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Sudden Cardiac Death in Young Adults with Long-Chain 3-Hydroxyacyl CoA Dehydrogenase Deficiency (LCHADD)

Ajesh Saini
Portland State University

SUDDEN CARDIAC DEATH IN YOUNG ADULTS WITH LONG-CHAIN 3-HYDROXYACYL COA DEHYDROGENASE DEFICIENCY (LCHADD)

By

Ajesh Saini

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Melanie Gillingham PhD, RD, LD

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Contents

Abstract

Long-chain 3-Hydroxyacyl CoA Dehydrogenase Deficiency (LCHADD) is an autosomal recessive defect in fatty acid oxidation that presents with hypoketotic hypoglycemia and/or hypertrophic cardiomyopathy in infancy, and recurrent rhabdomyolysis in adolescence, however, sudden cardiac death has not been a previously reported complication of LCHADD. We have conducted a case review study comparing young adult LCHADD patients who have experienced sudden cardiac arrest events (n=5) to similar patients who have not (n=5) for the purpose of evaluating associated cardiac risk factors. We reviewed medical records from ECG tests, hospitalization reports, acylcarnitine, and complete metabolic panels, clinic notes, and autopsy reports. Retrospective chart review has led to no certain etiology however, electrolyte derangements, low free carnitine and elevated total to free carnitine ratio have been noted upon hospitalization in sudden cardiac arrest cases. At the time of the sudden cardiac death event, only one subject was in a metabolic crisis with elevated creatine phosphokinase levels. Life-threatening ventricular arrhythmias appear to be a newly recognized life-threatening complication in the adolescent and young adult age groups of LCHADD patients. The exact mechanism underlying the sudden death events is not understood and there are no current therapies. Understanding the pathophysiology of inherited arrhythmias can be challenging due to the complexity of both the heart and LCHAD deficiency along with the lack of appropriate cellular and in vivo models. Recent advances in human-induced pluripotent stem cell (hiPSC) technology have provided extraordinary progress in understanding the mechanisms in generating human-induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). Future directions of this study seek to generate LCHAD deficient patient-derived iPSC-CMs in order to assess mitochondrial function, force of contraction, oxygen consumption rates, impacts of analytes, and

calcium regulation. In a paper published in the Nature Methods Journal, Burridge and colleagues establish an easily reproducible protocol for the chemically defined generation of human cardiomyocytes (Burridge, Matsa, & Shukla, 2014) from skin fibroblast-derived induced pluripotent stem cells. Our research lab will establish this method which utilizes three crucial components of a chemically defined medium consisting of the basal medium RPMI 1640, L-ascorbic acid 2-phosphate along with recombinant human albumin to generate cardiomyocytes with an immature phenotype. This chemically defined medium will provide reproducible, scalable methods for deriving cardiomyocytes from iPSCs however, they do remain immature in nature and future directions of the study seek to adapt newly engineered maturation protocols. Assessing the disruption of bioenergetics and mitochondrial function in hiPSC-CMs will provide a meaningful in vitro model to examine the potential pathways and biochemical mechanisms that contribute to cardiac arrhythmias observed in affected LCHADD patients who have experienced sudden cardiac death events. Ultimately, clinical trials will be needed to further characterize the pathophysiology of these severe cardiac manifestations in young adult LHCADD populations.

Introduction

Fatty Acid (FA) Metabolism

Metabolism is an essential process that occurs in every living organism. It's a term that describes all the chemical reactions that occur within living cells that function to either convert food into energy through a process of catabolism or to convert small nutrients into large molecules through anabolism. In living organisms, fatty acids (FAs) are critical and essential fuel sources in the body that consist of certain fats we obtain through the food we consume. When digestion occurs, the body breaks down fats into fatty acids which can then be absorbed into the blood and transported throughout the body. Fatty acids have many important functions in the body including energy storage, maintenance of cellular membranes, and as a major source of fuel. Lipolysis of fats in the body is a biochemical process that occurs in the cytoplasm of a cell where large FAs such as triglycerides can be broken down into smaller substituents of glycerol and three molecules of FAs which enter the mitochondria to further metabolism. Three types of fatty acid oxidation processes that occur in the body include mitochondrial-β oxidation, microsomal ω-oxidation, and peroxisomal β-oxidation which all differ vastly from each other. Mitochondrial beta-oxidation is one of the most crucial pathways in living organisms that oxidize the released FAs from triglycerides into two carbon units of acetyl CoA. Fatty acid oxidation (FAO) occurs through an orchestration of over 20 different enzymes that form the pathway and allow oxidation to occur. The movement and shuttling of FAs from the cytosol into the mitochondria for oxidation are credited to a set of enzymes known as carnitine palmitoyl-transferase shuttles. Long-chain fatty acyl-CoA enters the outer membrane of the mitochondria and is converted to a long-chain fatty acyl-carnitine by carnitine palmitoyl-transferase 1. The fatty acylcarnitine is transported into the mitochondria by carnitine palmitoyl-transferase and the

fatty acyl-CoA is reformed by carnitine palmitoyl-transferase II allowing for further oxidation and the crucial steps of beta-oxidation occur.

Mitochondrial β-Fatty Acid Oxidation

Beta oxidation produces a lot of ATP allowing living cells to meet high energy demands and, maintain internal homeostasis, especially during variations in internal or external environmental conditions. Conditions that increase cellular energy needs include disease, exercise and durations of fasting. Oxidation of fatty acids is necessary to meet these energy demands and requires a series of complex enzymes one of which is, the mitochondrial trifunctional protein (MTP). MTP is a multienzyme complex situated in the inner mitochondrial membrane that is constituted of four alpha subunits, and four beta subunits. The HADHA gene encodes the alpha subunit of the MTP complex and the HADHB gene encodes the beta subunit of the MTP complex which are responsible for catalyzing the last three steps of mitochondrial beta-oxidation of long-chain fatty acids (LCFAs). The HADHA gene is responsible for catalyzing the 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities whereas the HADHB gene is responsible for catalyzing the 3-ketoacyl-CoA thiolase activity. A number of fatty acid oxidation disorders (FAODS) are caused by genetic defects in the enzymes responsible for the transport of fatty acids in the mitochondria and for the oxidation of fatty acids in the mitochondria (Wilcken, 2010). A deficiency of any one of the enzymes listed above, could cause direct blocks in crucial pathways of fatty acid oxidation resulting in FAODs. Organs that particularly rely on FAO include the liver, eyes, muscle, and the heart which can become severely adversely affected by FAODs that block these processes from properly occurring (Wajner, 2016).

Fatty Acid Oxidation Disorders (FAODs)

FAODs are autosomal recessively inherited disorders which is one of several ways that a disease can be passed through families throughout generations. For autosomal recessive inheritance to occur, two copies of a gene in a cell are mutated and each parent will carry one copy of the mutated gene however, they typically do not present with any symptoms of the condition which implies that we can see the disease skip through generations in a family. Some of the common FAODs include short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), long-chain 3-hydroxyacyl CoA dehydrogenase (LCHAD), and very long-chain acyl-CoA dehydrogenase (VLACD) deficiencies. Of these enzyme deficiencies, the four most prevalent deficiencies in the long-chain fatty acid oxidation pathway are CPT-II, VLCAD, LCHAD, and MTP deficiencies. Figure 1 below includes the details of presentation and diagnostic criteria of the long-chain fatty acid deficiencies.

Fatty Acid Oxidation Disorder	Hypoglycemia	Liver Dysfunction	Cardiomyopathy	Skeletal Myopathy	Rhabdomyolysis	Acylcarnitne Elevations	Other Complications
Carnitine palmitoyltransferase type 1 defiecncy (CPT1D)	X	X	–	–	–	C0, C0/(C16+C18) ratio	Renal tubular acidosis
Carnitine palmitoyltransferase type 2 deficiency (CPT2D)	X	X	X	X	X	C16, C16:1, C18, C18:1	Renal cysts, facial dysmorphism
Very long-chain acyl-CoA dehydrogenase deficiency (VLCADD)	X	X	X	X	X	C12:1, C14:2, C14:1, C14, C16:1, C16	–
Long-chain 3-hydroxy acyl-CoA dehydrogenase deficiency (LCHADD)	X	X	X	X	X	C16:1-OH, C16-OH, C18:1-OH, C18-OH	Peripheral neuropathy, retinopathy
Medium-chain acyl-CoA dehydrogenase deficiency (MCADD)	X	X	–	–	–	C8, C10, C10:1	–
Trifunctional protein deficiency (TFPD)	X	X	X	X	X	C16:1-OH, C16-OH, C18:1-OH, C18-OH	Peripheral neuropathy, retinopathy

Figure 1. A table indicating the various long-chain fatty acid oxidation disorders and their typical presentations and diagnosis (Merritt, 2018)

Initial presentations of several FAODs are seen in the neonatal period with severe symptoms including hypoketotic hypoglycemia, cardiomyopathy, liver dysfunction, and retinopathy which are common and sometimes, also continue to present into infancy and early childhood. Episodic rhabdomyolysis is the breakdown of damaged skeletal muscle which causes the release of myoglobin. This release results in extreme pain in patients and is a frequent presentation during or after adolescence in those with long-chain FAODs (Merritt, 2018). Research suggests that individuals with FAODs accumulate toxic byproducts in the blood stream such as long-chain fatty and hydroxy acids, and fatty acyl-carnitines. Newborns with FAOD tend to experience multi-organ failure of varying severities which can become worse under stressed conditions that exacerbate energy use especially during times of fasting, illness or with prolonged exercise.

Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency

Long-chain 3-Hydroxyacyl-Coenzyme A Dehydrogenase deficiency (LCHADD) is part of the fatty acid oxidation disorders (FAOD) family of disorders. LCHADD is the result of a mutation in the alpha subunit of the MTP, HADHA. Mutation in the active site residue of the HADHA gene (1528G>C) is responsible for the loss of LCHAD enzyme activity. Classic symptoms of infants with LCHADD include recurrent hypoglycemia induced by fasting or physiologic stress, and recurrent rhabdomyolysis or exercise intolerance which can also induce overwhelming neonatal cardiomyopathy in young children and infants (Vockley, 2017). Newborn screening (NBS) for fatty acid oxidation disorders, including LCHADD, was started in Oregon in 2004. Today, most infants have the opportunity to become diagnosed at an early age allowing for prompt medical care before a catastrophic metabolic decompensation occurs (Frazier, 2006).

Prior to the advent of NBS, hypertrophic and dilated cardiomyopathy in infants was a frequently documented complication of LCHADD. Upon diagnosis and treatment, the cardiomyopathy typically resolves and patients have reported normal cardiac function in childhood. However, recently, there have been 7 known cases of sudden ventricular tachycardia (VT) in adolescent and young adults with LCHADD between 16 and 23 years of age. Acute VT in the absence of hypertrophic cardiomyopathy appears to be a late complication observed in some adolescent and young adults with LCHADD that is not currently described in the literature.

At a scientific meeting in 2013, our research group reported a short case series (3 subjects) of patients with LCHADD who had experienced sudden cardiac arrest events. Since then, 4 other cases have appeared. Medical records of 5 patients with LCHADD and sudden cardiac arrest have been reviewed. Of these patients, 4 were resuscitated and 1 was deceased. Three patients received an implantable cardioverter. Medical records of 5 patients with LCHADD of similar age who have not had a cardiac arrest were also reviewed and utilized for comparison. While electrolyte derangements are reported in the record, we hypothesize that unidentified underlying cardiac risk factors that increase the risk for sudden cardiac arrest may exist. To date, there are 7 known or suspected cases of sudden VT in adolescent and young adults with LCHADD in an estimated population of 60 patients >16 years of age in the U.S., thus becoming a significant medical problem for this specific patient population. Sudden cardiac arrest episodes have also become of concern to other patients with LCHADD and their families, who are now enquiring whether prophylactic implantable cardioverters should be considered for primary prevention of a sudden VT event in young adult LCHADD patients. A thorough cardiac evaluation has not been performed among these patients and specific risk factors have not yet been identified. A recent grant and IRB study submission to conduct more thorough cardiac

evaluation for patients described here was submitted and approved. Recruitment was about to start but due to the impacts of COVID-19, the study enrollment was postponed an indefinite amount of time. The lack of thorough cardiac data from this extremely rare and limited study population and the lack of an appropriate cellular model are two of the essential motivations behind the current line of research into sudden cardiac death in LCHADD patients.

Central Hypothesis, Purpose, and Specific Aims

Establishing a cellular model to further research sudden cardiac death in LCHADD patients is a major goal of this study which will also entail additional protocols and materials to generate cardiomyocytes with a mature phenotype that are capable of oxidizing fatty acids. In a paper published in the Nature Methods Journal, Burridge and colleagues establish a reproducible protocol for the chemically defined generation of human cardiomyocytes from skin fibroblast derived, induced pluripotent stem cells. This protocol was adapted by our lab to generate cardiomyocytes with an immature phenotype which was successful prior to the institution of modified research due to the COVID-19 pandemic.

The initial stage of cardiomyocyte generation involves three crucial components of a chemically defined medium consisting of the basal medium RPMI 1640, L-ascorbic acid 2-phosphate along with recombinant human albumin, and small-molecules responsible for cellular signaling. This chemically defined medium will provide a reproducible, scalable method for deriving cardiomyocytes from human induced pluripotent stem cells that begin immature and fetal like in nature. In another study, a research group from the University of Washington School of Medicine generated matured human induced pluripotent stem cell cardiomyocytes (hiPSC-CMs) by engineering a microRNA maturation cocktail known as MiMaC consisting of Let7i OE,

miR-452 OE, miR-122 KO, and miR-200a KO which greatly reduced the complete time of the protocol. One of the key differences during maturation is the switch from glycolysis to oxidative phosphorylation for energy production, including fatty acid oxidation. Once mature hiPSC-CMs have been generated, our study will aim to assess the functionality of hiPSC-CMs by measuring and comparing force of contraction, oxygen consumption rates, beat rates, and calcium regulation in LCHADD patient derived iPSC-CMs compared to normal healthy iPSC-CMs.

Other studies have also suggested that accumulation of long-chain hydroxyl acyl-CoA esters and/or the long chain 3-hydroxylated fatty acids (LCHFA) and particularly, 3-hydroxypalmitic acid (3HPA) in patients can have an effect on mitochondrial respiratory parameters measured by oxygen consumption in intact cell system consisting of heart fibers, cardiomyocytes, hepatocytes, and brain slices (Cecatto, Wajner, Vargas, 2018). This research has explored the potential effects of pathological concentrations of toxic byproducts (LCHFAs and 3HPAs) formed by LCHADD patients postulating toxicity as one particular mechanism of sudden ventricular death in this population. When the LCHAD enzyme is missing, accumulated levels of long-chain 3-hydroxylated fatty acids (LCHFA), particularly 3-hydroxypalmitic acid (3HPA) and their carnitine conjugates can be seen in patients' bloodstream. Diagnoses of mitochondrial fatty acid oxidation disorders like LCHADD are typically based on increased concentrations of long-chain 3-hydroxyacylcarnitines in plasma and excessive urinary excretion of 3-hydroxy-carboxlyic acids, especially during or after a metabolic decompensation (den Boer, 2002). Symptoms often develop in the presence of episodes of illness or fasting which affect organs that predominantly use long-chain fatty acids for means of energy such as the heart, liver, retinal, and skeletal muscle.

An additional avenue to the current study is to investigate the effects of LCHAD deficiency and the presence of long-chain 3-hydroxylated fatty acids, (which accumulates the most in LCHAD deficiency), on mitochondrial respiratory parameters measured by oxygen consumption, and cardiac electrophysiology in intact cell systems of human induced pluripotent derived stem cells that have been differentiated into cardiomyocytes. Measurement of other crucial mitochondrial functions including membrane potential, Ca^{2+} retention capacity, beat force and contraction rate, in cardiomyocytes derived from LCHAD patients who have and have not experienced severe cardiac death events will be useful in our exploration.

The answers to the following research questions is what the study seeks to explore:

- How are mitochondrial parameters such as oxygen consumption and various cardiac electrophysiological functions affected in LCHAD deficient patient derived, induced pluripotent stem cells differentiated into cardiomyocytes?
- How may the pathological concentrations of long chain 3-hydroxylated acids such as 3HPA affect bioenergetics and overall function of LCHAD deficient, mature cardiomyocytes?

Patients

Adolescent and young adult patients with confirmed LCHAD deficiency having consented, in compliance with the Helsinki Declaration, to the collection and use of their medical records for future research through our “Fatty Acid Oxidation Disorders Tissue Repository” (IRB #8561) were enrolled in this retrospective case study (IRB #18446). Medical records including results assessed from electrocardiogram (ECG) tests, hospitalization reports, acylcarnitine and complete metabolic panels, clinic and laboratory notes, and autopsy reports

were obtained for review. Medical records of 10 patients were reviewed and patients split into two groups according to whether they had a history of sudden cardiac arrest (SCA) (N=5) or had no history of SCA ('controls', N=5). In patients with a history of SCA, 4 were resuscitated and 1 is deceased following SCA. Age range at 1st cardiac event was 15-21 years, with current age at time of review of 19-27 years. In 'controls' without history of SCA, 2 died of cardiac abnormalities not related to SCA. Current age ranges of 'controls' at time of review was 19-25 years.

Methodology

Skin fibroblasts obtained from LCHADD patients in a separate IRB protocol (IRB#8561) can be used to generate induced pluripotent stem cells. Fibroblasts are reprogrammed in our lab by integration-free Sendai virus vectors which can then induce the formation of stem cells. hiPSCs can then be differentiated and used for further experiments. Our research lab has experience differentiating iPSCs to human retinal pigmented epithelial cells however, generation of hiPSC derived cardiomyocytes has not yet been attempted. It is our hope to establish a reliable protocol to differentiate hiPSCs to mature cardiomyocytes in order to carry out experiments that concern the electrophysiology and overall function of LCHAD deficient cardiomyocytes. This will provide an in vitro model to investigate potential mechanisms associated with arrythmias in human patients. Through the establishment of a cellular model, we hope this will provide a critical research tool that has the potential to further understand the mechanisms of arrythmias in patients.

Cell Cultures

Skin fibroblasts derived from patients and other similar somatic cell types lack high expression of the transcription Oct4, Sox2, Klf4, and c-Myc under normal condition. Once high levels of expression for these four genes are achieved, a fibroblast can become reprogrammed to pluripotent cell types. Induced pluripotent stem cells (iPSC) are genetically reprogrammed adult cells which exhibit a pluripotent stem cell-like state similar to embryonic stem cells (Takahashi et al., 2007). There are multiple ways of generating iPSCs which include retrovirus-mediated gene transduction and chemical induction. DNA-based vectors that include adenovirus, or plasmid vectors do not require integration into the hosts chromosomes and therefore allow the cells to reprogram with non-integrating episomal DNA vectors. Sendai virus is a single stranded negative-sense RNA virus that replicates in the cytoplasm and produces very high copy numbers of the target gene. The Sendai viral vector can be used to efficiently express all four factors stably without chromosomal integration and can be eliminated completely from reprogrammed skin fibroblast cells. Once iPSCs have been generated, cells can be inducted into three different potential layers that include the ectodermal (neuron, astrocytes, Retinal Epithelial cells (RPE)), endodermal (lung, thyroid, pancreatic cells), or into the mesoderm layer (cardiac and skeletal muscle cells, tubule cells of the kidney, or red blood cells) (Millipore Sigma, 2016).

Cardiomyocyte Differentiation

To further reveal the disease etiology of the sudden cardiac death events we will differentiate induced pluripotent stem cells (iPSCs) into cardiomyocytes which will inherit the genetics found in skin fibroblasts from LCHAD deficient patients that were enrolled into the study rendering these CMs as LCHAD deficient. In the past decade, many methods have been

developed that successfully differentiate iPSCs into cardiomyocytes. While this study seeks to establish an in vitro model to study potential mechanisms of SCD, this model is anticipated to still be largely limited in its implications to be made in relation to LCHADD patients who experience differences in physiology, course of medical history, and compliance to diet. Advantages of using hiPSC-CMs include their ability to replicate the genome of the patient donor to allow for characterization of numerous disease states and drugs, as well as their ability to contract which allows for a model of cardiac contractility and electrophysiology. Despite the ability to model biochemical mechanisms in one's body, this approach is still highly limited in being able to translate the results in human subjects.

hiPSC-CMs also have limitations due to their immaturity as they represent fetal cardiomyocytes instead of full-functioning, adult cardiomyocytes. A protocol adapted from Burridge and colleagues will be utilized in this experiment to generate an initial and immature cardiomyocyte model. Though this model has been adequately optimized to generate beating iPSC-CMs, the resulting cells remain highly immature in their nature in regards to their structure and function. These immature cardiomyocytes which rely on glycolysis to produce energy (instead of fatty acid utilization), experience reduced contractile and electrophysiological function and structure, and exhibit disorganized morphologies. Recent research has established that disease modelling using immature iPSC-CMs may be inaccurate and limited in their utility, particularly for investigating the impacts of a defect in fat oxidation. This weakness in the literature has been widely addressed and numerous groups have made efforts to establish methods of maturing cardiomyocytes. We will aim to review and replicate successful methods that have been developed for the maturation of human iPSC-CMs using small molecules, environmental manipulation, prolonged cell-culturing, or 3-dimensional growth approaches.

The same research group at the University of Washington recently published a paper addressing a successful method of maturing iPSC-CMs through addition of small molecules and environmental manipulation approaches of matured iPSC-CMs (Miklas, Clark, Levy, & Detraux, 2019). The research group engineered a microRNA maturation cocktail known as MiMaC consisting of mix of specific micro-RNAs that alter gene expression: Let7i OE, miR-452 OE, miR-122 KO, and miR-200a KO. Upon functional assessment of cardiomyocytes treated with MiMaC, an increase in parameters such as twitch force, power, cell area, and the ability to utilize palmitate more efficiently than control CMs was reported. Once mature phenotype exhibiting hiPSC-CMs have been generated that exhibit optimal mechanical and electrical stimulation, further studies of cell bioenergetics and mitochondrial function can be properly assessed.

Platforms for Functional Analysis of hiPSC-CMs

Using iPSC technology, we will produce a model of LCHADD patient specific cardiomyocytes to investigate potential mechanisms for sudden conduction arrhythmias. A crucial step for this procedure will be to characterize and confirm gene expression patterns and comparing mRNA levels in samples of cardiomyocytes that are produced. Measuring and characterizing the mRNA expression in cardiomyocytes will entail the use of quantitative polymerase chain reaction (qPCR). By using qPCR, protein expression analyses can be done to explore the physiological role of various channels in cardiomyocytes – in this case expression of calcium channels in LCHADD CMs will be assessed against normal, wildtype (WT) cells under normal or high fat media concentrations. In addition to this measure, the following list provides an overview of methodology that will be used to assess various patient specific iPSC-CM functionalities.

Patch Clamp: Patch clamps include high conformity intracellular electrical recordings from cardiomyocytes that can provide detailed and precise assessments of the electrophysiological properties of CMs. Sharp electrodes that can penetrate the membrane of CMs can measure membrane potential and thus, characterize action potentials.

Multielectrode Array: This method includes a noninvasive and nonterminal method that will allow for long-term measurements of extracellular field potential signals from monolayers of hiPSC-CMs. CMs can be plated on the walls of the MEA plate containing electrodes and then, field potential can be measured. These field duration potentials ultimately equate and correlate roughly to the QT interval in an in-vivo ECG and the action potential duration (APD) in-vitro (Garg & Shrestha, 2018).

Fluorescence Imaging: Fluorescence microscopy methods can use Ca^{2+} or voltage-sensitive dyes as a non-invasive method to measure intracellular ion fluctuations and voltage changes. A difficulty that exists with this method is the potential binding of dyes to intracellular molecules which can interfere with the normal functioning of the cell.

Impedance: Functional assays based on cellular impedance which is an indirect measure of cardiac contractility, offers a noninvasive, label-free, and high throughput analysis method (MF, SD, L, CW, & KL, 2015). Cells are seeded onto a multiwall cell culture plate that contains gold film electrodes embedded onto the bottom of the wells. The mechanical displacement of the cells during cardiomyocyte contraction is measured as variation in impedance otherwise measured as a resistance in a current which also directly correlates with beating frequency.

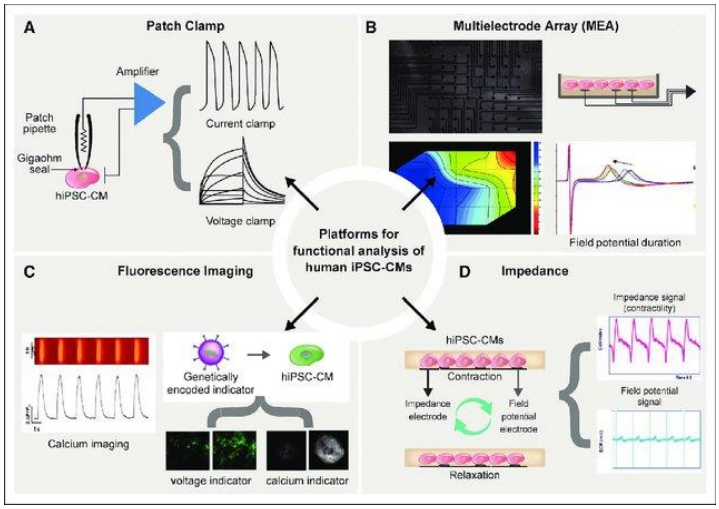

Figure 2. Platforms for functional analysis of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Graphical illustration summarizing different methods currently used for hiPSC-CM functionality analyses. Patch clamp (A); multielectrode array (B; MEA); fluorescence imaging (C); impedance (D). Representative MEA 78,79 and fluorescence imaging 80,81 traces (American Heart Association, 2015).

Without the presence of structural heart disease, this exploration aims to explore the life threatening ventricular arrests that can lead to sudden cardiac death in LCHADD patients especially in the younger adult population which can possibly arise as a result of concurrent defects found in specialized membrane proteins called ion channels as suspected by some other works of research (Garg, 2018). Changes in the function or expression of these channels in cardiomyocytes could potentially disrupt the highly intricate and orchestrated electrical activity of the heart which is why this study focuses on assessing the various functional platforms of cardiomyocytes.

Results

A case series study was done to evaluate for cardiac risk factors by comparing LCHADD patients who have experienced sudden cardiac arrest events (N=5) against those who have not (denoted ‘controls,’ N=5). A plethora of hospitalization and general medical records were reviewed including diagnostic testing, electrocardiograph (ECG) tests, heart rhythm strips,

plasma acylcarnitine levels, as well as the curation of a retrospective chart review of patient medical records including metabolic panels, clinician notes, electrolytes, and specific symptoms that were reported upon admission. The table below is followed by specific case details of each of the young adult patients that experienced sudden cardiac death in the study.

Patients	Gender	Cardiac Event	Age at Event	Current Age	Cardiac Arrhythmias	Significant Admission Notes	Course of Pregnancy	Other Medical History
Case #1	M	YES	21	27	**VF, VT, long QT, TdP**	4-5 days prior nausea,	--	Blindness, neuropathy
Case #2	M	YES	20	26	**VF, VT**	300,000 CK, acute kidney injury, seizure activity, was in rhabdo upon adm., 4-5 days prior nausea	--	300,000 CK, acute kidney injury, seizure activity, macular thinning
Case #3	M	YES	19	--	**VF, VT, long QT**	Anxiety and panic attacks upon pres.	ICU adm. @ 8mo hypoglycemia	Retinitis, blindness, anxiety, panic attacks
Case #4	M	YES	15	26	**VF, VT**	Found with seizure activity	Pre-mature birth	Retinopathy, hydrocephalus
Case #5	F	YES	19	--	**N/A**	Was found unconscious in basement and pronounced deceased	ICU adm. @ 4 months, mother w/ HELLP	Retinopathy, increased LFTs, rhabdomyolysis
Control #1	F	NO	--	23	**ST abnormalities, Diastolic dysfunction**	--	Coma @ 9mo	Rhabdomyolysis, periph. retinopathy and neuropathy
Control #2	M	NO	--	19	**PVCs, SVT**	Syncope, AVNRT, respiratory illness, S-ICD	Coma @ 10mo	Fatty liver,
Control #3	M	NO	--	24	**N/A**	--	ICU adm. @6mo hypoglycemia	Rhabdomyolysis, myopathy
Control #4	F	NO	--	26	**N/A**	--	ICU adm. @ 4 months, mother w/ HELLP	Rhabdomyolysis, retinopathy, ear infections
Control #5	F	NO	--	23	**N/A**	--	Premature 2mo, ICU adm.	Rhabdomyolysis

Figure 3. A general overview of LCHADD patient data divided by individuals who experienced sudden cardiac arrest and those who did not. Patients in red text indicate cases who experienced SCD, and those in black are 'controls' who have not yet experienced a SCD event.

Case #1 was 21 years of age at their first cardiac event which included episodes of ventricular fibrillations, sinus tachycardia, long-QT syndrome and potential suspicions of torsades de pointes. Other medical histories regarding classic symptoms of LCHAD include blindness, and neuropathy. This patient was diagnosed with LCHADD at 6 months of age following initial presentation to the emergency room with jaundice, failure to thrive, and

decreased activity. The diagnosis was confirmed by liver biopsy and subsequent fatty acid oxidation flux studies in cultured hepatocytes with low LCHAD enzymatic activity. His subsequent clinical history is remarkable for frequent recurrent rhabdomyolysis requiring hospitalization multiple times per year throughout his lifetime. CKs range from 4000 to >40,000 during these episodes. Prior to their admission, the patient had increased levels of CK at around 2,700 U/L, and had been vomiting for approximately 7 hours prior to their hospitalization. Upon admission, the patient was experiencing a significant decrease in cognition characterized by decreased comprehension, and expression of language with delayed response and decreased organization of language. The patient was found to be in torsade's de pointes after one cardiac event. An echocardiogram done on the patient showed ejection fraction down at 45-50%, with a mildly enlarged right ventricle, and mildly reduced RV systolic function. Right ventricular systolic pressure was noted to be calculated at 30.6 mmHg. Along with cardiac arrest-ventricular fibrillation, acute respiratory failure was reported which required intubation and mechanical ventilation. Additionally, the patient was found to be in sepsis secondary to aspiration pneumonia. Case #1 was prescribed MCT oil and levocarnitine and is presumed to have been compliant with this upon the time of admission.

Case #2 was 20 years of age at their first cardiac event which included ventricular fibrillation bursts followed by a ventricular tachycardia arrest. In prior history, the patient was known to have multiple hospital admissions for episodes of rhabdomyolysis. Before their event, case #2 had been experiencing 4-5 days of nausea prior to their admission similar to case #1. Of significant note was that case #2 was the only patient who was found to be in a severe case of rhabdomyolysis upon admission with CK levels upwards of 300,000 U/L. Upon review of an echocardiogram, the patient was noted to have systolic heart failure. The patient was also treated

for anemia, pleural effusions, antibiotic associated diarrhea, and epistaxis. Their ejection fraction demonstrated only mild decrease in systolic function and was suspected that some of their cardiomyopathy is related to metabolic disturbances exacerbated by rhabdomyolysis however, yet an unknown underlying result of LCHADD. The patient's arrests had occurred in the setting of acidosis and hyperkalemia related to rhabdomyolysis. Case #2 had been prescribed dietary supplements including MCT oil, levocarnitine, DHA, vitamin D, and magnesium/calcium but, was not compliant with these prescriptions at the time of the event.

Case #3 was 19 years of age at their first cardiac event. This patient had presented with an ICU admission at 8 months of age to be treated for hypoglycemia and was also diagnosed with LCHADD. In prior history, the patient has experienced retinopathy and blindness, generalized anxiety, panic attacks, and admitted for various hospitalizations due to episodes of rhabdomyolysis. The patient presented with an acute loss of consciousness and was found to be in ventricular fibrillation upon arriving to the emergency department at their first cardiac event. The patient was riding in a car as they complained of a headache, and then became unresponsive. They were given 2 rounds of epinephrine as well as cardioversion and then transferred to another medical center where the patient had several episodes of pulseless ventricular tachycardia which resulted in the administration of amiodarone and subsequently resulted in seizure activity. Following the seizures, the patient was administered fosphenytoin. An echocardiogram showed normal left ventricular size with moderate to marked depression of systolic function as well as normal right ventricular size with moderate depression of systolic function with no significant valvular or pericardial issues. Following an evaluation by the hospital's cardiology and electrophysiology unit, an automatic implantable cardioverter defibrillator was placed one week after their first admission. The patient had a past medical history of premature retinopathy and a

hydrocephalus which required a VP shunt. The patient was not on any home medications prior to hospitalization including any dietary supplements.

Case #4 was 15 years of age at their first cardiac event which presented with ventricular fibrillation and tachycardia arrest, and prolonged QTc interval. The patient had a pre-mature birth at approximately 10 months with a prior history of retinopathy and hydrocephalus. Upon admission, case #4 experienced an acute deterioration of EF from 50% to 20% over the course of 1 week during the admission which had ultimately led to cardiac arrest, 30 minutes of CPR, intubation for 11 days and ECMO therapy. They were hemodynamically stable since their extubation however the patient now has hoarseness and dysphagia to thin liquids. Upon hospitalization, the patient was noted to have a short run of VT (6 beats), and an evaluation of an ECHO that showed poor function. The patient had numerous hospitalization due to recurring cases of rhabdomyolysis. Their sudden cardiac arrest event was in the presence of an episode of rhabdomyolysis. The patient had also been experiencing retinitis, joint pain, blindness, and abnormality of gait. Of significant note is the patients recurring anxiety/diaphoresis/weakness and recent physical deterioration and decreased mobility. Case #4 was prescribed levocarnitine, MCT oil, and vitamin D and E tablets however it is unclear whether the patient was compliant with the medications upon hospitalization for their cardiac death event.

Case #5 was 19 years of age when they were unexpectedly found dead in the basement of their house. An autopsy report determined that the specific cause was unclear although due to an unknown, underlying condition of LCHADD. The patient had a classical presentation during early infancy with an ICU admission at four months of age with retinopathy and frequent episodes of rhabdomyolysis. The patient's mother had also died from increased LFTs due to

HELLP syndrome, Case #5 was prescribed MCT oil, carnitine, cod liver oil, and vitamin A, however, was not compliant with these in recent history.

Results for each of the sudden cardiac arrest events were compared against five 'controls' who were also young adult patients with LCHADD but, had not yet experienced any sudden cardiac arrest events besides control #3 who had experienced some premature ventricular contractions but no, complete cardiac death as experienced by all 5 cases in the study. All of the controls are of similar age to the cases who experienced sudden cardiac arrest however, there does not appear to be any significant differences in the course of the disease between the two populations. Both populations were prescribed and ordered to follow a strict diet and upon retrospective case review, 3/5 those who experienced sudden cardiac reported failure to keep up with their medications upon the time of admission for their cardiac events. Figure 4 illustrates the supplements that were prescribed to the two populations and their compliance. Both groups were prescribed similar dietary supplements and this does not appear to be influencing the nature of cardiac arrythmias in patients. To supplement this data, elevated carnitine levels can be seen in cases as indicated by the increased carnitine ratios in figure 4 that depict cases to have ratios >1 and controls with ratios <1. These results are limited in their ability to deduce sound conclusions of why cases are more likely than controls to experience sudden cardiac arrest due to the time upon when certain medical records were measured in patients; not all data points were retrieved upon initial hospital admissions and, some data points relied on prior test results which could have been extremely different to levels upon presentation of a cardiac arrest event.

Patients	MCT Oil	Levocarnitine	Docosahexaenoic Acid	Triheptanoin C7	Cholecalciferol (VitD)	Mg/Ca	Vit A, E, K, other sppl.
Case #1	*15 mL w/meals	1171 mg TID	Yes	--	2000 units QD	Yes	--
Case #2	Yes	Yes	--	--	--	--	Cod liver oil, VitA
Control #1	-	13 mL QD	--	--	--	--	VitE 200 IU/day, flaxseed & fish oil, Centrum tabs, 3 scoops lipistart, glycosade
Case #3	Yes	330 mg BID	Yes	--	2000 units QD*	Yes	Fish oil, taurine, Coenzyme Q10, Multivit tab, VitE & Primrose Oil
Case #4	--	--	--	--	--	--	--
Control #2	15 mL QD	660 mg qAM 330 mg qPM	Yes	Yes	1000 units QD	--	Multivitamin
Control #3	P	330 mg TID	650 mg QD	Yes	Yes	--	Fish oil, liquigen,
Case #5	15 mL QID	330 mg TID	200 mg	--	1000 units BID	Mag-Ox (adm)	VitE
Control #4	-	-	-	-	-	-	-
Control #5	-	-	-	-	-	-	-

Figure 4. A list of the prescribed supplements to be taken by the LCHADD patients included in the study.

Patients	C16-OH, 3-OH Palmitoyl	C16:1-OH, 3-OH Palmitoleyl	C18-OH, 3-OH-Stearoyl	C18:1-OH, 3-OH-Oleyl	C18:2-OH, 3-OH-Linoleyl	Free Carnitine	Total Carnitine	Carnitine Ratio
Case #1	0.21	0.11	0.17	0.37	0.12	9	19	1.1
Case #2	--	--	0.19	0.86	0.25	7	17	1.4
Control #1	--	--	--	--	--	--	--	--
Case #3	0.4	0.11	0.62	0.62	0.13	93	214	1.3
Case #4	--	--	--	--	--	--	--	--
Control #2	0.03	0.01	0.01	0.01	0.01	33	37	0.1
Control #3	--	--	--	--	--	12	21	0.8
Case #5	0.72	0.41	0.41	1.31	0.23	34.6	57.4	0.7
Control #4	0.52	0.21	--	0.62	0.23	40	50	0.3
Control #5	0.4	0.17	0.43	0.91	0.14	7	12	0.7

Figure 5. Various measures of carnitine levels in LCHADD patients (μMol/L). Patients in red are patients who are cases and those in blue are 'control' patients who have not yet experienced sudden cardiac death.

Anticipated Effects on Mitochondrial Function in iPSC-CMs & Discussion

Due to the impacts of COVID-19, further generation of cardiomyocytes was halted in the prior months prohibiting the advancement of this data acquisition. However, anticipated impacts, results and the rationale behind the exploration of various mitochondrial functions in LCHAD

deficient cardiomyocytes is described below. These various parameters and measurements are compared between LCHAD deficient cardiomyocytes and normal cardiomyocytes derived from wild type (WT) cells that are healthy.

For the exploration of bioenergetic impacts. we anticipate that LCHADD cardiomyocytes will have similar oxygen consumption and ATP production as WT cells under high glucose conditions. However, we hypothesize the LCHADD cardiomyocytes will have lower O2 consumption and ATP production compared to WT cells under conditions of low glucose/fatty acid supplemented media.

Following the completion of various functional assessment platforms, we anticipate that LCHADD cardiomyocytes will have similar functional parameters during patch clamp, MEA, fluorescent imaging and impedance as WT cells under high glucose conditions. However, we hypothesize the LCHADD cardiomyocytes will have altered functional analysis with lower contractile force compared to WT cells under conditions of low glucose/fatty acid supplemented media.

As described, some areas of research suggest that toxic byproducts can accumulate as a result of FAODs. With this consideration, we anticipate LCHADD cardiomyocytes will have elevated long-chain, 3-OH acylcarnitines in all conditions but that these metabolites will increase dramatically during low glucose/fatty acid supplemented media conditions. Elevated partially oxidized metabolites will be associated with increased ROS, acidosis, and increased lactate.

It is possible other mechanisms in addition to energetic deficiencies could contribute to the pathophysiology of the severe cardiac manifestations reported here in young adult LCHADD patients. Mitochondrial stress and accumulation of toxic metabolites such as long-chain hydroxy

FAs or carnitine due to inborn errors of metabolism that could potentially lead to severe cardiac death in LCHADD patients (Spiekerkoetter & Wood, 2010). Wajner summarized the potential mechanisms that affect energy homeostasis and suggests a potential explanation of the pathophysiology of the disease. Mechanisms included altered redox reactions, fatty acid accumulation, acidity, calcium and potassium homeostasis, as well as derangements in bioenergetics. Understanding the role of accumulated fatty acids metabolites as a crucial player in altered bioenergetics of the cells as well as its role in inducing mitochondrial dysfunction are crucial in understanding why LCHAD deficient patients may develop these dysregulations. In the study by Wajner, alterations were seen in biochemical processes in mice liver, muscle, and heart tissues. If cardiomyocytes with a mature phenotype were successfully developed, some anticipated results of the assessment of cardiomyocytes could include a decrease in force of contraction, decreased calcium retention capacities, erratic beating patterns, and severe mitochondrial defects.

Typically, upon diagnosis and treatment in the pre- and post-natal stages of LCHADD, cardiomyopathy is typically resolved and patients go on to report normal cardiac function in childhood. This exploration of sudden ventricular tachycardia in this young adult population with LCHADD ranging in ages between 16 and 23 years is one that is unique and not currently described in the literature. Following the retrospective case study review, we report minor differences in supplements and carnitine levels between cases and controls as well as minor electrolyte derangements that were seen. However, we hypothesize that unidentified underlying cardiac risk factors that increase the risk for sudden cardiac death continue to exist. It is important to consider that this study exemplifies that sudden cardiac arrest can occur in any setting and is not exacerbated through other severe symptoms often accompanied with LCHADD

patients including episodes of rhabdomyolysis or varied adherence to supplements and diets prescribed. Applications and exploration of this topic using stem cell technology to generate beating cardiomyocytes provides a promising model to investigate this severe cardiac manifestation and its potential molecular mechanisms. Future directions of this study will pick up where we left off before the spread of COVID-19 to produce phenotypically mature cardiomyocytes as well as to obtain thorough and complete data for this critically vulnerable population. Future anticipated clinical trials that have been approved by the IRB will include data from cardiac magnetic resonance imaging of the heart, results of ECG bicycle exercise stress tests, measures of acylcarnitine levels before and after these tests, and blood draws to perform thorough laboratory studies and measurement of patients' electrolytes, glucose, insulin, creatine kinase, free fatty acids, lactate, pyruvate, ketones and plasma/serum acylcarnitine levels. Following the acquisition of sound patient data through clinical trials along with the establishment of a mature cardiomyocyte model to explore potential mechanisms, our research group will further contribute to the limited understanding of this very severe cardiac manifestation exhibited in patients with FAODs like LCHADD.

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References

Burridge, P., Matsa, E., & Shukla, P. (2014). Chemically Defined and Small Molecule-Based Generation of. *Nature Methods*, 855-860.

Cecatto, C., Wajner, A., Vargas, C. R., Wajner, S. M., & Wajner, M. (2018). High vulnerability of the heart and liver to 3-hydroxypalmitic acid–induced disruption of mitochondrial functions in intact cell systems. *Journal of Cellular Biochemistry*, 7678-7686.

Corr PB, H. G. (1989). Mechanisms contributing to the arrhythmogenic influences of alpha 1-adrenergic stimulation in the ischemic heart. *American Journal of Medicine*, 19-25.

den Boer MEJ, W. R. (2002). Long-chain 3-hydroxyacyl-CoA dehydrogenase. *Pediatrics*, 99-104.

Frazier DM, M. D. (2006). The tandem mass spectrometry newborn screening experience in North Carolina. *Journal of Inherited Metabollic Disorders*, 76-85.

Garg, P., Garg, V., & Shrestha, R. (2018). Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes as Models for Cardiac Channelopathies . *Regenerative Cardiology Compendium*, 224-243.

Merritt, J. L., 2nd, Norris, M., & Kanungo, S. (2018). Fatty acid oxidation disorders. *Annals of translational medicine*, *6*(24), 473. https://doi.org/10.21037/atm.2018.10.57

MF, P., SD, L., L, G., CW, S., & KL, K. (2015). Human stem cell-derived cardiomyocytes in cellular impedance assays: bringing cardiotoxicity screening to the front line. *Cardiovascular Toxicology*, 127-139.

Miklas, J., Clark, E., Levy, S., & Detraux, D. (2019). TFPa/HADHA is required for fatty acid beta-oxidation and cardiolipin re-modeling in human cardiomyocytes. *Nature Communications*, 1-21.

Millipore Sigma. (2016). *Induced Pluripotent Stem Cell Differentiation Protocols.*

Spiekerkoetter, U., Lindner, M., Santer, R., Grotzke, M., Baumgartner, M. R., Boehles, H., Wendel, U. (2009). Management and outcome in 75 individuals with long-chain fatty acid oxidation defects: Results from a workshop. *Journal of Inherited Metabolic Disease*, 32, 488-497.

Ventura FV, R. J. (1195). Inhibition of oxidative phosphorylation by palmitoyl-CoA in digitonin permeabilized fibroblasts: implications for long-chain fatty aix beta-oxidation disorders. *Biochemical and Biophysics Acta*, 14-20.

Vockley J, B. M. (2017). Mitochondrial Fatty Acid Oxidation Disorders. *The Online Metabolic and Molecular Bases of Inherited Disease* .

Wilcken, B. (2010). Fatty acid oxidation disorders: Outcome and long-term prognosis. *Journal of Inherited Metabolic Disease*, 33, 501-506.