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CLINICAL AND GENETIC ANALYSIS OF RARE ION CHANNEL DISEASES

PhD Thesis

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Szeged
2024

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.

4. **Borbás J**, Takács H, Környei L, Katona M, Ördög B. Andersen–Tawil-szindrómás betegek EKG-jellegzetességeinek kvalitatív és kvantitatív elemzése. *Cardiologia Hungarica* 2019; 49: 344–348. doi: 10.26430/CHUNGARICA.2019.49.5.344

5. **Borbás J**, Erdős B, Katona M, Környei L, Ördög B. Congenitalis dysmorphiával, kamrai aritmiával és periodikus paralízissel járó ioncsatorna-betegség: Andersen-Tawil-szindróma. *Cardiologia Hungarica* 2019; 49: 358–364. doi: 10.26430/CHUNGARICA.2019.49.5.358

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.

Cardiac myocytes are specialized excitable cells capable of generating and propagating an action potential (AP), an electrical signal that culminates in myocardial contraction. Cardiac channelopathies arise from mutations in genes (either loss-of-function or gain-of-function) related to various cardiac membrane channels.

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.

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).

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.

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.

Mutations affecting the *CACNA1C* gene may exhibit a variety of clinical manifestations. These manifestations include the typical Timothy (Type 1) syndrome, 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. Atypical Timothy syndrome (Type 2) patients, who have no syndactyly but carry many of the other multisystemic manifestations of the disease. Further clinical manifestation of *CACNA1C* gene mutations include “cardiac only” Timothy syndrome (COTS), 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. 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.

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. 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.

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. Family history was negative for premature sudden cardiac death, arrhythmic disorders, syndactyly, facial dysmorphism, or autism. The pregnancy was uncomplicated. At 37 weeks gestation, intermittent fetal bradycardia (72 bpm) was noted, but the baby was born at 38 weeks via normal delivery with Apgar

scores of 9 and 10. Post-birth, the baby had 2:1 atrioventricular (AV) conduction and a prolonged QTc interval, which was managed with propranolol and mexiletine, stabilizing the QTc interval and resolving the AV conduction issue. Early echocardiography showed a patent foramen ovale and mildly dilated right ventricle, with no other significant heart defects. The baby developed normally, meeting milestones like sitting unassisted at 7.5 months and walking independently at 12 months, with no signs of autism or frequent infections. Despite a hypoglycemia episode at age 2 that required hospitalization, the child is now 3 years old, healthy, developing normally, and attending kindergarten without assistance.

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, a total of 134 patients were identified. 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.

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.

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. Seven patients met the pre-specified criteria (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. Patient L84.0 with the *KCNJ2* p.Val302del mutation was a novel variant. Patient L114.0 with the *KCNJ2* p.Glu293Lys mutation, cellular

electrophysiological analysis of the mutation provided a novel pathophysiological mechanism.

3.2 Methods

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

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.

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.

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. 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.

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. Studies eligible for inclusion were identified by using the following search query as full text search: "Timothy syndrome" OR ("LQT8 OR *CACNA1C*").

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.

Excluding reports on mosaic patients, a total of 34 publications comprising data of 134 patients were identified.

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. Among the qualitative abnormalities, the presence of ventricular extrasystoles (VES)/bigeminy, as well as couplets and NSVT (non-sustained ventricular tachycardia), were assessed. 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. 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

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; 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. 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.

4.2. 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

Altogether, 33 *CACNA1C* mutations were extracted from the reports. 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.

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)

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)

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.

4.2.3. 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)

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)

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; $p=0.004$).

4.3. 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.

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). Two mutations affected the N-terminal and 5 mutations affected the C-terminal part of the protein. Three of the mutations (p.Val302del, p.Glu293Lys, p.Cys54Tyr) were novel, while the others were previously reported mutations. All the variants were classified as pathogenic/likely pathogenic by Franklin.

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.

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

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.

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. 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%. 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.

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. 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. 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

Our systematic review examined *CACNA1C* gene mutation-associated Timothy syndrome (TS), "cardiac-only" Timothy syndrome (COTS), and isolated long QT syndrome 8 (LQT8), highlighting significant clinical differences among them. These differences can be defined based on either genotype or phenotype. The literature reveals controversy regarding the classification of Timothy syndrome, with several proposals since the original reports of TS1 and TS2. One proposal suggested classifying all TS phenotypes resulting from *CACNA1C* mutations as TS1 until another TS disease gene is discovered. Another proposal recommended that TS1 and TS2 should include only patients with the p.Gly406Arg mutation in exon 8A (TS1) or in exon 8 (TS2), while remaining alleles be called atypical TS. We found that aside from syndactyly or baldness, there are no major differences in clinical manifestations or outcomes between TS1 and TS2. Both subtypes show extreme QTc prolongation (median ≥600 ms) and high MACE rates, making the distinction between TS1 and TS2 potentially obsolete. Instead, using "classical TS" (with syndactyly) and "non-classical TS" (without syndactyly) may be more appropriate.

Timothy syndrome differs significantly from COTS and isolated LQT8, with earlier disease onset, more pronounced QTc prolongation, and higher mortality. Phenotypic differences form the basis for categorizing these disease forms, and our review demonstrates differences in ECG parameters and clinical outcomes for the first time. QTc prolongation in TS often exceeds 600 ms, while in COTS or LQT8, it is usually

less than 500 ms, explaining the higher rate of clinical complications in TS. These findings support the established categorization of TS, COTS, and isolated LQT8 based on clinical outcomes. Differences also emerge when comparing carriers of exon 8/8A mutations to non-exon 8/8A mutations. Exon 8/8A mutations account for 83% of TS cases but only 17% of COTS and 10% of isolated LQT8 cases, leading to younger disease onset, more prevalent TS phenotypes, pronounced QTc prolongation, and more severe clinical outcomes in exon 8/8A mutation carriers.

TS is genetically homogenous, with the p.Gly406Arg mutation in exon 8 or exon 8A responsible for 70% of cases, and mutations affecting codons 402-407 responsible for 85%. The strongest relationship is between the p.Gly406Arg mutation in exon 8A and "classical" TS, present in 93.5% of cases. COTS and isolated LQT8 are more genetically diverse, with causative mutations scattered throughout the gene. The cellular electrophysiological alterations caused by *CACNA1C* mutations alone do not explain the phenotypic differences between TS, COTS, and LQT8. While *CACNA1C* mutations lead to gain-of-function alleles prolonging the cardiac action potential and QT interval, the specific mechanisms of channel dysfunction vary among mutations.

The wide variations in phenotypic expression may be influenced by factors such as parental or individual mosaicism, where a "de novo" mutation arises during gametogenesis or embryogenesis. Additionally, the *CACNA1C* gene's complex transcript profile, with numerous novel exons and transcripts, suggests that different isoforms may impact phenotypic expression. Studies indicate that cells can tolerate a certain proportion of mutant *CACNA1C* channels, but beyond a threshold, action potentials become unstable and arrhythmogenic. The concept of "repolarization reserve," where other repolarizing currents compensate for impaired channels, may also play a role. These findings underscore the complexity of TS, COTS, and isolated LQT8 phenotypic expressions and the need for further research to fully understand their mechanisms.

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.

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

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.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. 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: (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 and catecholaminergic polymorphic ventricular tachycardia (CPVT), as the prognosis and treatment of ATS and CPVT differ. 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.

7. ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my supervisor Prof. Dr. Robert Sepp for the continuous support of my PhD study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

My sincere thanks also go to Professor András Varró, Professor Tamás Forster, Prof. Dr. Szili-Török Tamás who provided me an opportunity to join their institute as a PhD student, and who gave access to the laboratory and research facilities. Without their precious support it would not be possible to conduct this research. With gratitude and admiration, I pay homage to the life paths of Prof. Dr. Vince Varró and Prof. Dr. Miklós Csanády, without whose exemplary guidance I would not be here

I thank my fellow labmates (Dr. Beata Csanyi, Dr. Lidia Hategan) for their kind help during my PhD. I am especially grateful to Molnár Jánosné Klári, without her I would have been lost forever in the maze of lab work.

Last but not the least, I would like to thank my family, my parents, my wife and my friends for supporting me in every way possible throughout writing this thesis and my life in general.