AWARD NUMBER: W81XWH-19-1-0473

TITLE: Gene Therapy for Catecholaminergic Polymorphic Ventricular Tachycardia

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CONTRACTING ORGANIZATION: Children's Hospital Corporation (DBA Boston Children's Hospital)

REPORT DATE: OCTOBER 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2022	Annual progress report	1SEPT2021 - 31AUG2022

4. TITLE AND SUBTITLE

Gene Therapy for Catecholaminergic Polymorphic Ventricular Tachycardia

5a. CONTRACT NUMBER
W81XWH-19-1-0473

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a inherited arrhythmia syndrome characterized by life-threatening arrhythmias during times of stress or exercise. Dominant mutations in the intracellular calcium (Ca^{2+}) release channel RYR2 are responsible for the majority of clinical cases. Despite maximal medical therapy, patients continue to have breakthrough events or therapy related complications. To response to this unmet clinical need, we have developed a targeted gene therapy to suppress arrhythmias by inhibiting the Ca^{2+} regulated kinase CaMKII. Using adeno-associated virus (AAV) vectors we demonstrated efficacy in cellular and animal models of CPVT by targeted expression of CaMKII peptide inhibitors. This grant proposal is focused on the further refinement and testing of a clinical CaMKII peptide inhibitory vector in preparation for a human clinical trial. During this granting period we have determined the cardiac-specific promoter for optimal transgene expression and laid the foundation for refinement of the peptide inhibitor. We have also expanded our clinical network of CPVT patients and performed analysis of current healthcare costs for CPVT management.

15. SUBJECT TERMS NONE LISTED					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified	Unclassified	OF PAGES 20	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

	Page
1. Introduction	5
2. Keywords	5
3. Accomplishments	5-12
4. Impact	12-13
5. Changes/Problems	13-14
6. Products	15-16
7. Participants & Other Collaborating Organizations	17-21
8. Special Reporting Requirements	21
9. Appendices	

1. INTRODUCTION:

The purpose of this grant is to refine and optimize a novel cardiac gene therapy for the inherited arrhythmia disorder catecholaminergic polymorphic ventricular tachycardia (CPVT) in preparation for a first in human clinical trial. Mutations in the intracellular calcium (Ca^{2+}) release channel RYR2 are commonly associated with CPVT and lead to life-threatening ventricular arrhythmias during stress or exercise. Without any abnormality at baseline, activation of the Ca^{2+}/calmodulin dependent kinase II (CaMKII) in response to adrenergic stimulation is necessary to unmask the arrhythmogenic potential of RYR2 mutations. We have developed a gene therapy strategy to treat CPVT by targeted cardiac expression of CaMKII peptide-inhibitors using adeno-associated virus (AAV) delivery. Building on previous proof-of-concept research, this project encompasses the necessary steps to construct the optimal clinical vector, define the therapeutic dose, evaluate for possible toxicities, and identify eligible patients for potential treatment. In addition to being the first gene-specific treatment for an inherited cardiovascular syndrome, targeted inhibition of CaMKII may be more universally applicable because CaMKII dysregulation is implicated in several cardiac disorders.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Cardiac gene therapy
Catecholaminergic polymorphic ventricular tachycardia (CPVT)
Arrhythmia
Sudden cardiac death
Ventricular arrhythmias
Adeno-associated virus (AAV)

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1 – To optimize the final product design.	**Timeline**	**Percent Completed**
Major Task 1	Months	
Subtask 1: Submit documents for ACURO approval (mouse studies)	1-4	100%
Milestone # 1 ACURO approval obtained	4	100%

Subtask 2: Optimize inhibitory peptide	1-8	100%
Subtask 3: Optimize promoter	1-8	100%
Subtask 4: Optimize capsid	1-8	100%
Subtask 5: Compare self-complementary AAV to standard AAV	4-8	100%
Subtask 6: Develop an assay to detect transduced cells	4-8	100%
Milestone # 2 Finalized design of the clinical candidate	8	100%
Specific Aim 2 – To evaluate efficacy, dose-response, biodistribution, and safety of the clinical candidate in a murine CPVT model.		
Major Task 2.		
Subtask 1: Research grade vector production (AAV-nGFP then clinical candidate)	6-11	10%
Milestone #3a Delivery of research grade AAV-nGFP	9	10%
Milestone #3b Delivery of research grade clinical candidate	11	10%
Subtask 2: Dose-finding and biodistribution of AAV-nGFP	9-12	Pending
Milestone #4 Define the % cardiomyocyte transduction for a range of viral doses and biodistribution of the AAV-nGFP test vector	12	Pending
Subtask 3: Efficacy and dose-response study	13-18	Pending

Milestone #5 Define the effective dose, efficacy, safety, and biodistribution of the clinical candidate.	18	Pending
Specific Aim 3 – To develop safety data on the final therapy vector in a large animal model		
Major Task 3		
Subtask 1: Submit documents for ACURO approval (swine dose-finding studies at BCH)	6-18	100%
Milestone #6 ACURO approval obtained for swine	18	100%
Subtask 1: Pre-IND meeting	19-21	Pending
Milestone #7 Finalize design of the large animal safety trial	21	100%
Subtask 2: Production of clinical grade vector (AAV-GFP-P2A-IP1 then AAV-IP1)	13-24	10%
Milestone #8a Delivery of Hyperstack scale AAV-GFP-P2A-IP1	18	Pending
Milestone #8b Delivery of Hyperstack scale clinical candidate (no GFP-P2A)	24	Pending
Subtask 3: Large animal dose-finding and biodistribution using GFP-containing vector and clinically applicable delivery route and equipment. [2 pigs x 5 groups = 10 pigs]	19-24	Pending
Milestone #9 Define target vector dose to match efficacious dose found in mouse model	24	Pending

Subtask 4: Large animal safety and biodistribution study [12 pigs x 2 doses = 24 pigs]	25-34	Pending
Milestone #10 Completion of large animal safety and biodistribution study	34	Pending
Specific Aim 4 – To lay the groundwork for a First-in-Human clinical trial		
Major Task 4		
Subtask 1: Establish a CPVT Network	1-12	100%
Milestone #11 Hold a meeting of CPVT Network participating members	12	100%
Subtask 2: A retrospective chart review	8-24	100%
Milestone #12 Establish natural history and resource utilization of CPVT under the current standard of care.	24	75%
Subtask 3: Collect and test blood from patients with inherited arrhythmia for neutralizing antibodies	1-30	100%
Milestone #13 Determine the frequency of neutralizing antibodies amongst the target population	30	100%
Subtask 4. Finalize Phase I clinical protocol	25-32	Pending
Subtask 5. Prepare IND application	32-36	Pending
Milestone #14 Submission of FDA IND filing for a First-in-Human Phase I study	36	Pending

What was accomplished under these goals?

3.1 Major Activities

During this granting period, we have finalized the design of the clinical and test vectors and initiated their production. We have performed studies determining the presence of pre-existing antibodies in a cohort of CPVT patients. We have our large animal study approved and underway. We have uncovered a possibly novel mechanism of arrhythmia in CPVT based on differential CaMKII peptide inhibitor effectiveness.

3.2 Specific Objectives

Major Task 1

Selection of the optimal Capsid

In our original proof-of-concept studies, we used AAV9 as the delivery vector for transduction of the heart for our CaMKII inhibitory gene therapy. While AAV9 has a tropism for the cardiac tissue it also has significant transduction capacity of non-cardiac organs including the brain and liver. Inclusion of a cardiac-specific promoter in our therapeutic construct will aim to restrict expression to the heart, but additional safe-guards for extra-cardiac expression of our CaMKII inhibitory peptides would increase the safety and efficacy of our proposed therapy. Furthermore, any improvement in transduction efficiency will lower the effective dose and reduce both therapeutic cost and toxicity. Continued advances in AAV capsid engineering seek to produce novel recombinant capsids with increased transduction efficiency in target organs. Along with previously developed AAV capsids, we evaluated one such novel capsid, MyoAAV (PMID: 34506722) to determine the optimal viral delivery system for our CaMKII-inhibitory peptide. We packaged the fluorescent protein HaloTag driven by the cardiac troponin-T (cTnT) promoter with specific barcodes (BCs) within the 3' UTR, into selected AAVs including: AAV-9, MyoAAV, AAV-2i8, and AAV-rh10 (Fig. 1A). These viruses were then subcutaneously injected into P3 animals, each at a dose of $2x10^{10}$ viral genomes per gram (vg/g). After 4 weeks of expression, we isolated genomic DNA and performed amplicon-sequencing of the capsid-specific barcodes. Consistent with a previous report, MyoAAV demonstrated significantly improved cardiac transduction over other AAVs (Fig. 1B). To confirm the specificity of our optimized promoter, we isolated RNA and genomic DNA from multiple organs after transduction with our selected AAV capsid and performed amplicon-sequencing of the 3' UTR barcodes. The quantification of the RNA to DNA ratio revealed significant cardiac specificity, with minimal expression in non-cardiac organs (Fig. 1C). These data demonstrate that our selection of both an optimized promoter and capsid will increase on-target expression in the heart while minimizing non-cardiac transduction and expression.

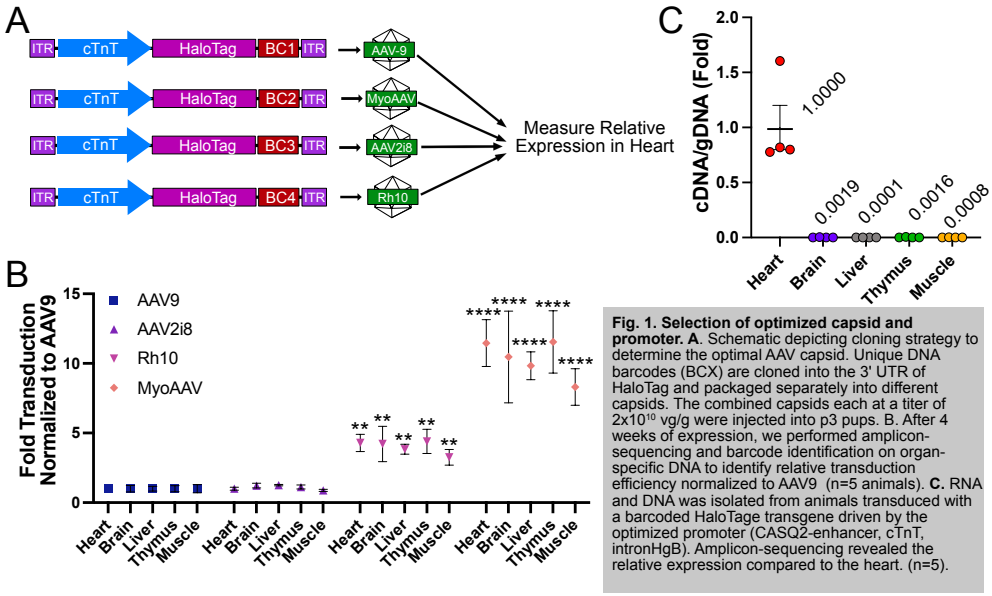

Fig. 1. Selection of optimized capsid and promoter. A. Schematic depicting cloning strategy to determine the optimal AAV capsid. Unique DNA barcodes (BCX) are cloned into the 3' UTR of HaloTag and packaged separately into different capsids. The combined capsids each at a titer of $2x10^{10}$ vg/g were injected into p3 pups. B. After 4 weeks of expression, we performed amplicon-sequencing and barcode identification on organ-specific DNA to identify relative transduction efficiency normalized to AAV9 (n=5 animals). **C.** RNA and DNA was isolated from animals transduced with a barcoded HaloTage transgene driven by the optimized promoter (CASQ2-enhancer, cTnT, intronHgB). Amplicon-sequencing revealed the relative expression compared to the heart. (n=5).

Major Task 2

Research grade vector production (AAV-nGFP then clinical candidate)

To perform both dose-finding efficacy testing in mice and transduction testing in swine, we plan to produce large quantities of AAV-AIPx5-P2A-mScarlet-nls and AAV-mScarlet-nls (Fig. 2A). Given an insert size of ~2.4kbp we will use a self-complementary approach to facilitate viral production (Fig. 2A). For the final clinical vector design, we will replace the chicken troponin-T promoter with the human sequence (hTnT) and include multiple AIPx5 inserts (Fig. 2B). To comply with current FDA guidelines the ampicillin resistance gene in the vector backbone will be replaced for kanamycin and packaged into a MyoAAV sub-type. We have already identified and partnered with a CRO to produce the AAVs in a GLP environment. We are currently in the process of producing the final plasmid clone for AAV production.

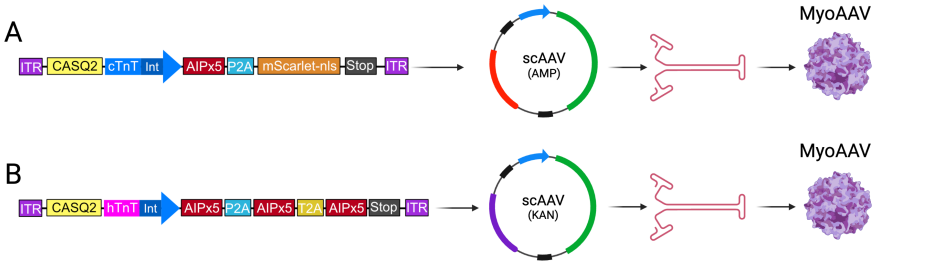

Fig. 2. Final vector design. A. AIPx5 is cloned with a nuclear-localized mScarlet as an expression marker for murine dose-finding studies and packaged into a self-complementary vector. **B.** For the final clinical vector, mScarlet will be removed and replaced with multiple AIPx5 inserts. To reduce potential immunogenicity, the chicken troponin-T (cTnT) promoter will be replaced with the human TnT promoter (hTnT), in a kanamycin-resistance backbone.

Major Task 3

Submit documents for ACURO approval

To ascertain the probable dose for a clinical trial we have proposed a small pilot study in a large animal model (swine) using our clinical vector. Given the lack of a large animal model for CPVT we will plan to perform a dose finding experiment in pigs to confirm restricted cardiac expression, and a target dose with at least 40% of transduced cardiomyocytes. We have obtained final ACURO approval and are preparing for the initiation of the swine studies.

Major Task 4

Establish a CPVT Network

To facilitate the identification of additional CPVT patients eligible for a potential clinical trial thereby increasing the clinical and molecular diversity of patients and better define the natural history of CPVT, we partnered with several institutions as part of the International CPVT network. We have been directly sharing samples and clinical data with British Columbia Children's Hospital, Simon Fraser University, and Children's Hospital of Atlanta. In addition, we are in the final steps of joining the CPVT registry run by Arthur Wilde from the Netherlands. This international registry has over 1000 patients and will enable increased access to clinical data and possible serum samples for neutralizing antibody testing.

As part of Milestone #11, we hosted a third CPVT consortium meeting on January 13, 2022 which focused on possible clinical trial design and establishment of a pipeline for obtaining patient sera for pre-existing immunity testing (see below). This network has been critical in the fostering of partnerships necessary for a future multi-center clinical trial

Collect and test blood from patients with inherited arrhythmia for neutralizing antibodies

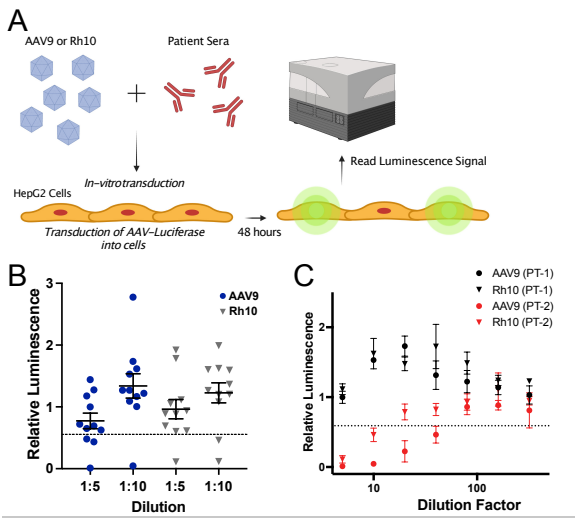

Fig. 2. Detection of neutralizing antibodies in CPVT patients A. The luciferase gene is packaged into either AAV9 or AAV-Rh10 (Rh10) capsids and mixed with diluted patient sera. After incubation, heptocellular cells (HepG2) are transduced and cultured for 48 hours prior to luminescence detection. **B.** Sera from 12 patients with clinical CPVT were diluted at 1:5 and 1:10 and subjected to analysis. A cutoff of 0.5 correlates with reduction transduction efficiency. **C.** One patient's sample was noted to have a substantial reduction in transduction efficiency and therefore additional dilutions were performed.

The presence of pre-exiting neutralizing antibodies (nAbs) for specific AAV capsids can limit the effective transduction efficiency and can completely prevent transgene delivery. After obtaining approval for our human studies, we isolated sera from CPVT patients. We combined diluted patient sera with either AAV9 or AAV-Rh10 (Rh10) capsids containing the luciferase gene and transduced HepG2 cells (Fig. 3A). After 48 hours, we measured the resulting luminescence as a measure of transduction efficiency. A reduction of luminescence corresponds to the presence of neutralizing antibodies (Fig. 3B). While most patient samples did not demonstrate significant inhibition, sera from one patient (PT-2) substantially inhibited transduction, AAV9 > Rh10 (Fig. 3C). These data support the hypothesis that the presence of nAbs is uncommon in our population, but we will collect additional samples to confirm this finding. We will also determine if the MyoAAV subtypes have lower immunogenicity than existing AAV capsids.

What opportunities for training and professional development has the project provided?

Dr. de la Serna Buzon continues with the T32 training fellowship grant and presented her work at the annual American Society of Gene and Cell Therapy (ASGCT) meeting in May 2022.

How were the results disseminated to communities of interest?

We recently presented our work at the recent ASGCT meeting and are preparing a manuscript for publication.

What do you plan to do during the next reporting period to accomplish the goals?

During the next granting period, we will produce the final therapeutic vector for testing in both small and large animal models. We will use our dose-finding experiments in mice to inform the dosing of swine to achieve at least 40% cardiomyocyte transduction. Evaluation of potential toxicity will be performed at a CRO with some initial testing performed at Boston Children's Hospital. We will complete the studies of pre-existing immunity in our patient population along with testing of the novel MyoAAV capsids.

To complete the swine studies, we have recently recruited a clinical fellow in electrophysiology, Dr. Robert Przbylski. He will lead the testing of our AAV vectors in our swine model to determine effective dose and possible electrophysiologic toxicity.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The discovery that multimerization of CaMKII inhibitory peptides and in particular AIP, enhance their effectiveness at suppressing arrhythmia in a mouse of CPVT, raises intriguing questions about the relationship of CaMKII inhibition and the pathophysiology of CPVT. While the pentameric form of AIP (AIPx5) was found to be most effective, AIP itself was superior to CN19o in suppressing arrhythmia despite the increased potency of CN19o by *in-vitro* kinase assays. To determine the effects of multimerization *in-vivo*, we transduced animals with our CaMKII peptide inhibitors and isolated whole heart lysates after adrenergic stimulation. Phosphorylation of phospholamban (PLB) by CaMKII was significantly increased with administration of isoproterenol and which was conversely inhibited by all the peptide inhibitors expect for AIP-FKBP12.6 (Fig. 4A). To directly determine the relative potency effect of multimerization, we performed in-vitro kinase assays with synthesized CaMKII peptide inhibitors. While there was a significant increase in potency with multimers of AIP, CN19o multimerization had a minimal effect (Fig. 4B). These data suggest that the mechanism of AIPx5's improved suppression of arrhythmia over derivatives of CN19o, is not explained entirely by CaMKII inhibition. We are currently exploring additional mechanisms which may explain this novel and unexpected finding.

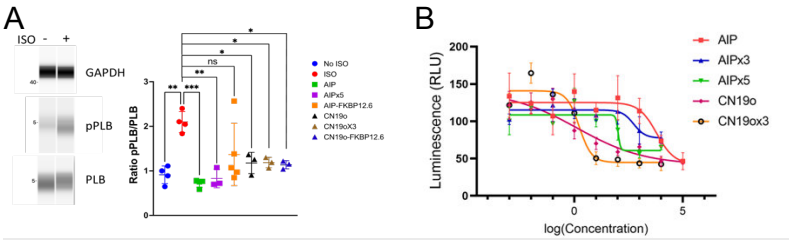

Fig. 4. Effects of multimerization on CaMKII inhibitory peptides. A. Whole heart lysates were isolated from animals injected with AAV capsids containing CaMKII peptide inhibitors as monomers, multimers or fused to the RyR2-binding protein FKBP12.6. Lysates were separated by capillary Western and stained for antibodies for phospholamban (PLB) and phospho-phospholamban (pPLB). Statistics by Kruskal-Wallis test, *P<0.05, **P<0.01, ***P<0.001. **B.** To determine the direction potency of the different CaMKII peptide inhibitors, recombinant CaMKIId was incubated with synthesized peptide inhibitors. Kinase activity was determined by the Promega ADP-Glo assay. One-way ANOVA EC-50: F (4,137) 23.89, p<0.0001, N = 5 per sample.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

We have an on-going partnership with a gene therapy company based on our proof-of-concept data for CaMKII inhibition to treat CPVT. We have filed for another patent based on the multimerization of AIP.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

5.1 Changes in approach

Our original proposal was to include nuclear-localized GFP (nGFP) as a marker for transduction in our final test vector and to remove it for our final clinical vector. We subsequently trialed HaloTag as an alternative fluorescent marker to GFP but discovered poor fluorescence in fixed cardiac sections. Attempts to design a high sensitivity RNA-based probe for AIPx5 are challenging because of the redundancy and short length of the transcript. Therefore, we will use nuclear-localized mScarlet along with a self-cleavage peptide (P2A), for the assessment of transduction efficiency in mouse and swine.

To minimize possible immunogenicity and optimize viral production, we will include additional AIPX5 inserts separated by either P2A or T2A (Fig. 2B). This will then be cloned along with the human TnT promoter into a self-complementary vector.

Actual or anticipated problems or delays and actions or plans to resolve them

5.2 Actual or anticipated problems or delays

The ongoing coronavirus pandemic continues to impact our productivity with delayed deliveries, back-orders of critical reagents, and work loss due to illness. However, we have continued to make significant progress by finding alternatives to back-ordered items whenever possible and hiring of a dedicated research assistant for the project. We have requested and received approval of a one year no-cost extension for which we expect to enable project completion.

Changes that had a significant impact on expenditures

The COVID pandemic and lab shutdown/slowdown delayed development work on the therapeutic candidate. We have finalized the design of our clinical vector, but the prolongation of the optimization has delayed initiation of production and final testing.

Significant changes in use or care of human subjects

There are no significant changes in the care or use of vertebrate animals, biohazards and/or select agents. There have also not been any changes in our approved IRB or the obtaining of patient samples for immunity testing.

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

We presented our work at the ASGCT meeting in Washington D.C. on May 18th 2022.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

We submitted a provisional US patent application on 7/6/2017 for our proposed gene therapy. A formal application was submitted on 1/2/2020 and currently is pending under number 16/628,162. We have partnered with a gene therapy company as part of a sponsored research agreement with an option for license. A second provisional patent was submitted for the multimerization of AIP and potential treatment for atrial fibrillation.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	William Pu, MD
Project Role:	PI
Researcher Identifier:	0000-0002-4551-8079
Nearest person month worked:	1
Contribution to Project:	Overall co-direction of the project along with Dr. Bezzerides
Funding Support:	Committed effort fully supported by this award

Name:	Vassilios Bezzerides MD, PhD
Project Role:	Co-investigator
Researcher Identifier:	0000-0003-0825-6580
Nearest person month worked:	1
Contribution to Project:	Overall co-direction of the project along with Dr. Pu
Funding Support:	Committed effort fully supported by this award

Name:	Dominic Abrams MBBS
Project Role:	Institutional Collaborator
Researcher Identifier:	0000-0003-0825-6580
Nearest person month worked:	1
Contribution to Project:	Assistance with establishment of CPVT network
Funding Support:	Departmental

Name:	Sofi de la Serna Buzon
Project Role:	Postdoctoral Fellow
Researcher Identifier:	NA
Nearest person month worked:	4
Contribution to Project:	Dr. de la Serna Buzon completed the promoter optimization and is performing the experiments to select the CaMKII inhibitory peptide. She will continue with the production of additional AAVs for dose-finding.
Funding Support:	Committed effort fully supported by this award

Name:	Suya Wang
Project Role:	Postdoctoral Fellow
Researcher Identifier:	NA
Nearest person month worked:	9
Contribution to Project:	Dr. Wang performed the comparison of self-complementary versus standard AAV. She also assisted with the design of testing the CaMKII peptides
Funding Support:	Committed effort fully supported by this award

Name:	Jasmine Feng
Project Role:	Research assistant
Researcher Identifier:	NA
Nearest person month worked:	12
Contribution to Project:	Ms. Feng assisted with construction of the AAVs for peptide and promoter testing.
Funding Support:	Committed effort fully supported by this award

Name:	Thomas Samenuk
Project Role:	Research assistant
Researcher Identifier:	NA
Nearest person month worked:	1
Contribution to Project:	Mr. Samenuk determined the lack of effect of microRNA targeting sequence for miR124. Currently improving on non-promoter expression specification.
Funding Support:	Committed effort fully supported by this award

Name:	Daisuke Yoshinaga
Project Role:	Postdoctoral Fellow
Researcher Identifier:	NA
Nearest person month worked:	12
Contribution to Project:	Dr. Yoshinaga assisted with imaging of cardiomyocytes along with other research members.
Funding Support:	Committed effort fully supported by this award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Organization Name: University of British Columbia
Location of Organization: (if foreign location list country) British Columbia, Canada
Partner's contribution to the project:
The University of British Columbia is part of international registry of CPVT patients providing clinical information for analysis.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*