrophysiol. 2018;4:459-66. |
| Gardner et al. 2018 | 2018 | New Zealand | 1 | LQT8 | Arg858His | Gardner et al. Mol Genet Genomic Med. 2019;7:e00476. |
| Kojima et al. 2018 | 2018 | Japan | 1 | COTS | K1580T | Kojima et al. J Cardiothorac Surg. 2017;12:118. |
| Kosaki et al. 2018 | 2018 | Japan | 1 | TS | Arg1024Gly | Kosaki et al. Am J Med Genet A. 2018;176:1657-1661. |
| Ozawa et al. 2018 | 2018 | Japan | 1 | TS | Ser643F | Ozawa et al. Heart Rhythm Case Rep. 2018;4:273-77. |
| Seo et al. 2018 | 2018 | South-Korea | 1 | COTS | Arg518Cys | Seo et al. Ann Lab Med. 2018;38:54-8. |
| Tunca Sahin et al. 2018 | 2018 | Turkey | 1 | TS | Gly406Arg | Tunca Sahin et al. Ann Noninvasive Electrocardiol. 2018;23:e12522. |
| Walsh et al. 2018 | 2018 | UK | 5 | TS | Gly406Arg | Walsh et al. Europace. 2018;20:377-85. |
| Ye et al. 2018 | 2018 | USA | 1 | LQT8 | E1115K | Ye et al. Circulation. 2017;136:A15885 |
| Colson et al. 2019 | 2019 | France | 1 | TS | Glu407Ala | Colson et al. Eur J Med Genet. 2019;62:103648. |

Table 1: Main characteristics of the reports included in the study

| | *CACNA1C* exon 8/8A mutation carrier *n* = 52 | *CACNA1C* non-exon 8/8A mutation carrier *n* = 33 | *p* | TS *n* = 59 | COTS *n* = 6 | LQT8 *n* = 20 | *p* |
|---|---|---|---|---|---|---|---|
| ***Demographics*** | | | | | | | |
| Age at diagnosis, months [median (IQR)] | **2 (0–30)** | **144 (33–270)** | **<0.001** | **1 (0–30)** | **180 (30–534)** | **174 (144–318)** | **<0.001** |
| Diagnosis at birth, *n* (%) | **19/39 (48.7)** | **5/31 (16.1)** | **0.005** | **23/46 (50.0)** | **1/4 (25.0)** | **0/20 (0.0)** | **0.000** |
| Diagnosis in first year of life, *n* (%) | **27/39 (69.2)** | **6/31 (19.4)** | **<0.001** | **32/46 (69.6)** | **1/4 (25.0)** | **0/20 (0.0)** | **<0.001** |
| Sex (M/F) | 19/21 | 13/18 | 0.643 | 24/21 | 3/3 | 5/15 | 0.096 |
| ***Disease manifestation*** | | | | | | | |
| Extra cardiac manifestation, *n* (%) | **49/52 (94.2)** | **10/31 (32.3)** | **0.000** | | | | |
| Syndactyly, *n* (%) | **39/52 (75.0)** | **5/28 (17.9)** | **0.000** | | | | |
| Baldness, *n* (%) | **20/32 (62.5)** | **3/23 (13.0)** | **0.000** | | | | |
| Facial abnormality, *n* (%) | 15/31 (48.4) | 7/27 (25.9) | 0.106 | | | | |
| Seizures, *n* (%) | 5/14 (35.7) | 6/26 (23.1) | 0.469 | | | | |
| Neuro developmental delay, *n* (%) | **18/28 (64.3)** | **6/26 (23.1)** | **0.003** | | | | |
| Autism/ASD, *n* (%) | 0/5 (0.0) | 2/21 (9.5) | 1.000 | | | | |
| Recurrent infections, *n* (%) | **5/10 (50.0)** | **1/21 (4.8)** | **0.007** | | | | |
| Dental abnormalities, *n* (%) | **15/18 (83.3)** | **2/21 (9.5)** | **0.000** | | | | |
| Hypocalcemia, *n* (%) | 1/3 (33.3) | 1/20 (5.0) | 0.249 | | | | |
| Hypoglycemia, *n* (%) | **11/24 (45.8)** | **2/25 (8.0)** | **0.004** | | | | |
| Orthopedic disorder, *n* (%) | **4/6 (66.7)** | **4/23 (17.4)** | **0.033** | | | | |
| ***ECG and arrhythmia manifestations*** | | | | | | | |
| Max. QTc, ms [median (IQR)] | **606 (570–654)** | **498 (475–534)** | **<0.001** | **603 (566–655)** | **490 (480–500)** | **480 (451–498)** | **<0.001** |
| QTc > 500 ms, *n* (%) | **36/39 (92.3)** | **12/33 (36.4)** | **0.000** | **43/46 (93.5)** | **1/6 (16.7)** | **4/20 (20.0)** | **<0.001** |
| AVblock, *n* (%) | **38/51 (74.5)** | **6/18 (33.3)** | **0.002** | **43/56 (76.8)** | **1/2 (50.0)** | **0/11 (0.0)** | **<0.001** |
| Syncope, *n* (%) | 5/7 (71.4) | 10/13 (76.9) | 1.000 | 7/9 (77.8) | 0/2 (0.0) | 8/9 (88.9) | 0.101 |
| T wave alternans, *n* (%) | 20/31 (64.5) | 5/9 (55.6) | 0.705 | 22/33 (66.7) | 1/2 (50.0) | 2/5 (40.0) | 0.437 |
| Documented major arrhythmia NOT leading to ACA/SCD/ICDD, *n* (%) | 16/34 (47.1) | 6/23 (26.1) | 0.114 | **20/40 (50.0)** | **1/4 (25.0)** | **1/13 (7.7)** | **0.011** |
| ***Devices and interventions*** | | | | | | | |
| PM, *n* (%) | 15/24 (62.5) | 2/7 (28.6) | 0.198 | 16/28 (57.1) | 1/1 (100.0) | 0/2 (0.0) | 0.196 |
| ICD/AED, *n* (%) | 24/37 (64.9) | 12/15 (80.0) | 0.340 | 28/43 (65.1) | 2/3 (66.7) | 6/6 (100.0) | 0.214 |
| LCSD, *n* (%) | 4/37 (10.8) | 3/10 (30.0) | 0.155 | 5/42 (11.9) | 0/1 (0.0) | 2/4 (50.0) | 0.154 |
| ***Outcome*** | | | | | | | |
| Death, *n* (%) | **17/52 (32.7)** | **3/33 (9.1)** | **0.017** | **19/59 (32.2)** | **1/6 (16.7)** | **0/20 (0.0)** | **0.006** |
| Age at death, months [median (IQR)] | 2 (1.4–26) | 12 | 0.926 | 3 (1.1–29) | 1.5 (1.5–1.5) | | 0.560 |
| Major adverse cardiac event (death/ACA/SCD/ICDD), *n* (%) | **37/52 (71.2)** | **11/32 (34.4)** | **0.001** | **40/58 (70.7)** | **2/6 (33.3)** | **6/20 (30.0)** | **0.004** |
| Age at MACE, months [median (IQR)] | 24 (2–53) | 60 (20–147) | 0.156 | 27 (2–54) | 385 (2–768) | 138 (120–156) | 0.111 |

Table 2: Comparison of clinical characteristics and outcome data in the index patients in *CACNA1C* gene associated disease forms.

## 3. Identification of known and novel mutations of the *KCNJ2* gene in Hungarian patients with Andersen Tawil syndrome.

3.1. Electropherograms of some of the identified *KCNJ2* gene mutations in the study

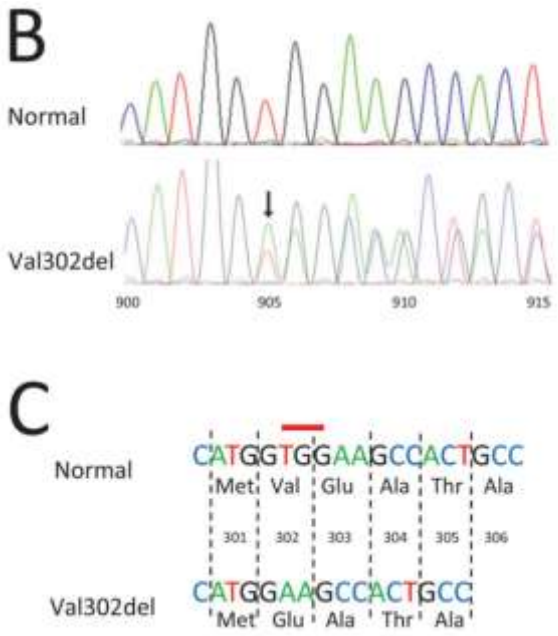



Figure 2: Sequence chromatogram of the relevant region of the *KCNJ2* gene in a normal control and the proband (Panel B). Sequencing revealed a heterozygous deletion of the nucleotides TGG between positions 905 and 907 of the *KCNJ2* coding region in the proband leading to a one amino acid deletion (p.Glu302del, Panel C).

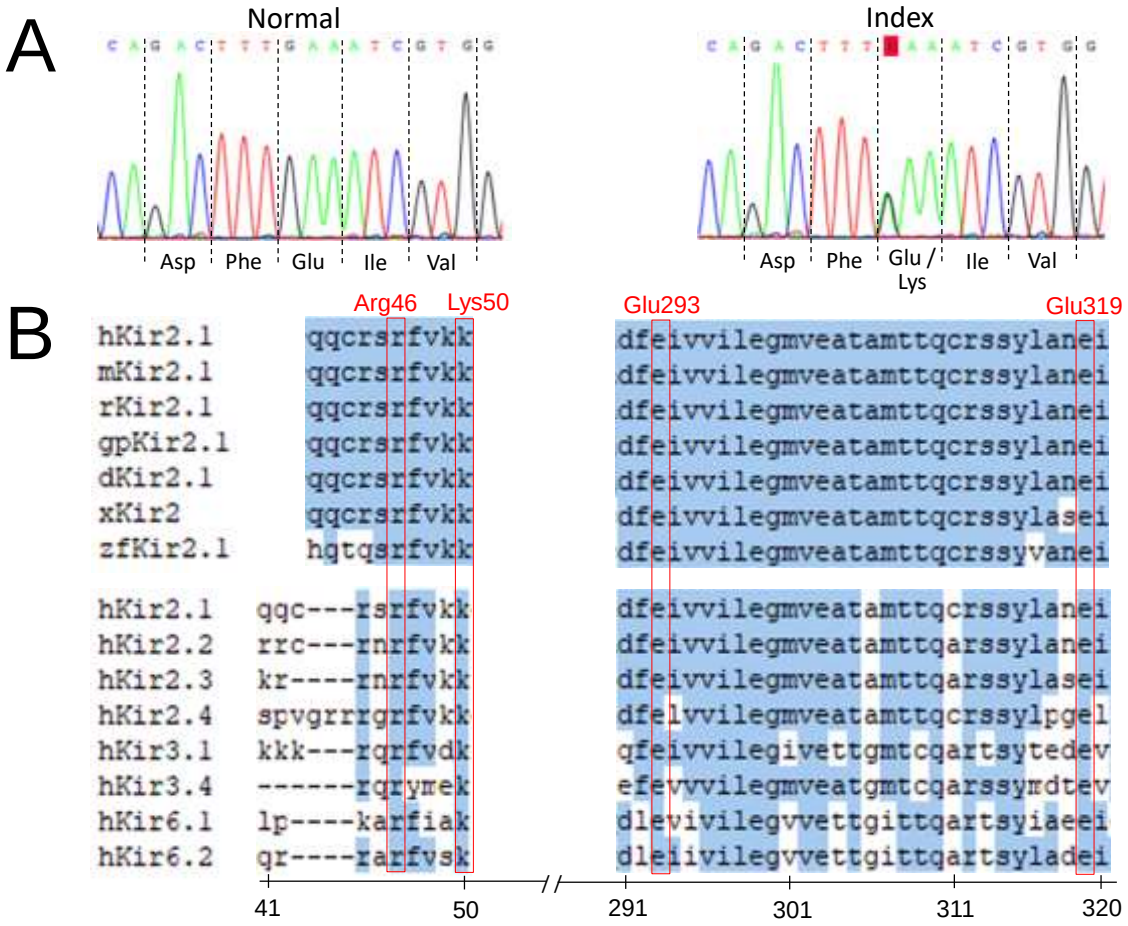
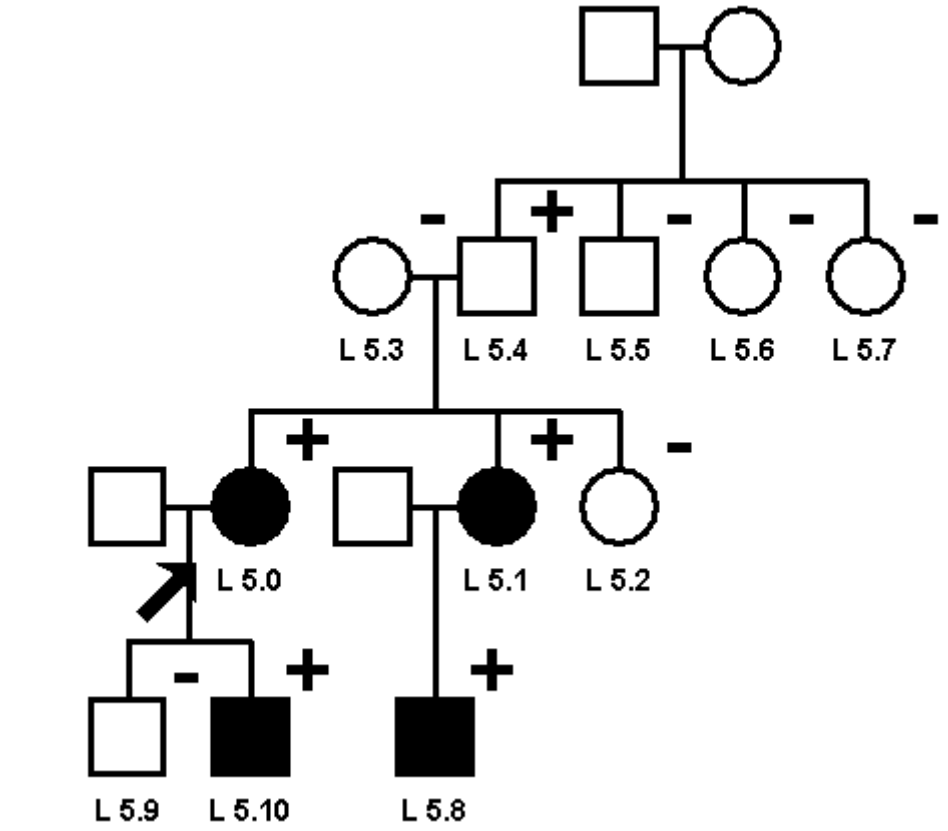
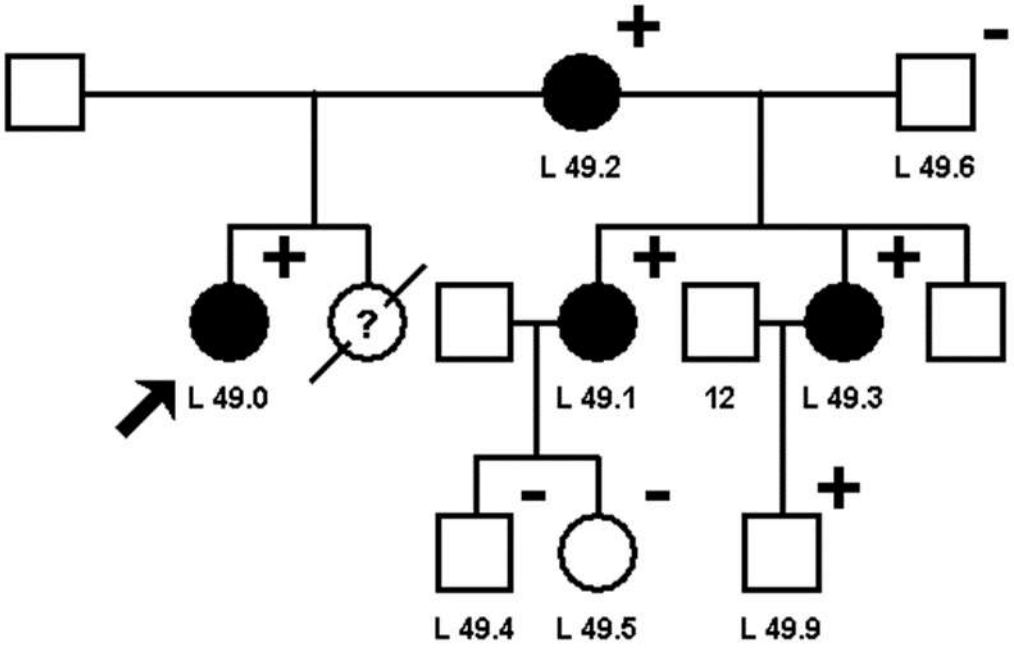
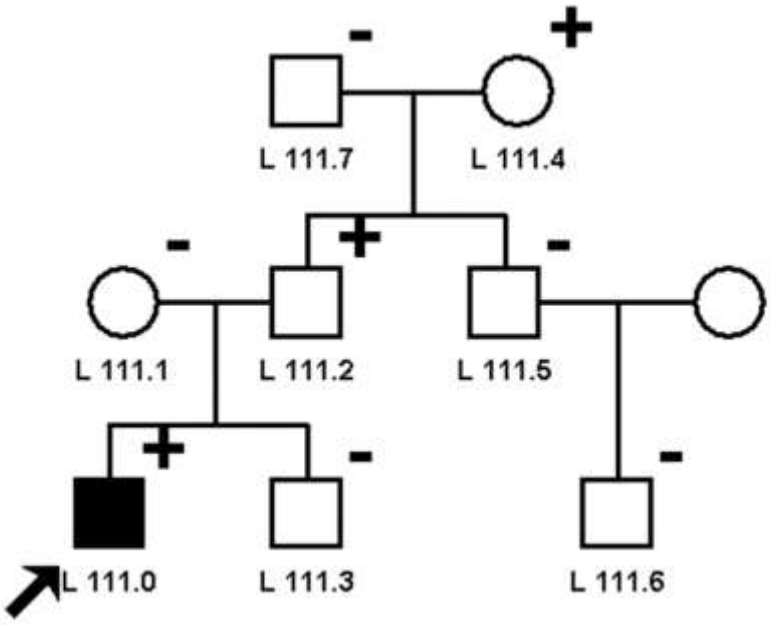



Figure 3. Panel A: Sequencing chromatograms from a normal genotype (left) and the index patient, showing a G to A transition in codon 293 of the *KCNJ2* gene (right). Panel B: Alignment of amino acid sequences of human (h), mouse (m), rat (r), guinea pig (gp), dog (d), Xenopus tropicalis (x) and zebra fish (zf) Kir2.1. Lower panel: alignment of amino acid sequences of human inward rectifier potassium channel subunits. Positions indicated are relative to the amino acid sequence of human Kir2.1, conserved amino acids are shown in blue shading. The highly conserved Arg46, Lys50, Glu219 and Glu319 amino acids, participating in an intersubunit network of salt bridges, are highlighted in red. Glu293 is highly conserved in Kir2.1 from vertabrate species and in the human inward rectifier potassium channel subunit proteins.

Family trees of the families with the identified *KCNJ2* gene mutations in the study

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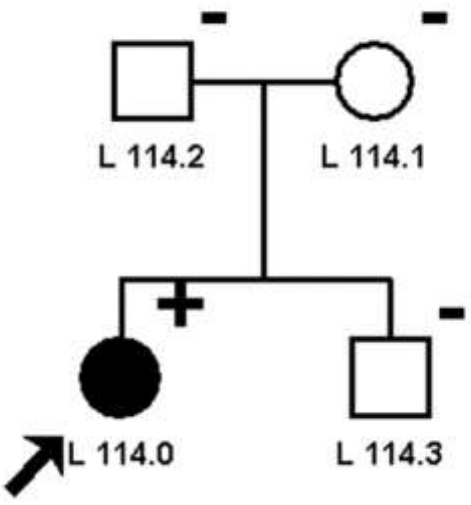
-
-
L 114.2
L 114.1
+
-
L 114.0
L 114.3

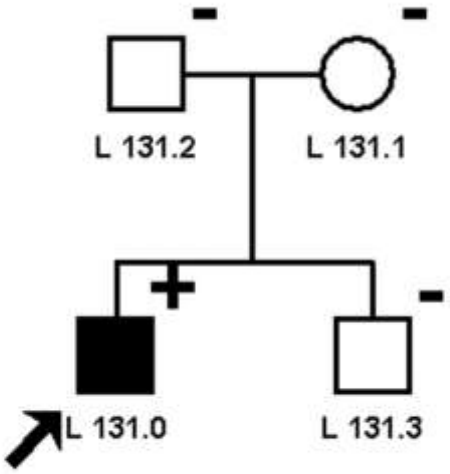


Figure 4. Pedigrees of families L5, L49, L111, L114, and L131 with Andersen-Tawil syndrome with different *KCNJ2* gene mutations identified in the study. Squares and circles denote males and females, respectively; filled symbols indicate clinically affected family members. Arrow point to the index patient. Deceased individuals are slashed. Carriers of the different *KCNJ2* mutations are labelled with a plus (+) sign, and non-carriers with a minus (–) sign. The clinical characteristics of the mutation carrier family members are given in Table. Probands in family L84 and L154 were adopted, and therefore family trees were non-contributory.

# Declaration of co-authorship

As the principal investigator, I declare that dr. János Borbás and Szilvia Ladányi Lászlóné Déri PhD candidates contributed equally and substantially as shared first authors to our below-cited research study entitled ‘Impaired cytoplasmic domain interactions cause co-assembly defect and loss of function in the p.Glu293Lys KNCJ2 variant isolated from an Andersen-Tawil Syndrome patient’ as follows:

**János Borbás** designed and carried out genetic and bioinformatic analysis, contributed to clinical diagnosis and constructed case history.

**Szilvia Déri** designed and carried out molecular cloning, heterologous expression, immunostaining, patch-clamp and protein:protein interaction experiments.

Balázs Ördög
principal investigator

Deri S, Borbas J, Hartai T, Hategan L, Csanyi B, Visnyovszki A, Madacsy T, Maleth J, Hegedus Z, Nagy I, Arora R, Labro AJ, Kornyei L, Varro A, Sepp R & Ordog B. (2021). Impaired cytoplasmic domain interactions cause coassembly defect and loss of function in the p.Glu293Lys KNCJ2 variant isolated from an Andersen-Tawil syndrome patient. Cardiovasc Res 117, 1923-1934.

# Co-author certification

I, myself as a corresponding author of the following publication(s) declare that the authors have no conflict of interest, and János Borbás M.D. Ph.D. candidate had significant contribution to the jointly published research(es).

The results discussed in his thesis were not used and not intended to be used in any other qualification process for obtaining a PhD degree.

Szeged, 2024.07.01.

.....

author

The publication(s) relevant to the applicant's thesis:

Sepp R, Hategan L, Bácsi A, Cseklye J, Környei L, **Borbás J**, Széll M, Forster T, Nagy I, Hegedűs Z. Timothy syndrome 1 genotype without syndactyly and major extracardiac manifestations. *Am J Med Genet A* 2017; 173(3):784-789. doi: 10.1002/ajmg.a.38084. (Q2, IF: 2.264)