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Final Summary Overview

NIJ FY 15 Basic Science Research to Support Forensic Science

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Enhancing Molecular Autopsies through Function Assays and Family Studies of Cardiac Arrhythmogenic Variants in Sudden Unexplained Deaths



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This is the Final Summary Overview on the grant-**awarded project, entitled** “Enhancing Molecular Autopsies through Function Assays and Family Studies of Cardiac Arrhythmogenic Variants in Sudden Unexplained Deaths” (award# 2015-DN-BX-K017). The document addresses the purpose of the project, project design and methods (including project subjects and data analysis), project findings, and implications for criminal justice policy and practice in the United States.

1. PURPOSE OF THE PROJECT

Molecular autopsies have an increasingly important role in modern forensic practice¹. Our previous study results² supported by NIJ FY2011 Basic Research Grant project (2011-DN-BX-K535) showed that in a large cohort of people who died suddenly, all had comprehensive forensic investigations and complete autopsies at the New York City Office of Chief Medical Examiner (NYC OCME) and whose deaths remained still unexplained, approximately 15% are due to inheritable cardiac arrhythmia or cardiomyopathy. We also reported approximately 40% of decedents carried rare non-synonymous variants (minor allele frequency <0.05%) in cardiac arrhythmogenic genes. These **variants are classified as “variants of uncertain significance”**. In this grant, we aimed to determine the significance of these putative cardiac arrhythmogenic variants identified in decedents and will 1) characterize the effect of the genetic variants on cardiac ion channel functions through electrophysiological studies and 2) utilize clinical tools to examine the inheritance of the genetic variants (i.e. de novo variants, which are present only in the deceased, or pathogenic variants, which co-segregate with the clinical phenotype of biologically related family members).

The overall goal of **this proposal is to enhance molecular autopsies by evaluating genetic variants of “uncertain significance” through functional assays and family studies mediated** by genetic counseling. This is a joint grant by Dr. Yingying Tang (Director of the Molecular Genetics Laboratory of NYC OCME, CAP-accredited) and Dr. William Coetzee (Professor of Pediatrics, NYU School of Medicine), who has the capacity to determine how the variations of ion channel genes participate in sudden death. This grant also supports the recruitment of the first-ever certified genetic counselor in the NYC OCME, who provides professional genetic counseling services to the families of the decedents and connects them to an appropriate genetic clinic for further medical cares.

The proposed studies will improve our understanding of the underlying causes of sudden deaths and enhance the postmortem molecular diagnostic capabilities available to medical examiners, thereby, improving their ability to determine causes of death in cases which would otherwise remain “undetermined”. **More importantly**, timely genetic testing results disclosure to the families of the decedents and prompt clinical referral mediated by our certified genetic counselor enables the identification of family members (parents and siblings) who are potentially at risk of sudden death and who are eligible for appropriate treatment.

It is noteworthy that this project is the third of four grants which NYC OCME has received from NIJ on the topic of molecular autopsy. Most importantly, each NIJ-awarded grant not only produced scholarly publications based on the funded studies but also served as validation of the positive value of our work and affirmation of the influencer role NYC OCME plays in the field of forensic sciences as it established a model in the United States for the incorporation of state-of-art and high-throughput molecular autopsy in routine death investigations. Specifically, the first grant (2011-DN-BX-K535) allowed NYC OCME to acquire the next-generation sequencing technology, bioinformatics pipeline, and variant interpretation expertise; the second grant (2014-DN-BX-K001) allowed the validation of cardiac-focused genes testing panel to be implemented routinely in sudden death investigation in NYC OCME; the third grant (this grant) enabled the first-ever genetic counseling program to be established in NYC OCME as well as collaborative functional studies of genetic variants to be conducted; the fourth grant (2018-DU-BX-0204) enabled the establishment of comprehensive molecular testing panels for disease-causing genes associated with various underlying genetic conditions associated with sudden death, such as epilepsy, aortopathy, thrombophilia, and non-cardiac channelopathy, adding to the cardiac-focused testing panel implemented through previous grants. Through **NIJ’s continuous grant funding support, NYC OCME was able to be an example of high standards to the nation with high-quality molecular autopsy and unwavering commitment to forensic science, criminal justice and the surviving families which we serve.**

2. PROJECT DESIGN AND METHODS (including project subjects and data analysis)

Functional Studies of Genetic Variants: We introduced the genetic variants as mutations in the human channel cDNAs using site-directed mutagenesis and standard molecular biology techniques. We transiently transfected a

mammalian cell line (e.g. HEK-293 cells) with the mutant or wild-type cDNA. To examine accessory subunits of a channel (e.g. KCNE1), we co-expressed with the relevant primary channel subunit (e.g. KCNQ1). Cells were co-transfected with either EGFP or mCherry cDNAs, which allows for positive selection of successfully transfected cells by their fluorescence. We allowed protein expression to occur for 48 hours before studying the channel function. Not each cell is successfully transfected when performing transient transfections of mammalian cells, which leads to a heterogeneous cell population (some cells express the protein, and others do not). In our case, the successfully transfected cells can be easily identified by fluorescence since the cells were co-transfected with EGFP or mCherry cDNAs. Mutations may not only lead to alterations in channel function, but often also cause defects in channel trafficking, which reduces the number of channels expressed on the cell surface. The Flow Cytometry methods can also be used to measure the number of surface channels in live cells. The principle involves combined flow cytometric analysis of a surface antigen (e.g. an antibody specifically directed at an extracellular epitope) and an intracellular marker (e.g. EGFP or mCherry to identify transfected cells). For example, we have successfully used the anti-Nav1.5 monoclonal IgM antibody (clone 4G8:1G7, Novus) to detect surface Nav1.5 levels. Surface-targeted antibodies are not available for all channels. In such cases, we have developed constructs in which an extracellular peptide epitope has been directed (e.g. a myc-epitope or the avi-epitope that allows the specific attachment of extracellular biotin, mediated by the E. Coli enzyme, BirA). We transiently co-transfected a mammalian cell line with the channel cDNA and with EGFP or mCherry cDNAs to allow for positive selection of transfected cells. Cells were studied 48h after transfection. We used standard flow cytometry methods to analyze the surface expression of channels. We used **the “Port-a-Patch” (Nanion Technologies Inc.) patch clamp system for efficient patch clamp screening, which is available in Dr. William Coetzee's laboratory. It is a miniaturized patch clamp system supporting giga-seal recordings from one cell at a time and offers fast and easy access to high quality patch clamp data with minimal training. Our system features optional add-ons, such as the Internal Perfusion and Temperature Control to allow recordings at physiologically relevant temperatures. A typical workflow to study each of the mutants is as follows: The electrodes are chlorinated. This is a one-time operation to be performed each day. A disposable chip is filled with intracellular solution, placed in the chip mounting station and cells are added. Patch automation occurs through control software. Data are recorded using a patch clamp amplifier and automated analysis is performed**

using custom-designed software. A full patch clamp analysis was only performed on variants where marginal effects are observed.

Genetic Counseling and family referral: When a medical examiner determines molecular analysis is required in cases of sudden unexplained infant deaths, the turn-around time for this is up to 8 weeks following the request. Upon completion of variant curation and report writing, the report is returned to the medical examiner who can make a referral for the genetic counselor to meet with the families in cases deemed appropriate. The genetic counselor then schedules face-to-face or telephone genetic counseling sessions that last approximately one hour. Each session includes grief counseling, offering appropriate support services, educating the families on the testing results generated by the Molecular Genetics Laboratory at the NYC OCME, constructing a pedigree analysis and referring the family to a local cardiac genetics program. These programs include NYU Medical Center, New York Presbyterian Hospital - Columbia University Medical Center, and Montefiore/Einstein Medical Center, with which OCME has partnerships, where Clinical Genetics evaluation protocols, including family consent, are provided. This is also the opportunity for high risk family members to be clinically examined and treated, if indicated. Any relevant results from these family studies are returned to NYC OCME after a synthesis of clinical and scientific discovery by the genetic counselor. This process is meant to assist in the revision of the interpretation of variants of uncertain significance so **that enhanced molecular autopsies can improve medical examiner's service to the families as well as the impact on** public health records and law enforcement through death certification. The timeline from the point of the test request from the medical examiner to the time in which the family studies are returned to the cardiac genetics clinic is approximately 7 months.

3. PROJECT FINDINGS

A. *Scholarly publications*

Collectively, eight PubMed indexed articles have been published in peer-reviewed journals as results of this grant³⁻¹⁰.

1: Dong J, Williams N, Cerrone M, Borck C, Wang D, Zhou B, Eng LS, Subbotina E,

Um SY, Lin Y, Ruiters K, Rojas L, Coetzee WA, Sampson BA, Tang Y. Molecular autopsy: using the discovery of a novel de novo pathogenic variant in the *KCNH2* gene to inform healthcare of surviving family. *Heliyon*. 2018 Dec 8;4(12):e01015. doi: 10.1016/j.heliyon.2018.e01015. eCollection 2018 Dec. PubMed PMID: 30582040;

Forensic investigation and molecular autopsy were performed on an 18-year-old female who died suddenly and unexpectedly. Co-segregation family study of the first-degree relatives and functional characterization of the variant were conducted. We identified a novel nonsense variant, NP_000229.1:p.Gln1068Ter, in the long QT syndrome type II gene, *KCNH2*, in the decedent. This finding correlated with her ante-mortem electrocardiogram findings. Patch clamp functional studies using transfected COS-7 cells show that hERG- Δ Q1068 has a mixed phenotype, with both gain- (negative voltage shift of steady-state activation curve, the positive shift of the steady-state inactivation curve, and accelerated activation) and loss-of function (reduced current density, reduced surface expression and accelerated deactivation) hallmarks. Loss of cumulative activation during rapid pacing demonstrates that the loss-of-function phenotype predominates. The wild-type channel did not rescue the hERG- Δ Q1068 defects, demonstrating haploinsufficiency of the heterozygous state. Targeted variant testing in the family showed that the variant in *KCNH2* arose de novo, which eliminated the need for exhaustive genome testing and annual cardiac follow-up for the parents and four siblings. Molecular testing enables accurate determination of natural causes of death and precision care of the surviving family members in a time and cost-saving manner. We advocate for molecular autopsy being included under the healthcare coverage in US.

2: Dong J, Subbotina E, Williams N, Sampson BA, Tang Y, Coetzee WA. Functional reclassification of variants of uncertain significance in the *HCN4* gene identified in sudden unexpected death. *Pacing Clin Electrophysiol*. 2018 Dec 22. doi: 10.1111/pace.13593. [Epub ahead of print] PubMed PMID: 30578647.

The *HCN4* gene encodes a subunit of the hyperpolarization-activated cyclic nucleotide-gated channel, type 4 that is essential for the proper generation of pacemaker potentials in the sinoatrial node. We reported the in vitro functional characterization of four rare variants of uncertain significance (VUS) in *HCN4*, identified through testing a cohort of 296 sudden unexpected natural deaths. The variants are all missense alterations, leading to single amino acid changes: p.E66Q in the N-terminus, p.D546N in the C-linker domain, and both p.S935Y and p.R1044Q in the C-

terminus distal to the CNBD. We also identified a likely benign variant, p. P1063T, which has a high minor allele frequency in the gnomAD, which is utilized here as a negative control. Three of the HCN4 VUS (p.E66Q, p.S935Y, and p.R1044Q) had electrophysiological characteristics similar to the wild-type channel, suggesting that these variants are benign. In contrast, the p.D546N variant in the C-linker domain exhibited a larger current density, slower activation, and was unresponsive to cyclic adenosine monophosphate (cAMP) compared to wild-type. With functional assays, we reclassified three rare HCN4 VUS to likely benign variants, eliminating the necessity for costly and time-consuming further study.

3: Tang Y, Williams N, Sampson BA. Genetic testing in sudden unexpected natural death in the young: New York City Office of Chief Medical Examiner's experience and perspective. *Forensic Sci Med Pathol*. 2018 Dec 7. doi: 10.1007/s12024-018-0068-6. [Epub ahead of print] PubMed PMID: 30535908.

This is a review article. Postmortem genetic testing is a diagnostic tool that is becoming increasingly utilized. The benefits and limitations of genetic testing in cases of sudden, unexpected death in the young (≤ 40 years old) are reviewed from the perspective of the Office of Chief Medical Examiner of the City of New York, whose Molecular Genetics Laboratory, accredited by College of American Pathologists, has had 15 years of postmortem testing experience. Challenges to the interpretation and communication of testing results are highlighted, and opportunities for improving testing yield are discussed for age groups across the lifespan, from infancy to adulthood.

4: Subbotina E, Williams N, Sampson BA, Tang Y, Coetzee WA. Functional characterization of TRPM4 variants identified in sudden unexpected natural death. *Forensic Sci Int*. 2018 Dec;293:37-46. doi: 10.1016/j.forsciint.2018.10.006. Epub 2018 Oct 24. PubMed PMID: 30391667.

The TRPM4 gene encodes the subunit of the Ca²⁺-activated nonselective cation channel, which is enriched in the specialized cardiac conduction system and Purkinje fibers. We reported the functional effects of previously uncharacterized variants of uncertain significance (VUS) that we have found while performing a "genetic autopsy" in individuals who have suffered sudden unexpected death (SUD) in the New York City area. We have identified thirteen uncommon missense VUS in TRPM4 by testing 95 targeted genes implicated in channelopathy and cardiomyopathy in 330 cases of SUD. In several cases there were co-existing VUS in one or more other genes that

were tested. We selected four TRPM4 VUS (C20S, A380V, L595V and I1082S) for functional characterization, since these cases lacked detectable variants in other genes of our testing panel. Two of the cases were infants, one was a child and one an adult. RNA-seq data analysis showed that the longer TRPM4b splice variant is predominantly expressed in adult and fetal human heart. We therefore used site-directed mutagenesis to introduce these variants in a TRPM4b cDNA. HEK293 cells were transfected with the cDNAs and patch clamping was performed to assess the functional consequences of the TRPM4 mutants. The TRPM4 current was recorded in excised patches and was significantly reduced by each of the mutants. The total protein level of TRPM4-C20S was markedly decreased, whereas the A380V and L595V mutants exhibited decreased surface expression. The TRPM4-A380V current rapidly desensitized following patch excision. Each of the VUS tested caused a defect in TRPM4 channel function via distinctly different mechanisms, hence, it lays the foundation for further co-segregation family studies and animal studies of the TRPM4 variants.

5: Williams N, Marion R, McDonald TV, Wang D, Zhou B, Eng LS, Um SY, Lin Y, Ruitter K, Rojas L, Sampson BA, Tang Y. Phenotypic variations in carriers of predicted protein-truncating genetic variants in *MYBPC3*: an autopsy-based case series. *Cardiovasc Pathol*. 2018 Nov - Dec;37:30-33. doi:10.1016/j.carpath.2018.09.001. Epub 2018 Sep 13. PubMed PMID: 30282064.

We characterized predicted protein-truncating variants (PTVs) in *MYBPC3*, the gene most commonly associated with hypertrophic cardiomyopathy (HCM), found in a series of autopsied HCM cases after sudden unexpected cardiac death. All cases underwent death scene investigation, gross and microscopic autopsies, toxicological testing, a review of medical records, and a molecular analysis of 95 cardiac genes. We found four pathogenic PTVs in *MYBPC3* among male decedents. All variants were previously submitted to ClinVar without phenotype details. Two PTVs were located in the cardiac-specific myosin S2-binding (M) motif at the N-terminus of the *MYBPC3*-encoded cMyBP-C protein, and two PTVs were in the non-cardiac-specific C-terminus of the protein. The carriers of two cardiac-specific M-motif PTVs died at 38-years-old; their heart weight (HW, g) and body mass index (BMI, kg/m²) ratio were 34.90 (890/25.5) and 23.56 (980/41.6), respectively. In contrast, the carriers of two non-cardiac-specific C-terminal PTVs died at age 57 and 67 years, respectively; their HW and BMI ratio were 14.71

(450/30.6) and 13.98 (600/42.9), respectively. A detailed three-generation family study was conducted in one case. This study showed age-at-death variations among *MYBPC3* PTVs carriers in adult males.

6: Ivan Gando, Nori Williams, Glenn I. Fishman, Barbara A. Sampson, Yingying Tang, William A. Coetzee. Functional Characterization of SCN10A Variants in Several Cases of Sudden Unexplained Death. Forensic Science International Available online 29 May 2019. <https://doi.org/10.1016/j.forsciint.2019.05.042>

Patch clamp studies were performed to assess effects of the variants on whole-cell Nav1.8 currents. We also performed RNA-seq analysis and immunofluorescence confocal microscopy to determine Nav1.8 **expression in heart. We show that four of the five rare 'variants of unknown significance' (L388M, L867F, P1102S and V1518I)** are associated with altered functional phenotypes. The R756W variant behaved similar to wild-type under our experimental conditions. We failed to detect Nav1.8 protein expression in immunofluorescence microscopy in rat heart. Furthermore, RNA-seq analysis failed to detect full-length SCN10A mRNA transcripts in human ventricle or mouse specialized cardiac conduction system, suggesting that the effect of Nav1.8 on cardiac function is likely to be extra-cardiac in origin. We have demonstrated that four of five SCN10A variants of uncertain significance, identified in unexplained death, have deleterious effects on channel function. These data extend the genetic testing of SUD cases, but significantly more clinical evidence is needed to satisfy the criteria needed to associate these variants with the onset of SUD.

7: E Subbotina, HQ Yang, I Gando, N Williams, BA Sampson, Y Tang, William A Coetzee. Functional characterization of ABCC9 variants identified in sudden unexpected natural death. Forensic science international 2019 May; 298, 80-87

We performed genetic testing of cases of sudden expected death in the New York City metropolitan area and found four rare or novel variants in ABCC9, which codes for the regulatory SUR2 subunit of KATP channels. All were missense variants, causing amino acid changes in the protein. Three of the variants (A355S, M941V, and K1379Q) were in cases of infants less than six-months old and one (H1305Y) was in an adult. The predicted pathogenicities of the variants were conflicting. We have introduced these variants into a human SUR2A cDNA, which we coexpressed with the Kir6.2 pore-forming subunit in HEK-293 cells and

subjected to patch clamp and biochemical assays. Each of the four variants led to gain-of-function phenotypes. The A355S and M941V variants increased in the overall patch current. The sensitivity of the **KATP channels to inhibitory 'cytosolic' ATP was repressed for the M941V, H1305Y and K1379Q variants.** None of the variants had any effect on the unitary KATP channel current or the surface expression of KATP channels, as determined with biotinylation assays, suggesting that all of the variants led to an enhanced open state. All four variants caused a gain-of-function phenotype. Given the expression of SUR2-containing KATP channels in the heart and specialized cardiac conduction, vascular smooth muscle and respiratory neurons, it is conceivable that electrical silencing of these cells may contribute to the vulnerability element, which is a component of the triple risk model of sudden explained death in infants. The gain-of-function phenotype of these ABCC9 variants should be considered when assessing their potential pathogenicity.

8: Williams N, Manderski E, Stewart S, Bao R, Tang Y. Lessons learned from testing cardiac channelopathy and cardiomyopathy genes in individuals who died suddenly: A two-year prospective study in a large medical examiner's office with an in-house molecular genetics laboratory and genetic counseling services. *J Genet Couns.* 2019 Aug 22. doi: 10.1002/jgc4.1157. PMID: 31436011.

This is a comprehensive review and analysis of 254 cases tested consecutively in the in - house College of American Pathologist - accredited molecular genetics laboratory within the New York City Office of Chief Medical Examiner between October 2015 and February 2018, using a multigene cardiac panel composed of 95 genes associated with cardiac channelopathy and cardiomyopathy. Demographics, autopsy findings, medical history, and postmortem genetic testing results were collected for each case. The majority of decedents were adults (>25 years old, 52.7%), followed by infants (<12 months, 25.6%), young adults (19–25 years old, 11.4%), and children (1–18 years old, 10.2%). There were more males (64.2%) than females (35.8%). The racial/ethnic composition of decedents was 40.2% Black, 29.9% Hispanic, 22.4% White, 5.1% Asian/Pacific Islander, and 2.8% mixed/unspecified. Overall, 45.7% of decedents had a negative autopsy, and the remaining had one to four cardiac findings (cardiac hypertrophy, dilation, atherosclerosis, and fatty change). Twenty - seven pathogenic/likely pathogenic variants (P/LP) and 99 variants of uncertain significance (VUS) were identified in 10.6% and 39% of

decedents respectively. P/LP and VUS were found in 51 cardiac genes of the total 95 genes, where MYBPC3, TTN (predicted truncating variants), KCNH2, RYR2 and DSP genes had more than two P/LP variants identified. Among the 73 decedents who were suspected of having cardiac arrhythmia or cardiomyopathy, 20.3% had P/LP variants and 47.9% had VUS; among 23 decedents who had hypertensive cardiovascular diseases and 20 decedents with a history of substance use, 13% and 30% had P/LP variants, respectively. There were 26 referrals from medical examiners for genetic counseling and the outcomes are discussed. The study demonstrates characteristics of the diverse population typically seen by medical examiners in an urban center and our results support a broader implementation of molecular testing in sudden death.

4. IMPLICATIONS FOR CRIMINAL JUSTICE POLICY AND PRACTICE IN THE UNITED STATES

The design of the project is the culmination of extensive discussion between medical examiners, molecular diagnostic laboratory, and basic research scientists who are driven by one of the most vexing challenges in the practice of forensic pathology within the criminal justice system: to determine the inexplicable cause of sudden death in infants and young adults. With rapidly improving sequencing technologies, our ability to sequence medically relevant genes faster and cheaper has become a reality. This leads to the discovery of a large number of rare or novel highly suspicious genetic variants by sequencing. Understanding their contribution to sudden unexplained death is now the limiting factor and this aspect of forensic medicine has become a major new challenge. Two roadblocks limit our understanding of the role of the genetic variants i) the lack of an effective functional assay to characterize this huge amount of genetic variants within a reasonable time and at reasonable costs; and ii) the lack of a family-based study to delineate the roles of genetic variants within a biologically related family. Both obstacles are being directly addressed in this study and will significantly advance forensic medicine. Implementation of our proposed studies, and their successful completion, would be expected to significantly advance both molecular autopsy and the practice of forensic pathology.

With the completion of the proposed studies we have improved our knowledge and the understanding of the underlying causes of sudden infant deaths; we have developed tools and technologies for better characterization of large number of genetic variations, and we enhanced the postmortem molecular diagnostic capabilities and the

medical examiner's capacity in certifying otherwise "undetermined" cases. More importantly, through our family studies, siblings and family members at risk have had the opportunities to be evaluated clinically by programs of cardiogenetic practices throughout New York City and treatment have initiated to prevent possible recurrent sudden deaths in the family.

Finally, through our publications in forensic science journal, forensic pathology journal, medical genetics journal, and health science journal with broader readers, we have reached the audience beyond medical examiners' community.

5. REFERENCES

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