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# Mitigating Mitochondrial Dysfunction in the Insulin Resistant Phenotype: A Ketogenic Diet and Intermittent Hypoxia

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# MITIGATING MITOCHONDRIAL DYSFUNCTION IN THE INSULIN RESISTANT PHENOTYPE: A KETOGENIC DIET AND INTERMITTENT HYPOXIA

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## ABSTRACT

Chronic disease currently affects 45%, nearly half, of all persons in the United States (Raghupathi & Raghupathi, 2018). A systemic, cellular etiology of chronic disease has been proposed: literature supports the mechanism of a universal cell danger response (CDR) (Naviaux, 2019). The hallmark of the CDR, the shift in metabolism to anaerobic glycolysis, is initiated by altered functionality of the mitochondria (Naviaux, 2019).

Current research on mitochondrial dysfunction has demonstrated that a hypoxic intervention has successfully increased longevity in mice with mitochondrial dysfunction, but such an intervention has not, to our knowledge, been attempted in humans (Jain et al., 2016). If a hormetic intervention such as intermittent hypoxia were successful in humans, medical providers would need to be interested in and equipped to understand and implement the clinical intervention. In this study, a pre-medical instructor surveyed students following researchers' educational presentation, and found that students had increased confidence in understanding mitochondrial pathology in the CDR, and additionally were moderately to extremely interested in learning more about the topic in their graduate education (Table 1). Further studies are needed to determine whether the hypothesized interventions could have significant effect, and whether the virtual presentation style could be utilized to successfully educate medical students and professionals.

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# Chapter 1: Introduction

## Introduction

One hundred years ago, the top causes of death in the United States were pneumonia, tuberculosis, organic diseases of the heart, acute nephritis, cancer, and cerebral hemorrhage (Principle Causes of Death, 1920). Today, the top causes of death of persons in the United States are heart disease, malignant neoplasms, accidents, chronic lower respiratory diseases, cerebrovascular diseases, Alzheimer's disease and Diabetes Mellitus (Heron, 2019). It is notable that while all of the top causes of death 100 years ago were acute, many of the top causes of death today are non-acute, and arise from chronic etiologies. Chronic disease currently affects 45%, nearly half, of all persons in the United States (Raghupathi & Raghupathi, 2018). Physicians have traditionally focused on the management of acute illnesses; however, in 2018 physicians spent the majority of their time and effort managing chronic disease (Naviaux, 2019). The pathology of disease has been shifting in past decades, but medicine has not simultaneously adapted a successful medical model to address the rise in chronic disease.

> While it has been tempting to treat this rising tide in chronic disease by using the principles that have proven so successful in acute care medicine, a growing literature supports the conclusion that every chronic disease is actually a whole body disease- a systems problem- that cannot be solved using an old paradigm. (Naviaux, 2019)

A systemic, cellular etiology of chronic disease has been proposed: literature supports the mechanism of a universal cell danger response (CDR) (Naviaux, 2019). When in danger, due to pathogens, toxins, or other assaults, the cell moves through three distinct phases before returning to normal function (Naviaux, 2019). However, if the cell is being continually assaulted, or

maladaptively freezes in the CDR, chronic disease may develop as a result of cell function shift to energy conservation, preservation, promotion of inflammation, and pathogen defeat, at the expense of normal cell function (Naviaux, 2019). The hallmark of the transition into, and maintenance of, the CDR, is a shift in metabolism into anaerobic glycolysis. Unfortunately, immunometabolism demonstrates that the same chronic maladaptive metabolism that is created by the CDR increases cellular susceptibility to assault (Hotamisigil, 2017). In other words, the study of overlapping immune and metabolic pathways demonstrates that maladaptive metabolism increases risks for poor immune responses to other pathogens. Therefore, chronic disease development via sustained CDR can be initiated by either cellular immune activation generating maladaptive metabolism, or by maladaptive metabolic pathways decreasing immune function.

The observation that the chronic disease cycle of the CDR has multiple possible initial triggers begs the question: where in this cycle should a treatment be targeted? The hallmark of the CDR, the shift in metabolism to anaerobic glycolysis, is initiated by altered functionality of the mitochondria (Naviaux, 2019). Indeed, anaerobic glycolysis begins when the mitochondria shifts from the Krebs cycle to the production of ROS via reverse electron transport, in order to provide both quick energy and as a defense mechanism (Naviaux, 2019). The proposed pathophysiology in the CDR provides an explanation for the finding of mitochondrial dysfunction in many chronic diseases, such as Type 2 Diabetes Mellitus (T2DM) and Alzheimer's Disease, and suggests that mitochondrial dysfunction is present in many more diseases than are currently acknowledged (Sizer & Phillips, 2018). Could treatments aimed at returning the mitochondria to aerobic metabolism shift the cell out of the CDR and back to

healthy cellular function? If such treatment existed, it would be of immeasurable benefit in the battle against chronic disease.

**Background**

Recent studies of mitochondrial disorders have led researchers to look more closely at the different roles that mitochondria play in cellular function. One unique role is the regulation of the CDR. Like other immunological responses in the body, such as the adaptive immune system, the CDR is an effective deterrent to infection when it is utilized in short intervals. However, when the acute response becomes chronic, it can induce cellular damage and lead to systemic malfunction (Naviaux, 2019). Additionally, if the mitochondria themselves are injured during the CDR, the assault can freeze the cell in the CDR, creating a continuous vicious cycle which supports the development of chronic disease (Swerdlow et al., 2014).

**Problem Statement**

Type 2 Diabetes Mellitus is the most common example of a common disease in which mitochondrial dysfunction occurs, and is one of the top three most expensive health care costs in the United States (Sizer & Phillips, 2018) (Waters & Graf, 2018). In 2016, the United States spent $1.1 trillion, or 6% of the GDP on management of chronic disease (Waters & Graf, 2018). The majority of T2DM costs are due to pharmacologic medications and complications of disease (Economic Cost, 2018). As the cost of chronic disease is an increasing burden, there is a critical need for affordable interventions which could not only slow the progression of, but halt and reverse the development of T2DM altogether (Waters & Graf, 2018).

The concept of treatments targeting mitochondrial dysfunction as a means to treat T2DM is relatively novel. For example, one specific intervention, targeted hypoxia exposure, which has

been successful in improving longevity of mice with mitochondrial dysfunction, has not yet been attempted in humans (Jain et al., 2016). Additionally, another novel treatment for T2DM, a ketogenic diet, likely produces benefits via improving mitochondrial dysfunction, but this physiological process needs further detailed evaluation (Cox et al., 2019).

**Purpose**

The purpose of this study is to trial two interventions directed at reversing mitochondrial dysfunction occurring in chronic disease, specifically in T2DM. According to the CDR, alteration of pathologic mitochondrial function will result in a cell shift from pathologic metabolism back to healthy maintenance cell metabolism (naviaux, 2019). A healthy cell state has three characteristics: the mitochondrial utilization of oxygen for oxidative phosphorylation, the balance of mitochondrial fission and fusion, and the ability of the mitochondria to communicate with the nucleus via retrograde signaling (Sizer & Phillips, 2018) (Naviaux, 2019). The two interventions are designed to restore these three characteristics. Induced hypercapnia via hypoxia triggers retrograde signaling, which may restore healing of the cell. A ketogenic diet bypasses maladaptive glycolysis, increases oxidative phosphorylation, and induces epigenetic shifts which assist in cellular healing (Gibas, 2017; Vasudevan et al., 2003).

**Significance of the Study**

The impact of chronic disease on the United States population cannot be underestimated: currently, chronic disease plagues nearly half of the population and is a factor in seven of every ten deaths (Partnership to Fight Chronic Disease, 2007). Type 2 Diabetes Mellitus is among the most prevalent chronic diseases in the United States, affecting nearly one in ten citizens (*Statistics About Diabetes | ADA*, 2019). Even more alarming, by 2050, it is projected that one in

three persons will be affected (Boyle, Thompson, Gregg, Barker & Williamson, 2010). Persons with T2DM play a role in over 330,000 U.S. deaths each year (*Statistics About Diabetes | ADA*, 2019). Additionally, every 30 seconds, a limb is amputated as a result of T2DM (Partnership to Fight Chronic Disease, 2007). Chronic disease is also financially costly, both to the individual and to the larger economy. Chronic diseases account for 81% of hospital admissions and 76% of visits to a healthcare provider (Partnership to Fight Chronic Disease, 2007). Additionally, studies during the current COVI - 19 pandemic have demonstrated persons with T2DM are at increased risk of prevalence and mortality (Misra et al., 2020). Type 2 Diabets comes with a cost: loss of life, loss of quality of life, and loss of economic resources for the individual and for the U.S. economy.

Insulin injections are the current treatment for T2DM; injections are used to control blood glucose levels (Cefalu et al., 2018). Patients are dependent on insulin for control of the disease, yet there is concern for the affordability of insulin due to rising cost. Between 2002 and 2013, insulin prices jumped from $4.34 per milliliter to $12.92 per milliliter (Hua et al., 2016). Several factors contribute to increasing insulin prices. First, a limited number of companies produce insulin, which results in less competitive pricing (Cefalu et al., 2018). For example, without enough competition to check the company, Humalog prices increased by 138% from 2009 to 2015 (Langereth, Keller, & Cannon, 2016). Additionally, from 2001 to 2016, the Novolog Flex pen's listing price jumped 353% (Hobbs, 2016). A major concern of increasing medication cost is that it may be a hindrance to access for individuals: persons who are insulin dependent but not getting enough insulin are at high risk for serious adverse events, such as diabetic ketoacidosis (Emmett & Hirsch, 2021).

The cost of T2DM also affects the U.S. economy on a large scale; the U.S. spent $327 billion on Diabetes in 2017 (Economic Costs of Diabetes in the U.S. in 2017, 2018). Patients with T2DM cost an average of 2.3 times more than a patient without diabetes annually (*Economic Costs of Diabetes in the U.S. in 2017*, 2018). As the prevalence of diabetes increases by 1.5 million people per year, the parallel increases in cost must be considered (*Statistics About Diabetes | ADA*, 2019)(*Economic Costs of Diabetes in the U.S. in 2017*, 2018). One study found that from 2012 and 2017, the U.S. experienced a 26% increase in the cost of caring for diabetes (*Economic Costs of Diabetes in the U.S. in 2017*, 2018).

Of the 30.3 million U.S. citizens suffering from diabetes, only 4.1% have Type 1 Diabetes Mellitus (*Statistics About Diabetes | ADA*, 2019 ). Therefore, 95.9% of Diabetes cases may be avoidable, or may be improved by lifestyle interventions. The Center for Disease Control (CDC) estimates that changes could impact 80% of T2DM cases, via diet improvement, smoking cessation, and sedentary lifestyles alteration (Partnership to Fight Chronic Disease, 2007). The proposed study was conceived on a shared notion with the CDC: the best way to combat increasing prevalence and cost of diabetes is to stop development, halt progression, or even reverse the disease via interventions designed to treat the root cause of the disease. If the interventions proposed in this study prove to restart healthy metabolism, as evidenced by utilization of oxidative phosphorylation (OXPHOS) in the mitochondria, the interventions have the potential to provide persons with T2DM with an alternative treatment protocol, and to therefore reduce personal and economic strain associated with the disease.

**Research Questions**

1) Can the modulation of metabolic pathways via mitochondrial targeted interventions correct aberrant immune activity occurring in the CDR?
2) Can mitochondria be shifted from M1 back into M2 via hormetic interventions?
3) Is induced hypoxia/hypercapnia alone enough to bring diseased mitochondria back into a healthy state?
4) Is adherence to a ketogenic diet alone enough to bring diseased mitochondria back into a healthy state?
5) Will a combination of hypoxia/hypercapnic and ketogenic interventions be more effective than monotherapy?

**Limitations of the Study**

Limitations of this study include assumed adherence of the individuals involved to the assigned interventions. To address this limitation, education will be provided prior to and throughout the interventions. Participants in the hypoxia/hypoxemia masked group will be required to log their compliance. Compliance to the ketogenic diet will be determined via level of beta-hydroxybutyrate in the blood. Additionally, lifestyle changes made by the participants outside of the prescribed interventions have the potential to affect the results of the study. Finally, the study duration is 12 weeks. It may be that the interventions are capped before changes manifest, or that long term side effects are not determined.

Delimitations of the study include a population of only 30 participants. This cap was set to assure that each participant would receive adequate attention from the researchers in the form of maintenance appointments throughout the course of the study. Additionally, subjects have assumed mitochondrial deregulation in the form of T2DM. While this limits application to

persons with T2DM and mitochondrial dysfunction, it limits confounding variables, which would be present if multiple forms of mitochondrial dysfunction were evaluated.

**Definition of Terms**

AMPK- Activated protein kinase- A metabolic enzyme pathway which determines whetehr the cell will utilize fat or glucose to meet energy requirements (Winder, Holmes, Rubink, Jensen, Chen, Hollosz, 1985).

CDR- Cell Danger Response- An acutely adaptive state which the cell enters when it is threatened (Naviaux, 2019).

CDR1- Stage 1 of the Cell Danger Response (Naviaux, 2019).

Hormesis- The application of an element which is damaging at higher concentrations at lower concentrations in order to induce an adaptive response (Mattson, 2008).

HSP- Heat Shock Proteins- Part of a major chaperone family that help maintain mitochondrial function (Hu & Liu, 2011).

Metabokine- A Product of metabolism that acts both as an energy source and as signaling molecule (Naviaux, 2019).

OXPHOS- Oxidative phosphorylation- An a daptive system used by mitochondria to efficiently produce energy in the presence of oxygen.

ROS- Reactive Oxygen Species- Free radicals that cause damage within cells (*NCI Dictionary of Cancer Terms*, 2019).

SIRTS- Silent Information Regulators- Signaling molecules which protect the genome in order to increase cell survival in times of stress (Haigus & Sinclair, 2010).

T2DM- Type 2 Diabetes Mellitus.

NLRP3- Inflammatory signaling molecule (Jo, Kim, Shin & Sasakawa, 2015).

Warburg Effect- When a cell utilizes aerobic glycolysis to quickly produce ATP at the expense of efficiency (Liberti & Locasale, 2016).

**Conclusion**

This study explores possible preventative and curative interventions for mitochondrial dysfunction. Recent research has indicated that mice with dysfunctional mitochondria can experience improved function and significantly prolonged life expectancy (Mootha & Chinnery, 2018). The goals of this study are: to determine if the proposed interventions can successfully reverse mitochondrial dysfunction in humans, and to determine whether the interventions can be applied as a cost effective and minimally invasive manner. Chapter 2 will provide an overview of the literature pertaining to the pathophysiology of mitochondrial dysfunction, interventions for mitochondrial dysfunction, and the relationship between mitochondrial dysfunction and T2DM.

# Chapter 2: Literature Review

## Introduction

While the study of mitochondrial dysfunction originated with a focus on rare genetic disorders, the scope of study has broadened as research demonstrates the prevalence of mitochondrial dysfunction in common diseases of civilization: Type 2 Diabetes Mellitus (T2DM), Alzheiemer's disease, Cardiovascular disease and stroke (Sizer & Phillips, 2018). How could such seemingly disparate diseases be connected? Dr. Robert Naviaux, founder of the Mitochondrial and Metabolic Disease Center, has proposed the existence of a universal cellular healing cycle, which follows a common pathway in human cells (Naviaux, 2019). The state of the mitochondria is a major determinant of whether an injured cell heals or progresses to chronic disease (Naviaux, 2019). Fortunately, researchers such as Dr. Vamsi Mootha have been evaluating mitochondrial dysfunction for years, and have observed successful interventions for mitochondrial dysfunction in mice (Jain et al., 2017). Meanwhile, there is extensive research available on signaling molecules and pathways related to mitochondria and cellular healing. By understanding the mitochondria's role in chronic disease and cellular healing, we may design targeted interventions, which treat common diseases at the cellular level.

## Mitochondria, Immunometabolism & The Cell Danger Response

Many chronic diseases may be interconnected via concurrent mitochondrial dysfunction that disrupts the universal cellular healing cycle (Naviaux, 2019). Naviaux stated:

> Although the circular nature of healing seems obvious from daily experience with cuts, scrapes and the common cold, the extension of this notion to a unified theory to explain

> the pathophysiology of chronic complex disease has only recently become possible. Technological advancements in mass spectrometry and metabolomics have permitted the characterization of 4 discrete stages in the healing cycle. (2019, p. 280).

Naviaux (2019) suggests evidence to support both a normative health cell cycle and a Cell Danger Response (CDR). The CDR includes 4 metabolically and energetically unique stages, which are determined by observing mitochondria (Naviaux, 2019). An alteration in mitochondrial metabolism accompanies each stage of the CDR. In the healthy cycle, mitochondria are in the M2 form, and utilize oxygen for oxidative phosphorylation (OXPHOS). However, when the Cell Danger Response Stage 1 (CDR1) is triggered by a threat to homeostasis, mitochondria shift to the M1 form. In M1, mitochondria decrease ATP production by the Krebs Cycle, instead utilizing the Krebs intermediates for cellular repair, and utilizing the electron transport chain for ROS production (Naviaux, 2019). The decrease in oxidative phosphorylation in the mitochondria generates a shift to anaerobic glycolysis (Naviaux, 2019). Indeed, this phenomenon is also demonstrated in immunometabolism, and was first described as the crabtree effect (Hotamisigil, 2017). As a defense mechanism, rather than using oxygen for OXPHOS, oxygen is used to create oxylipin signaling molecules and reactive oxygen species (ROS). (Naviaux, 2019) (Gabbs, Leng, Devassy, Monirujjaman & Aukema, 2015). The glycolytic system operates under aerobic glycolysis, or the Warburg principle, and serves as a lactate reserve during immune activation (Gibas, 2017). This alteration in metabolism, triggered by an immune response, ultimately leads to a systemic inflammatory response via activation of the NLRP3 inflammasome, of type 1 interferons and the antiviral response, and is described by immunometabolic studies (Naviaux, 2019)

(Hotamisigil, 2017).

The function of CDR1 is the activation of innate immunity, intruder and toxin detection and removal, damage control, and containment (Naviaux, 2019). The innate immune system is activated by an increase in gap junctions, bacteria, virus, fungi, protozoa or toxins. *** Additionally, the innate immune response generates the release of extracellular adenosine triphosphate (ATP), which is is a metakokine: it serves a dual function as an energy source and as a metabolite, or signaling molecule. Metabolically, increases in extracellular ATP are another signal which promotes sustenance of the pro-inflammatory M1state (Naviaux, 2019). The CDR1 response functions optimally to address acute infections or injuries for 1-3 days (Naviaux, 2019). However, extended periods of the cellular danger response put the mitochondria at an increased risk for reactive oxygen species (ROS) induced mitochondrial DNA (mtDNA) damage. Likewise, CDR activation may become a self-perpetuating cycle, thereby freezing the cell in the CDR1 response (Swerdlow et al., 2014). Additionally, conditions of excess nutrition in the context of sluggish or depleted enzymes perpetuate dangerous hydroxyl free radical overproduction via the up-regulation of NADH and FADH2 electron donors with sluggish electron transport chains (Brownlee, 2005).

**Mitochondria and Cellular Healing**

Although the precise control of transitions between stages of CDR is not fully understood, it is known that the cross-talk between the nucleus and mitochondria of a cell plays a key role in whether the cell remains in CDR or transitions back to a healthy cycle (Naviaux, 2019). For cells to be restored back to healthy cycles, the mitochondrial dysrgulation must be addressed. Four specific mechanisms may aid in restoring the function of the mitochondria: an

increase in utilization of OXPHOS, a reduction in damage associated with ROS, enhancement of mitochondria-nucleus cross-talk, and regulation of mitochondrial fission and fusion (Naviuax, 2019; Hu & Liu, 2011; Brown, Murphy, Scott & Youle, 2010 ).

Healing from CDR1 is characterized by an overall increase in beta fatty acid oxidation, restoring aerobic respiration and maximum ATP synthesis (Nauviaux, 2019). The activated protein kinase (AMPK) pathway is a universal pathway, which regulates substrate utilization. The pathway is activated in states of ATP utilization, including fasting, calorie restriction (CR) and exercise, via the ATP by-products ADP and AMP (Tanag & Mauro, 2017). AMPK is a potent activator of OXPHOS. Specifically, AMPK's coactivator proliferator-activated receptor-γ-coactivator-1 (PGC-1 alpha), induces mitochondrial biogenesis and respiration (St-Pierre et al., 2003). In addition, AMPK activates succinic dehydrogenase (SDH) in the TCA cycle itself, which directly increases OXPHOS (Winder et al., 1985).

Silent Information Regulators (SIRTs) also induce OXPHOS, and are also activated by mild forms of hormesis, a mild stressor inducing a beneficial defense response in the cell via retrograde signaling (Mattson, 2008). Hormetic activators of SIRTs include calorie restriction (CR), increased temperature (37 degrees Celsuis) and nitrogen restriction (Haigas & Sinclair, 2010). Sirtuin 1 (SIRT1), Sirtuin 2 (SIRT2) and Sirtuin 3 (SIRT3), have been shown to induce transcriptional changes, which shift the cell away from glycolysis and increase OXPHOS in the mitochondria (Haigas & Sinclair, 2010). SIRT1 has the capability of switching mitochondrial substrate from glucose to fatty acid oxidation via regulation of the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1alpha) activity, which specifically triggers the transcription of genes regulating oxidative phosphorylation (Canto &

Auwerx, 2009). SIRT1 works in collaboration with AMPK: both SIRT1 and AMPK ultimately act on PGC-1alpha. In addition, SIRT2 is actively increased by up-regulation of the PNC1 gene, whichup-regulates via multiple forms of hormesis (Lin, Ford, Haigis, Liszt & Guarente, 2004).

SIRTS can also assist the cell in healing from ROS damage. Sirtuin 3 is a NAD+ dependent protein deacetylase that has the ability to regulate mitochondrial oxidative stress by decreasing reactive oxygen species (ROS) production (Ansari et al., 2017). In order to be activated, SIRT3 must travel to the mitochondria to be processed (Ansare et al., 2017) Once activated, SIRT3 can begin to regulate cellular metabolism by activating enzymes that help reduce reactive oxygen species (ROS) (Ansari et al., 2017). Reduction of ROS is crucial to ending the assault of damage to mitochondria so that healing can be initiated. Additionally, studies have shown that a decrease in SIRT3 is specifically associated with the pathogenesis of diabetes and obesity, due to SIRT3's ability to restore mitochondrial function, decrease ROS production,oxidative stress, and increase insulin sensitivity (Jing et al., 2011).

Heat shock proteins are molecular chaperones, which allow communication from the mitochondria directly to the nucleus in what is referred to as retrograde signaling (Hu & Liu, 2011). Heat shock proteins are activated in the cellular stress response, and once activated, are cytoprotective (Zeng, Tan Lu, Lu & Hu, 2013). For example, the HSP alphaB-crystallin is protective against oxidative cell damage (Zeng et al., 2013). During oxidative conditions, alpha-B crystallin specifically protects cytochrome c from damage; its overexpression during oxidative stress is key to preserving the mitochondrial membrane potential (Zeng et al., 2013). Heat shock proteins such as alpha-B crystallin have the ability to protect the mitochondria from oxidative damage common to metabolic pathologies including T2DM (Zeng et al., 2013). Heat

shock proteins are suggested to play a key role in immunometabolic restoration of the healthy M2 cycle via retrograde signaling (Zeng et al., 2013). In review: HSPs, SIRTs, and AMPK are activated by hormesis, and activate mitochondrial fuel partitioning by fluxing substrate away from aerobic glycolysis and toward OXPHOS, thereby transitioning cells out of a danger response to restore the health cycle.

In addition to the upregulation of OXPHOS, activation of SIRTs and AMPK increases mitochondrial fusion to aid mitophagy (Naviux, 2019). Mitochondrial fusion is the process of two independent mitochondria joining, and excising parts, which are not functioning optimally (Brown, Murphy, Scott & Youle, 2010). Fission is the division of a mitochondria into two daughter mitochondria (Brown, Murphy, Scott & Youle, 2010). Fusion and fission must be in balance for appropriate mitochondrial morphology; balance of these processes requires tight regulation (Brown, Murphy, Scott & Youle, 2010). Without regulation via hormetic induction, a decrease in fusion with accelerated fission results in mitochondrial fragmentation, which is associated with degenerative diseases, T2DM and obesity (Rovira-Llopis, Banuls, Diaz-Morales, Hernadez-Mijares, Rocha & Victor, 2017).

**Mitochondria, Metabolism and Oxygen Utilization**

A recent study by Harvard professor and mitochondrial expert, Dr. Vamsi Mootha, revealed an intriguing scenario in patients with mitochondrial dysfunction: during exercise, patients with mitochondrial dysfunction showed a significant deficit in oxygen extraction from the blood when compared to controls (Delaney, Sharma, Tadvalkar, Clish, Haller & Mootha, 2017). Decreased oxygen extraction from the blood occurred in spite of equal cardiac output (Cheng et al., 2017). One proposed mechanism for the altered oxidative capacity in

mitochondrial disorders is impaired TCA flux (Delaney et al., 2017). The TCA cycle is fed by Acetyl-CoA (Figure 1a). Glycolysis produces Acetyl CoA via the irreversible conversion of Pyruvate to Acetyl-CoA by the Pyruvate Dehydrogenase Complex (PDC) (Figure 1b). In anaerobic conditions pyruvate shunts to lactate via Lactate Dehydrogenase (LDH-A), thus conserving the systemic pool of pyruvate (Figure 1c). Under oxygen deficient conditions, such as intense or prolonged exercise, lactate accumulates due to increases in anaerobic glycolysis. However, in healthy individuals, lactate levels decline when exercise ceases; healthy controls quickly flux back to oxidative respiration for the generation of maximum ATP (Delaney et al., 2017). However, in cells with mitochondrial dysfunction, lactate remains elevated at rest, indicating the cells are using aerobic glycolysis for ATP generation, even in the presence of oxygen; the aforementioned dysregulated cells express a CDR1 driven Warburg phenomenon, suggesting an epigenetic, phenotypic shift in cellular respiration away from OXPHOS and toward aerobic glycolysis (Naiaux, 2019).

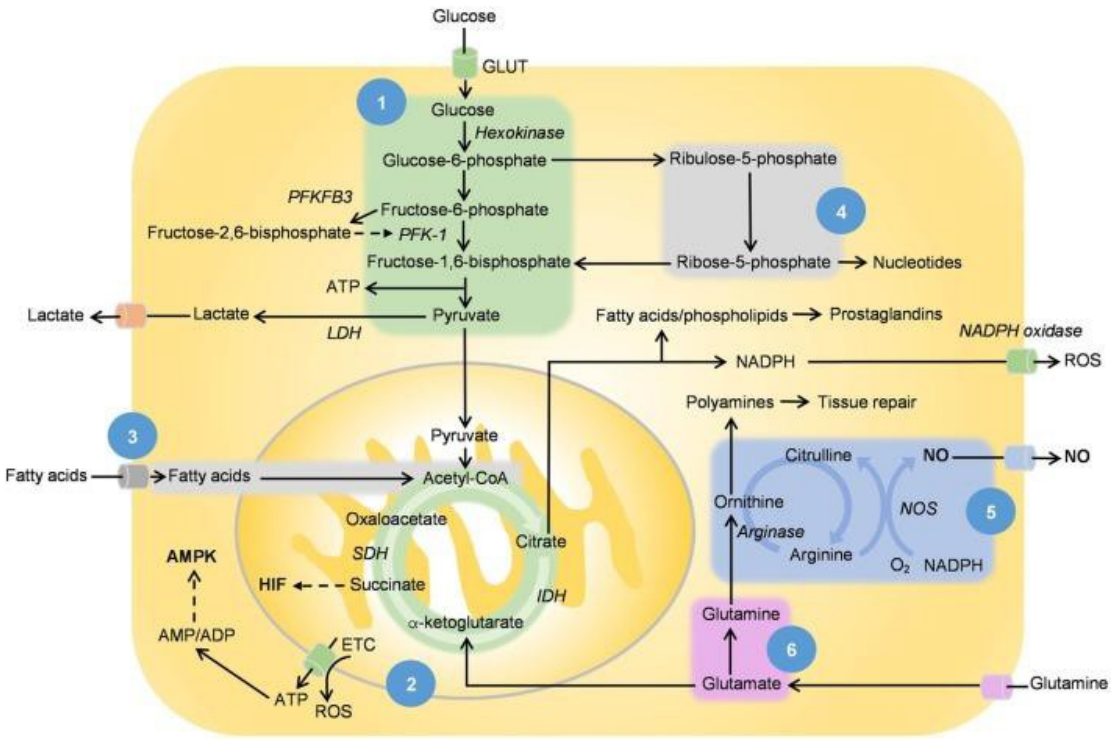


Figure 1. The TCA Cycle, Glycolysis and activation of AMPK. a) The TCA cycle is fed by Acetyl-CoA. b) The conversion of pyruvate to Acetyl-CoA is irreversible. c) In anaerobic conditions, LDH converts pyruvate to lactate. d) Fatty acids bypass glycolysis and enter the TCA cycle (Tanag & Mauro, 2017).

In 1929, Herbert Crabtree discovered a similar effect in tumour cells: despite the presence of oxygen, high concentrations of glucose caused cells to generate ATP via aerobic glycolysis rather than OXPHOS (Crabtree, 1929). He coined this effect the Crabtree Effect. One hypothesis explained the CrabTree Effect as a time demand for ATP:

> While the ATP yield is the amount of ATP produced per unit of substrate, the rate of ATP production is the amount of ATP produced per unit of time. A trade-off between ATP rate and yield means that ATP can either be produced fast (i.e., at high rate and low yield) or efficiently (i.e., at low rate and high yield) (Pfeiffer & Schuster, 2005).

It is possible that cells with mitochondrial dysfunction are choosing an evolutionarily similar option; as a part of the CDR1, sustained glucose elevation processed by anaerobic glycolysis generate ATP quickly but not efficiently. Meanwhile, TCA intermediates are utilized for cellular repair rather than OXPHOS. Pathology ensues when cells fail to return to their healthy, oxidative cycle (Naviaux, 2019). Cells are not equipped to maintain ATP generation via glycolysis for extended periods; in healthy cells, the utilization of glycolysis along with OXPHOS produces an excess of NAD+, a cofactor for SIRT activation, which induces increased OXPHOS and -Delta G or a favorable, spontaneous state (Naviuax, 2019; Haigas & Sinclair, 2010). However, in extended aerobic glycolysis, inefficient generation of both ATP and NAD+ due to inhibition of OXPHOS increases cellular dependency on glucose, while increasing the ATP/ADP ratio to sustain an unfavorable cellular state (Naviaux, 2019). This predisposes the patient to dysregulations in systemic glucose homeostasis common to insulin resistance and T2DM (Hu & Liu, 2011). In the case of yeast cells, when the Crabtree Effect occurs, oxygen utilization plateaus, or declines; this is the same phenomenon that Mootha observed in mammalian mitochondrial dysfunction (Delaney, Sharma, Tadvalkar, Clish, Haller & Mootha, 2017) (Jouhten et al., 2008).

There appears to be two physiological mechanisms by which oxygen utilization and efficiency may decline due to the reduced flux of OXPHOS. Mitochondrial utilization of the OXPHOS pathway serves as an “oxygen sink” pulling oxygen down a concentration gradient to the fourth protein chamber of the electron transport chain. In chamber IV, $O_2$ becomes the final electron acceptor as it reduces to water e (Mootha, 2019). Simultaneously, OXPHOS generates $CO_2$, which diffuses into the blood causing the Bohr shift,to displace $O_2$ from

Oxyhemoglobin allowing it to diffuse into the cells. If the rate of OXPHOS is decreased, cells lose the pull of the "oxygen sink," while at the same time inhibit the shift to deoxyhemoglobin, resulting in decreased diffusion of oxygen and tissue oxygenation with sustained PO2 saturation (Figure 2) (Mootha, 2019).

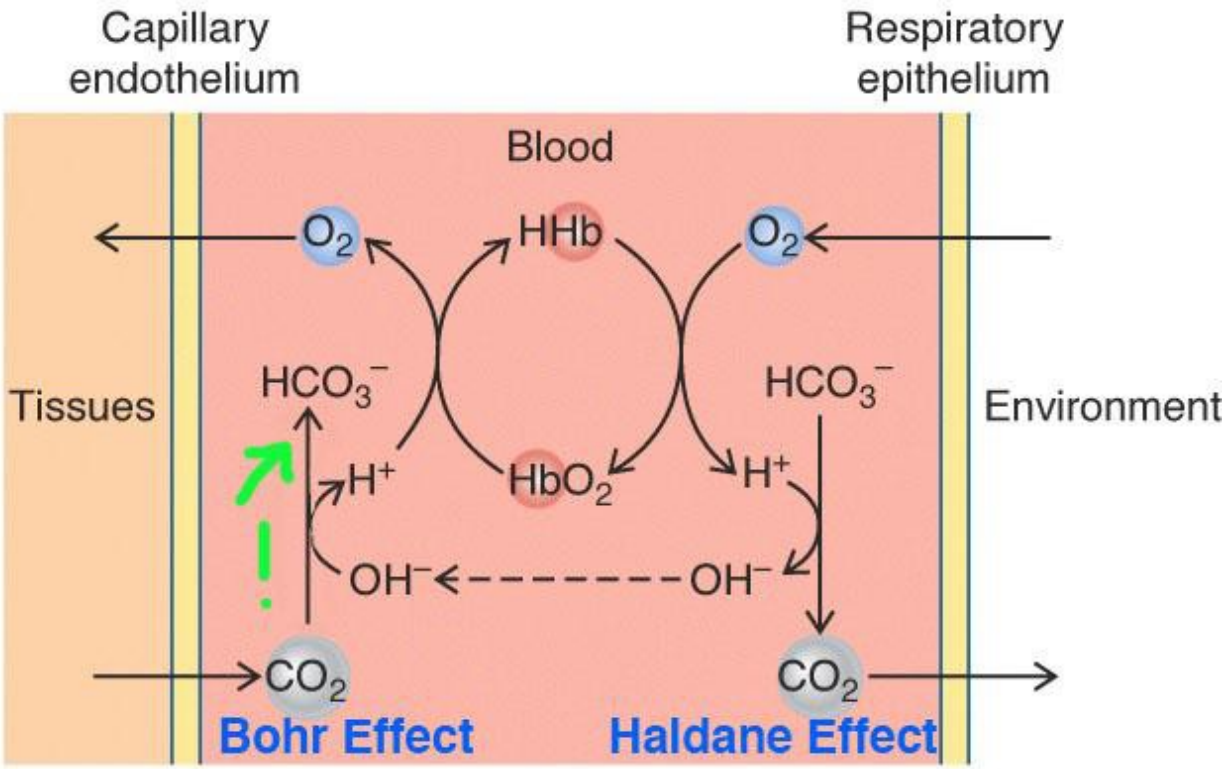


Figure 2. Bohr Effect (USMLEFastTrack, 2013).Two significant lab values in patients with mitochondrial deregulation support the hypothesis of decreased OXPHOS in the mitochondrial matrix: elevated lactate and elevated long chain triacylglycerols (LCTG) (Delaney et al., 2017). Patients with mitochondrial dysfunction show significantly increased levels of lactate at rest (Delaney et al, 2017). A decrease in oxygen consumption coupled with increased lactate production is synonymous with conditions of CDR1 (Naviaux, 2019). "With M1 polarization, energy-coupled mitochondrial oxygen consumption drops, and cellular

energy production switches to glycolysis and lactate production" (Naviaux, 2019, p. 282). While the polarization of M2 to M1 occurring in CDR1 is adaptive for acute infection or injury, chronic M1 response becomes maladaptively destructive when the restoration of the M2 stage is hindered (Naviaux, 2019. The cell remains stranded in the CDR1. Mitochondrial dysfunction in chronic disease states reflects the phenomena of M1 mitochondria stuck in CDR1: oxygen consumption is decreased via oxylipin signaling molecules as cellular respiration is shunted into anaerobic glycolysis; the mitochondria switch from utilizing oxygen for OXPHOS to over-producing ROS (Naviaux, 2019). An additional abnormal lab value in mitochondrial dysfunction is the elevation of specific long chain triglycerides (LCTG). In Mootha's study, the resting long-chain triacylglycerols (LCTG) correlated with the severity of impairment of OXPHOS as determined by the PO2 levels in the blood (Delaney et al, 2017). The inverse correlation between LCTG and OXPHOSwassuggested as a novel tool to determine severity of mitochondrial deregulation (Delaney et al., 2017). Fatty acid oxidation is dependent on the utilization of oxygen in OXPHOS. If the capability of the mitochondria to perform OXPHOS is hindered or epigenetically down-regulated, the ability to oxidize fatty acids will also be hindered. A decrease in utilization could ultimately lead to elevated levels of blood LCTG in patients with mitochondrial dysfunction.

**Mitochondria and Type 2 Diabetes Mellitus**

Dr. Vamsi Mootha, Professor of Systems Based Biology and Medicine at Harvard, identified a decrease in respiratory chain activity in the mitochondria ofpatients with T2DM (Mootha, 2019). It is notable that lactate and lactate dehydrogenase A (LDH-A) are commonly elevated in T2DM, as they are in mitochondrial disorders, indicating impaired flux

of the TCA cycle and reduced oxidative respiration (Crawford et al., 2010). T2DM may induce mitochondrial dysfunction, or mitochondrial dysfunction may exacerbated T2DM (Crawford et al., 2010). As stated above, conditions of excess nutrition, such as T2DM, have been shown to trigger ROS-overproduction via the up-regulation of oxylipin molecules (Brownlee, 2005). T2DM is associated with highly fragmented mitochondria and mtDNA damage (Fetterman et al., 2016). Deficient mitochondrial mitophagy has been associated with decreased responsiveness to insulin and feedback inhibition in pancreatic beta cells (Sizer & Phillips, 2018). In addition, ER stress has been shown to induce pancreatic beta cell apoptosis (Sizer & Phillips, 2018). As T2DM induces mitochondrial dysfunction via decreases in the respiratory chain, and mitochondrial dysfunction induces ROS, which interfere with the insulin response, it is likely that the genesis of disease begins with either T2DM or with mitochondrial deregulation; the original disease state induces the second disease state, leading to a vicious cycle.

**Proposed Study Interventions**

This study intends to explore the therapeutic potential of two interventions on patients with mitochondrial dysfunction in the form of T2DM. To coax the dysfunctional mitochondria out of the maladaptive CDR1 state and reactivate a dormant TCA cycle, one group of patients will receive a hormetic application of CO2 administered in the form of a hypoxia inducing mask and another group will abide by a ketogenic diet (KD). A third group will partake in both interventions.

The inspiration for the hypoxia inducing mask stems from the 2019 article *Oxygen in mitochondrial disease: Can there be too much of a good thing?* by Vamsi K. Mootha and Patrick

F. Chinnery. The researchers addressed the mishandling of oxygen levels in patients suffering from mitochondrial dysfunction; namely, patients with mitochondrial dysfunction may be over administered oxygen treatment (Mootha & Chinnery, 2018). A recent meta-analysis found 50-84% of acutely ill patients became hypoxemic in emergency settings after oxygen was supplied by providers (Chu et al., 2018). Even for patients without mitochondrialdysfunction, the repercussions of hyperoxemia were dire (Chu et al., 2018). “Vasoconstriction, inflammation, and oxidative stress” were inflicted on the pulmonary, cardiovascular, and neurological systems” (Chu et al., 2019, p. 1702). Ultimately, the meta-analysis determined “liberal oxygen therapy” corresponded with raised mortality rates (Chu et al., 2019). Therefore, Dr. Mootha and Dr. Chinnery developed a counterintuitive treatment to mediate oxygen abnormalities in persons with mitochondrial dysfunction; he tested the treatment on mice (2018).

In situations where patients with mitochondrial dysfunction are treated for hypoxia or lactic acidosis, Mootha and Chinnery side with the United Mitochondrial Disease Foundation (UMDF), suggesting that administering oxygen to patients with mitochondrial dysfunction risks inflicting further damage from ROS (Mootha & Chinnery, 2018). While addressing the implementation of Hyperbaric Oxygen Therapy for mitochondrial diseases, the UMDF in their 2010 newsletter stated “it is contra-intuitive to believe that putting more oxygen into this system would be helpful” because these patients are inherently unable to “reduce molecular oxygen” (United Mitochondrial Disease Foundation, 2010, p. 2). A 2016 study took mice with the mitochondrial disease Leigh syndrome and exposed them to 21% oxygen (ambient), 11% oxygen (simulated 4500 m above sea level), and 55% oxygen for a hyperoxic state (Jain et al., 2016). Ambient air meant poor development and an early death (median 60 days) for the affected mice.

However, at 11% oxygen,, the lifespanof the mice wasextended,up to 170 days and many diseases were avoided. Conversely, mice subjected to 55% oxygen died within 11 days of entering hyperoxia (Jain et al., 2016). Researchers in the Leigh mouse study hypothesized that by limiting the amount of oxygen arriving in the deregulatedmitochondria causes less ROS to form and the organism is spared from toxicity (Jain et al. 2016). Researchers in the Leigh mouse study also hypothesized that improvements in mice exposed to hypoxia may be connected to the activation of an "evolutionarily conserved adaptive program that allows mammals to cope with limited oxygen levels… [which] decreases an organism's reliance on oxidative metabolism" (Jain et al., 2016, p. 60). The presence or absence of oxygen is the key difference in this adaptive anaerobic glycolysis versus aerobic glycolysis, which occurs in mitochondrial disease (Mootha & Chinnery, 2018). Researchers in this study believe that mitochondrial diseases stem from a mismatch in oxygen delivery and oxygen utilization (Mootha & Chinnery, 2018). The mitochondria are not getting hypoxic signals as they would in anaerobic conditions, and therefore mitochondria do not signal the nucleus to induce epigenetic adaptive changes (Mootha & Chinnery, 2018). Inducing hypoxia, as occurs in elevation or during anaerobic exercise, may induce the retrograde signaling needed to initiate adaptive changes and healing (Mootha & Chinnery, 2018).

To deduce if similar benefits can be found in humans with mitochondrial dysfunction, oxygen deprivation masks will simulate a hypoxic state. Like Leigh's syndrome, Type 2 Diabetes was identified as another disease of"quantitative decline in the activity of the mitochondrial [respiratory chain]" (Jain et al., 2016, p. 54). To assess the impact of the induced hypoxic

therapy, several values will be collected before and after intervention to reveal whether reducing oxygen curbed ROS accumulation and relieved oxidative stress in the patients.

The second intervention consists of adherence to a KD. Ketones induce cellular healing via three powerful mechanisms: ketones upregulate OXPHOS while bypassing glycolysis; ketones induce mitochondrial signaling molecules; and ketones epigenetically regulate the genome by acting as histone deacetylase inhibitors (HDACi) (Gibas, 2017; Elamin, Ruskin, Masino & Sacchetti, 2018; Vasudevan et al., 2003). Ketonesassist in cellular healing via exerting a metabolic effect; ketones limit glycolysis while up-regulating OXPHOS (Gibas, 2017). Ketone bodies and fatty acids directly enter the TCA cycle thereby bypassing glycolysis (Figure 1d). Therefore, the mitochondria is forced to utilize OXPHOS to generate ATP. Cessation of glycolysis is a potent first step to inhibiting the CDR. In addition, a ketogenic diet has been shown to increase cellular insulin sensitivity in T2DM by reducing blood insulin levels and sensitizing the tyrosine kinase insulin receptors (Cox, Gibas, Salisbury, Gomer & Gibas, 2019).

In the mitochondria, ketones are a potent activation of SIRTs, via up-regulation of the necessary cofactor NAD+ (Elamin, Ruskin, Masino & Sacchetti, 2018). As previously discussed, SIRTS 1, 2 and 3 exert a cytoprotective and healing affect. Studies have shown that a decrease in SIRT3 is specifically associated with the pathogenesis of diabetes and obesity, due to SIRT3's ability to restore mitochondrial function, decrease ROS production, oxidative stress, and increase insulin sensitivity (Jing et al., 2011).

Finally, ketones regulate cellular healing at the level of the genome (Vasudevan eet al., 2003). Histone acetylation is an important epigenetic modulator of the genome; acetylation of lysine at the base of chromaffin influences whether genes are accessed for transcription (Marks,

Richon, Miller & Kelly, 2004). Normal cell growth requires a balance of histone acetylation and deacetylation (Marks, Richon, Miller & Kelly, 2004).Overactive histone deacetylation has been associated with maladaptive regulation of the cell cycle; maladaptive regulation of the cell cycle is a part of the CDR present in chronic diseases like T2DM (Vasudevan et al., 2003; Naviux et al., 2004; Rovira-Llopis et al., 2017). Ketones have been shown to act as potent HDACi (Vasudevan et al., 2003). Therefore, via inhibition of HDAC, ketones may restore the balance of acetylation and deacetylation allowing the cell to return to a state of adaptive regulation.

**Proposed Study Measurements**

In order to measure the effects of the interventions, several metabolites will be considered as well as the patient's Metabolic Equivalent of Task (MET). One MET, considered a standard metabolic rate at rest, is equivalent to a VO2 of 3.5 mL/kg/min (Taivassalo, 2003). Measuring oxygen consumption during rest will illuminate whether a patient is adaptively utilizing aerobic respiration via OXPHOS or pathologically using glycolysis for energy in a resting state (Naviaux, 2019). Patients with mitochondrial myopathies have been shown to express low VO2max measurements, some scarcely above 1 MET (Taivassalo, 2003). Recording the participants MET at rest pre and post intervention will reveal whether the mitochondrion have returned to the adaptive M2 state, which utilizes oxygen for OXPHOS.

Several metabolites will be measured pre and post intervention. Mitochondrial dysfunction hidden at rest can be exposed under the stress of exercise in the form of aerobic capacity and metabolic measurement (Delaney et al., 2017). As "the classic marker of mitochondrial dysfunction," serum lactate levels will be monitored in the T2DM patients (Delaney et al., 2017, p. 8403). In T2DM, the impairment of OXPHOS results in reliance on

glycolysis for energy production (Ishitobi et al., 2019). This anaerobic process produces lactate, which is known to be elevated in patients with T2DM (Ishitobi et al., 2019). The metabolic profiling of lactate as a biomarker of disease, and for mitochondrial disease in particular, has been employed by Delaney and colleagues, in their study of McArdle Disease and Mitochondrial Myopathy (Delaney et al., 2017). Delaney et al (2017) relied on serum lactate to gain insight on metabolic pathophysiology of the diseases and measure the efficacy of their exercise interventions (Delaney et al., 2017). When applied to the case of T2DM, lactate levels will reveal thedegree of OXPHOS inhibition(Delaney et al., 2017).

In addition to lactate, measurements of the enzyme Lactate Dehydrogenase (LDH) will also be recorded pre and post intervention. In an anaerobic state, LDH-A converts pyruvate to lactate (Liang et al., 2016). In an adaptive individual, that same anaerobic state, likely exercise, prompts PGC-1 alpha to upregulate the *ldhb* gene, which codes for LDH-B (Liang et al., 2016). This enzyme is responsible for converting lactate back to pyruvate once oxygen is available for OXPHOS (Liang et al., 2016). LDH-B has been identified as a biomarker of glucose oxidation, making it a useful marker to evaluate a patient's utilization of OXPHOS for ATP production (Liang et al., 2016). Increased LDH-A expression can be attributed to extended periods of hypoxia. Increased expression of LDH-A leads to increased lactate levels, which drive down blood pH resulting in lactic acidosis (Liang et al., 2016). This type of maladaptation has also been found in cancer and is associated with poor cancer survival rates (Liang et al., 2016). In T2DM, the underlying mitochondrial deregulation drives the cell toward reliance on glycolysis and the synthesis of lactate;this is reinforced by hypoxia. Measuring LDH-A and LDH-B before

and after intervention will provide further data regardingthe metabolic alterations (Liang et al., 2016).

Elevated serum triglycerides are observed in patients with mitochondrial dysfunction and have been associated withimpaired fatty acid beta oxidation (Clarke et al., 2013). Recently, serum triglyceride levels have been shown to correlate more closely with the severity of mitochondrial disease than the more common biomarker, lactate, which is used to identifythe presence of disease (Delaney et al., 2017). Elevated triglycerides correlate with a maladaptive VO2 difference at peak exercise. If this finding can be reinforced, it may both help negate the need for intense VO2max testing on patients with mitochondrial disease and provide a simple method to determine the severity of mitochondrial deregulation (Delaney et al., 2017).

Hemoglobin A1c (HbA1c) will ultimately serve as the Gold Standard value indicating if the T2DM patient has restoredtheir mitochondrion and insulin sensitivity. If the proposed interventions cause the mitochondrion to facilitatehealthy mitophagy, the increased insulin sensitivity will be reflected in the decreasing levels ofcirculating glycated hemoglobin. Measuring participants' HbA1c provides an average forblood glucose levels over the previous 3 month period (van Raalten et al., 2019).

**Conclusion**

In review, the key to healing mitochondrial deregulation may lie in the ability of the mitochondria to transition away from the CDR1 M1 state and return to the healthy M2 state (Naviaux, 2019). TheM2 state is characterized by the utilization of oxygen for OXPHOS, healing of ROS damage, balancing of fission and fusion, and the ability of the mitochondria to communicate with the nucleus via retrograde signaling (Naviuax, 2019; Brown, Murphy, Scott &

Youle, 2010; Hu & Liu, 2011). The two aforementioned interventions were designed to restore these characteristics. Induced hypercapnia mimics exercise and may increase oxygen availability for OXPHOS (Jain et al., 2016). Induced hypoxia mimics the effects of hypoxia at altitude or intense exercise, and may initiate the mitochondria to induce retrograde signaling to the nucleus forepair and biogenesis (Zeng et al., 2013; Haigas & Sinclair, 2010). Ketone bodies demand the utilization of OXPHOS, activate SIRTs, and induce epigenetic alterations, which favor cellular healing of the genome (Gibas, 2017; Elamin, Ruskin, Masino & Sacchetti, 2018; Vasudevan et al., 2003). Chapter 3 will introduce the study design including methodology with a description of the interventions, and details about the attainment, recording and analysis of variables.

# Chapter 3: Methodology

## Introduction

The purpose of this study is to examine whether mitochondrial dysfunction in the insulin resistant phenotype can be mitigated via lifestyle modifications focused on improving cellular oxygen uptake and utilization. Lifestyle modifications include a clinically prescribed KD, intermittently induced hypoxia with hypercapnic rebreathing, or a combination of the two aforementioned interventions. This study specifically aims to answer the following questions:

1. Can the modulation of metabolic pathways via mitochondrial targeted interventions correct aberrant immune activity occurring in the CDR?
2. Can mitochondria be shifted from M1 back into M2 via hormetic interventions?
3. Is induced hypoxia/hypercapnia alone enough to bring diseased mitochondria back into a healthy state?
4. Is adherence to a ketogenic diet alone enough to bring diseased mitochondria back into a healthy state?
5. Will a combination of hypoxia/hypercapnic and ketogenic interventions be more effective than monotherapy?

This chapter describes the population evaluated, study duration and design, protocol, equipment and methodology utilized to collect data, validity and reliability, statistical analysis and limitations.

## Sample Population

Participants will be recruited via referral from their Optometrist and via informative fliers (Appendix A) that will be distributed at participating optometry clinics. Persons who express

interest in the study will be encouraged to contact research investigators by phone or email, and at that time will be provided with a copy of the informed consent (Appendix B). Participants will be adults (18 years of age or older) previously diagnosed with T2DM or prediabetes, defined by HgA1c measurements of > 6.5% and 5.7% - 6.4%, respectively. Exclusion criteria include a diagnosis of a restrictive or obstructive pulmonary disorder, or failure to gain medical clearance from a primary care physician.

**Study Design**

A true experimental design will be used to determine the mitochondrial dysfunction. Twenty to thirty participants will be randomly assigned to one of three interventions for 10 weeks: a clinically prescribed ketogenic diet (KD); 70 minutes/week of respiratory training device (RTD) utilization to induce low grade hypoxia with hypercapnic rebreathing (Porcari et al., 2016); or a combination of the aforementioned KD and RTD protocol. A KD has normalized HgA1c in patients with T2DM, and hypoxic conditions have significantly increased the lifespan of mice with mitochondrial dysfunction (Cox et al., 2019)(Jain et al., 2016).Therefor, the interventions of a KD and RTD use will serve as independent variables.

Mitochondrial dysfunction has been associated with T2DM and other common chronic diseases (Sizer & Phillips, 2018). Mitochondrial impairment is evidenced by an accumulation of elevated long chain triglycerides, elevated serum LDH and decreased arterial oxygen uptake/utilization both at rest and during exercise (Delaney et al., 2017). In chronic disease, the shift of cellular respiration away from fatty acid beta-oxidation in the matrix of the mitochondria and toward glucose dependent aerobic glycolysis, with concomitant elevations in oxidative stress, is clinically characterized by an accumulation of blood triglycerides and a decreased

uptake/utilization of arterial oxygen (Naviaux, 2018)(Delaney et al., 2017). Therefore, the dependent variables will include participants' pre-intervention and post-intervention POC triglycerides, lactate dehydrogenase (LDH), HgA1c, fasting insulin, fasting blood ketones, body composition and gas exchange data (VO2/VCO2, MET and RMR).

**Protocol**

The KD intervention group will adhere to a clinically prescribed ketogenic diet, which will be bioindividually designed by licensed Functional Medicine practitioners (CFMP) at *Bristlecone Health, Inc.,* based on the participants' lean mas, body fat percentage, and HgA1c (Gibas, 2019). Adherence to intervention protocol will be determined by at home, POC measurements of blood ketones (> .5 mmol/l), taken by Abbott Labs Precision Xtra Ketone Meters supplied to the participants for the 10 week study. Ketone measurements will be recorded in a HIPAA compliant Google Document shared with the research investigators and saved using a non-identifiable numerical system. The RTD only intervention group will use the Expand-a-lung Breathing Device for 10 minutes daily; the Expand-a-lung devices will be supplied to participants. Adherence will be measured via daily, self-reported logs of RTD times in a HIPAA compliant Google Document shared with the research investigators and saved using a non-identifiable numerical system. The *KD and RTD group* will adhere to the aforementioned protocol for the KD outlined above and utilize the RTD as previously described; adherence for this group will be measured using the same HIPAA compliant format previously outlined. The keys to the numerical systems for all interventions will be kept on the investigators private, locked computers; only members of the research team will have access to the files.

Pre-intervention (week 0) and post intervention (week 10) labs, including serum LDH, fasting insulin, and HgA1c, will be collected via venous draw obtained at North Memorial Lab in Maple Grove, MN. Serum LDH will also be measured via venous blood draw at North Memorial Labs on weeks 0, 6 and 10. At the initiation of the study (week 0), all participants will attend a 30-minute, intervention-specific educational session delivered by the investigators, designed to teach participants how to adhere to their designated intervention(s), and to educate participants on the physiology of their assigned intervention. Participants will be instructed how to log their data weekly (Appendix D). For all groups, on weeks 0 and 10, participants will meet investigators at the Bethel University Biokinetics lab to obtain the following measurements: gas exchange Resting Metabolic Assessment (VO2/VCO2, MET and RMR) as measured by Indirect Calorimetry using a ventilated hood system with Metabolic Cart (Medgraphic CR Diagnostics Ultima Series Metabolic System), body composition analysis via Bioelectrical Impedance Analysis (BIA) and POC triglycerides via fingerstick. On weeks 2/4/6/8, participants will meet with investigators at Bristlecone Health, Inc. or Bethel University for brief, 30-minute check-in sessions, which will include: a short educational review of the physiology with time for questions/answers about the intervention(s), POC assessment of blood ketones via Abbott Precision Xtra meters, and calculation of body composition analysis via BIA. On weeks 1/3/5/7/9, participants will do a brief check-in session with investigators via Telehealth's HIPAA compliant *Secure Video* platform. These real time, interactive videoconferences will be hosted on Bristlecone Health Inc.'s contracted secure account with Telehealth and consist of a short educational lesson/review with time for questions/answers about the assigned intervention(s). Subjects will receive an invitation email from the researchers, select "Yes" if they wish to

confirm their intentions to attend the scheduled session, and select “Join Session” when the meeting time arrives. To log out, they will select “End Meeting.” Secure Video boasts a Business Associate Agreement with the account holder, encrypted signaling, media stream, administration, and an encrypted database protected by Bitlocker. Additionally, role based security ensures access to data is permitted only to the researchers involved in the study. The investigators will be available for questions via email throughout the duration of the study, with communicated understanding of a 48 hour response time.

**Data Collection**

Specific instruments integral to the execution of the study include: the Expand-a-Lung Respiratory Training Device (RTD); Medgraphic CR Diagnostics Ultima Series indirect calorimetry and Metabolic Cart System; Bioelectrical Impedance Analysis (BIA); Abbott Labs Precision Xtra Ketone/Glucose meter; CardioCheck point of care (POC) fingerstick blood assessment device by pts Diagnostics; Google Documents; and the services of North Memorial Lab in Maple Grove, MN for the venous measurement of the fasting lipid panels and LDH.

A ventilated hood system (Medgraphic CR Diagnostics Ultima Series Metabolic System) will be utilized to obtain Resting Metabolic Assessment (VO2/VCO2, MET and RMR). Investigators will calibrate the metabolic system prior to participant arrival. Participants will meet the investigator at the Biokinetics Laboratory, and will be lead to a private, dimly lit and quiet room. The participant will receive a mouth mask and be connected to the ventilating system. The participant will be asked to relax for 25 minutes while sitting in supine position in a reclined chair. The participant will be asked to breathe as normally as possible, avoid fidgeting,

and let the investigator know if they begin to feel discomfort. After 10 minutes, the machine will begin recording oxygen consumption. Data will be collected for 30 minutes.

**Validity and Reliability**

Employing a KD to mitigate mitochondrial dysfunction has been shown to reverse T2DM via epigenetic modulation of the genome; in fact, recent studies suggest defects in mitochondrial respiration may be driven by nutrient excess and energy oversupply common to T2DM, but most important, these abnormalities are proving to be reversible (Sergi et al., 2019). The intermittent induction of hypoxia with hypercapnic rebreathing has been shown to be hormetic for mice with mitochondrial dysfunction in studies conducted by Harvard Medical School Professor, Dr. Vamsi Mootha (Jain et al., 2016). Measurement and analysis of gas exchange (inspired VO2/expired VCO2) at rest via indirect calorimetry is an accepted proxy for mitochondrial efficiency; the production of chemical energy (ATP) via the proton gradient flowing into the mitochondrial matrix is proportional to gas exchange (Taivassalo, 2003). Serum lactate levels preside as the "classic marker of mitochondrial dysfunction" (Delaney et al., 2017, p. 8403). Landmark research by Pere Llinàs-Arias et al., (2019) suggests elevated lactate, as measured by serum LDH, is a predictive biomarker for phenotypic reversion of cellular respiration away from OXPHOS (oxidative phosphorylation) toward glucose dependent, aerobic glycolysis. Likewise, elevated fasting triglycerides are a reliable marker of disturbed, fatty acid beta oxidation and HbA1c is the clinical Gold Standard for the diagnosis of diabetes (T1DM/T2DM) and pre-diabetes (Clarke et al., 2013)(van Raalten et al., 2019).

**Confidentiality**

To maintain HIPAA compliance, measures will be taken to secure the documented information of the participants. Bethel University, Saint Paul, Minnesota will provide a locked file to protect participant records. A HIPAA Google Document, accessible only to participants and investigators, will hold the self-reported participant logs. Participants will be assigned a random numerical identity under which they will post their log entries. During data collection and analysis, the key to these identities, along with informed consent and clinical data measures, will be secured on password-protected computers owned by the researchers: Bethany K. Tangen, William C. Grillo, Joshua W. Pinson, Christopher K. Carroll M.Ed., PhD., Kelly J. Gibas LPCC, CFMP, DBH and Julie A. Gomer LPCC, CFMP, DHB. The collected data will be reported as averages instead of individual identifiers. On completion of study results, any data on paper will be shredded by a confidential shredding service affiliated with Bethel University. After completion of the study, the data will be kept on an external storage device locked in the PA program office for a minimum of five years, per securing requirements for Bethel University's Physician Assistant Program. (IRB approval- added later, after approval)

**Statistical Analysis**

SPSS Statistical Analysis Software will be utilized to perform statistical analysis. Significant changes in the pre-intervention and post-intervention dependent variables, including POC triglycerides, lactate dehydrogenase (LDH), HgA1c, fasting insulin, fasting blood ketones, body composition and gas exchange data (VO2/VCO2, MET and RMR), will determined by a paired-samples t test analysis. Further exploratory analysis evaluating the above mentioned variables will be objectively evaluated using analyses of variance (ANOVA). Descriptive

statistics and p values, percent difference between groups will be calculated to allow comparison with published literature.

**Limitations/Delimitations**

Documentation of participant adherence to the three interventions is dependent on participant compliance and integrity. As 24-hour surveillance is impractical, the following steps will be taken to best ensure the individuals adhere to the protocol. The participants will receive a 30-minute educational session prior to beginning the study. Every other week, participants will attend a 30-minute group appointment, modeled after the Cleveland Clinic's Shared Medical Appointment (SMA) format, with an investigator to reiterate protocol, answer questions, and address concerns. Between face-to-face meetings, participants will have Virtual Appointments with investigators to answer questions and address concerns. Daily, participants will log their adherence to the assigned intervention on a secured Google Document shared with investigators. Please see Appendix E for details. Additionally, investigators will available for questions via email throughout the study. The daily Google Document adherence logs in conjunction with email contact with investigators will be crucial to ensuring participant integrity. The dependence on technology for logging, however, may be an obstacle for participants who are not familiar with the programs needed to communicate, or perhaps cannot afford the equipment required to access these programs. The logged ketone measurements will indicate whether the participant is adhering to the prescribed KD. Obstacles to adherence of the KD include personal taste, expenses assumed with the diet adjustment, and other dietary constraints like food allergies or special nutritional demands. Clearance by a primary care physician is required for participant safety. Adherence to the RTD will also be logged daily, and records are also contingent on the

participant's integrity; there is no POC measurement able to confirm compliance, and participants will be responsible for timing their duration of usage. Obstacles to adherence of use of the RTD may include claustrophobia, as the sense of inhibited breathing may generate anxiety. Participants will be instructed what to do if they experience discomfort; please refer to Appendix E. As the interventions will be randomly assigned, comfort with a particular intervention cannot be considered upon assignment. It is notable that the voluntary nature of the experiment and the hope of potential health benefits of the interventions may predispose the participants to initiate other healthy habits simultaneously. While lifestyle changes outside of the assigned interventions could skew results independently from the intervention, they cannot be controlled for. It is possible that 10 weeks of the interventions is too short of an amount of time for significant results to be observed. Certain measurements of this study require the participant to have their finger pricked. In the cases of ketone measurements and POC finger prick assessments, communication between investigator and participant will be key for maintenance of sterile technique to avoid infection and any discomfort potentially experienced by the participant. Additionally, serum LDH does not exclusively respond to shifts in mitochondrial functionality. Blood conditions, cancers, ischemia, trauma to muscle tissue, and liver disease also may elevated serum LDH levels ("Lactate dehydrogenase test | Allina Health", 2020)

Delimitations of this study include the capping of participants at 30 and using MET as a proxy for OXPHOS. The population of 30 was set to ensure each participant received adequate attention from the limited amount of investigators, of which there are three. Participants must meet specific criteria to qualify, such as being prediabetic or having T2DM while being void of

obstructive or restrictive pulmonary disorders. MET will be used to assess oxygen consumption, rather than VO2max, as attaining a MET via RMR is less strenuous on the participant.

**Conclusion**

In summary, 20-30 participants will be randomly assigned to one of three intervention groups: a clinically prescribed KD, utilization of a RTD 70 minutes weekly, or a combination of KD and RTD protocol. Compliance will be documented via intervention-specific, self-reported logs in a HIPPA compliant Google Document. Participants will meet with investigators via group appointment or Virtual Appointment on alternating weeks. Measurements, including, body composition analysis, POC ketones, gas exchange data (VO2/VCO2, MET and RMR), POC triglycerides, LDH, fasting insulin and HgA1c, will be obtained on weeks 0 and 10, with LDH also measured on week 6. Results of the study and statistical analysis will be presented in Chapter 4, with a discussion of the results to follow in Chapter 5.

# Chapter 4: Results

## Alterations

The methodology above was proposed and IRB approved in January of 2020 (Appendix C). However, following approval, a pandemic caused by the virus SARS-CoV-2 led to the implementation of social distancing and limitations on human interactions. Due to elimination of nonessential human contact, the proposed methodology was no longer feasible. Therefore, the researchers altered the methodology of the study.

Rather than performing a clinical intervention based on Dr. Vamsi Mootha's findings, researchers designed an educational presentation proposing the physiological methods behind Dr. Vamsi Mootha's findings. The researchers were graduate level students, who had not had prior exposure to the majority of physiology which allowed for understanding and application of the referenced studies, most significantly in regards to the CDR. Therefore, researchers were curious whether pre-medical students would be both interested in and able to comprehend basic pathophysiology of mitochondrial metabolism and the CDR. The purpose of the presentation was to educate undergraduate students on the physiology of mitochondrial metabolism and the pathophysiology of the CDR.

Additionally, the instructor of the pre-medical students' course utilized a pre and post survey to evaluate the effectiveness of the presentation. Specifically, the survey evaluated whether and to what extent the presentation was comprehended, and whether and to what extent the students were interested in the content presented.

**Presentation**

Presenters delivered an 85 minute interactive Zoom presentation to the Advanced Lab Students of the Biokinetics Department at Bethel University in Saint Paul, MN. The presenters included Physician Assistant students Bethany Tangen, William Grillo, and Joshua Pinson. Permission was obtained from Biokinetic professor Dr. Kelly Gibas. The presentation included a review of cellular and mitochondrial metabolism, an overview of the pertinent literature, and a proposed physiological explanation for the findings of Dr. Vamsi Mootha. Physiologic review included: the Warburg Effect, the Bohr Effect, and The Cell Danger Response. Literature Review included: The Cell Danger Response; Metabolic Profiles of Exercise in Patients with McArdle Disease or Mitochondrial Myopathy; Hypoxia as a Therapy for Mitochondrial Disease (Naviaux, 2019) (Delaney et al., 2017) (Jain et al., 2016). Before the presentation and following the presentation, a five question survey was sent via email to all participants (Appendix F).

**Survey**

The survey consisted of six questions which were utilized by the instructor to evaluate pre-medical students' confidence in their comprehension of the material presented, confidence in interpretation of current literature, and interest in learning more about the materials in the future (Appendix F). One survey was emailed by a researcher to each pre-medical student pre and post presentation. Responses were gathered anonymously by a google form, to which only the three researchers had access. No identifying information was gathered. Students were informed that their participation was voluntary. Students were not given incentive to participate, and were informed that their grades would not be affected by their responses or participation.

**Findings**

Pre and post survey responses were averaged (Table 1). After participating in the presentation, students' confidence in comprehending the pathophysiology of mitochondrial dysfunction, comprehending research regarding the cell danger response, and applying the research findings, increased by 2.4 - 3.5 points. Additionally, students became more interested in learning about mitochondrial dysfunction in their post baccalaureate programs, increasing from an interest level of 7.6/10 to 9.2/10. Finally, students expressed a 9.6/10 likelihood of allowing cellular pathology research to impact their future practice.

| **Survey Question** | **Pre Presentation Average** | **Post Presentation Average** |
|---|---|---|
| 1.How confident are you with the pathophysiology of mitochondrial dysfunction in the cell danger response? | 5.4 | 8.4 |
| 2.How confident are you in comprehending research regarding the cell danger response? | 5.6 | 8 |
| 3.How confident are you in applying research findings regarding the cell danger response? | 4.9 | 8.4 |
| 4.How interested would you be in learning more about cellular pathology in your post baccalaureate program? | 7.6 | 9.2 |
| 5.How likely are you to allow current research on cellular pathology to impact your practice or profession? | 9 | 9.6 |

Table 1. Survey Responses

# Chapter 5: Discussion

## Introduction

The current epidemic of metabolic disease, as evidenced by the alarming ever increasing prevalence of diseases such as Type 2 Diabetes Mellitus, will continue to be an immense challenge not only to the persons affected, but to the nation's economy and to the healthcare system (Boyle, Thompson, Gregg, Barker & Williamson, 2010). Current treatment protocols can delay the many complications of Type 2 Diabetes Mellitus, but are not curative, and additionally come with compliance and expense issues (Cefalu et al., 2018). In order to decrease the burden on the healthcare system, economy, and individual persons, researchers must continue to explore treatment options which aim to cure, not only delay, the disease process. For treatment to have an effect on the population at large, the option must also be accessible and affordable. In addition, in order to evaluate and implement interventions, providers must demonstrate understanding of both the disease pathophysiology and of relevant research.

## Summary of Results

This study demonstrated that following one presentation, pre-medical student felt increased confidence in understanding of both mitochondrial pathophysiology in the CDR and current research publications regarding the CDR (Table 1). Post presentation, each pre-medical responded that they would be moderately to extremely interested in learning more about cellular pathology in their post-baccalaureate program (Table 1).

## Limitations

Though students were instructed that their responses were anonymous and would not impact their grades, responses could still be biased. Pre-medical students may have responded

more favorably to survey questions under the perception of judgement by the PA student researchers. Pre-medical students may also have exaggerated interest in order to generate favor with the PA student researchers. The findings in this survey were utilized by the instructor to gauge students confidence and interest in the material, and cannot be utilized to gauge actual knowledge regarding the topics presented.

**Further Research**

Further research is needed to evaluate the specific effectiveness of increasing knowledge regarding mitochondrial pathology and the CDR via a single virtual lecture. Additionally, further research could be conducted in graduate level students themselves. Further research could also be conducted in providers, in order to evaluate the impact of the presentation on clinical practice.

**Conclusion**

This study demonstrated that, following one presentation, pre-medical students experienced increased confidence in comprehension and interest in mitochondrial pathology and the CDR. The presentation in this study may serve as a template of a virtual education model both for educational material for graduate level students and for current providers. Such a presentation, which is both easily accessible and captures the interest of medical personnel, will be necessary if the initial hypothesis should prove to be correct. After further research on the presentation is completed, the model of presentation in this study may serve adjunctively to study results to allow for the results to be implemented in clinical practice.

The initial methodology proposed in the Methodology section, previous to the SARS-CoV-2 pandemic, can still be carried to fruition by future researchers. If the hypothesis is demonstrated to be correct, providers will have confirmation of effectiveness for a cost-effective,

at-home intervention for mitochondrial disease. The combination of this intervention and the aforementioned educational presentation could be a powerful duo approach to equipping healthcare providers to fight back against the epidemic of metabolic disease.

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## Appendix A- Recruitment Flier

BETHEL UNIVERSITY

# MITIGATING MITOCHONDRIAL DYSFUNCTION IN THE INSULIN RESISTANT PHENOTYPE: A KETOGENIC DIET AND INTERMITTENT HYPOXIA

## What will you do?

**A 10 Week Study:**

Group 1- Low carb diet

Group 2- Respiratory Training Device- worn for a short period of time daily

Group 3- Low carb diet + Respiratory Training Device

## What's this about?

Research has demonstrated that a low carb diet can reverse Type 2 Diabetes.

Research has demonstrated that altering oxygen levels has increased lifespan in mammals with mitochondrial dysfunction.

**Space is Limited!**
**Call Us Today:**
**218-242-0818**

## Why join?

*Complementary Diabetes education from graduate level Physician Assistant Students.*

*Complementary Labs*!

*Free, individual interpretation of your metabolic equivalent rate*!

## Appendix B- Informed Consent

# BETHEL UNIVERSITY PHYSICIAN ASSISTANT PROGRAM
# For MINIMAL RISK Medical Human Subject Research

***Principle Investigators:*** Bethany K. Tangen, William C. Grillo & Joshua W. Pinson, Department of Physician Assistant Studies
***Organization:*** Bethel University Physician Assistant Graduate Program
***Protocol Director:*** Kelly Gibas LPCC, CFMP, DBH
***Proposal Name:*** *Mitigating Mitochondrial Dysfunction in The Insulin Resistant Phenotype: Nutritional Ketosis and Intermittent Hypoxia*

We are students at Bethel University in the Physician Assistant Graduate Program. In collaboration with Dr. Kelly Gibas at Bethel University, we are conducting a research study to evaluate the effects of a ketogenic diet and/or the daily utilization of a respiratory training device (RTD) on metabolic health in Type 2 Diabetics or pre-diabetics as measured by the following physiological biomarkers: triglycerides, lactate dehydrogenase (LDH) hemoglobin A1c (HgA1c), fasting insulin, fasting lipid panel, gas exchange data from a Resting Metabolic Assessment.

We are inviting your participation, which will involve the collection of the following data over the next 10 weeks: *point of care triglycerides, lactate dehydrogenase (LDH) and fasting lipid panel (via venous draw @ North Memorial lab in Maple Grove, MN), hemoglobin A1c (HgA1c), fasting insulin, Resting Metabolic Assessment.* The study involves completing one of three randomly prescribed interventions: a sustained 10-week ketogenic diet (KD), the daily use of a Respiratory Training Device, or a combination of KD and RTD. If you choose to participate in the study, you will be randomly assigned to one of the aforementioned groups. The previously described clinical measures will be assessed in each participant over the 10 weeks regardless of group selection.

Approximately 30 adults will participate in the study. If you choose not to participate or to withdraw from the study at any time, there will be no penalty. Your participation in this study is completely voluntary.

**********

**DESCRIPTION:** You are invited to participate in a research study on the clinical implications of a ketogenic diet or intermittently induced hypoxia/hypercapnia using a respiratory training device (RTD) or a ketogenic diet together with intermittently induced hypoxia/hypercapnia using the RTD. All procedures are evidence-based and non-experimental. You will be randomly assigned to either the ketogenic diet only, the Respiratory Training Device only, or a ketogenic diet and the Respiratory Training Device. During the course of the study, you will participate in the following biomarker measurements at week 0 and week 10: *Point of Care triglycerides, hemoglobin A1c (HgA1c) and Resting Metabolic Assessment (RMR, MET & VO2/VCO2) data.* You will be asked to visit North Memorial Lab in Maple Grove, MN for

venous blood draws to measure lactate dehydrogenase (LDH) and fasting lipid panel prior to weeks 0, 6 and 10.

You will also be asked to meet with investigators at *Bristlecone Health, Inc.*, Maple Grove, MN or *The Anderson Center of Bethel University*, Saint Paul, Minnesota 6 times throughout the course of the study on weeks 0, 2, 4, 6, 8 and 10 to obtain the aforementioned clinical measures and receive biological education on your assigned intervention. Appointments will be in small groups and will last for approximately 30 minutes. You will need to arrive at each appointment in a fasted state (a minimum of 4 hours hours). Investigators will be available on weekends and weekdays for your convenience. Below are the sites for the clinical measurements and meetings:

1. *Bristlecone Health, Inc.,*
   *13700 Reimer Drive N., Ste. 220*
   *Maple Grove, MN 55311; (763) 424-2474; www.bristleconefitness.com*

2. *The Anderson Center of Bethel University*
   *2 Pine Tree Drive*
   *Arden Hills, MN 55112*

**RISKS AND BENEFITS:** There are no foreseeable risks associated with this study. No costs are associated with participation, nor will your decision to participate affect your employment/medical care or insurance status. Discomfort associated with study participation may include short-term general malaise commonly associated with dietary changes and the reduction of dietary carbohydrates. If you are experiencing discomfort at any time throughout the study, please contact us at (218) 452-0818 or *Bristlecone Health, Inc.* @ 763-913-4600 for dietary counsel and consideration.

The benefits which may reasonably be expected to result from this study are clinical reductions in fasting triglycerides, HgA1c and LDH values with increased oxygen utilization and caloric consumption as measured by gas exchange via the Resting Metabolic Assessment. *We cannot and do not guarantee or promise you will receive any specific benefits from this study.*

**TIME INVOLVEMENT:** Your participation in this study will require you to record either one or two numerical values daily: blood ketones and/or the log of your daily usage of the Respiratory Training Device. In person, small group appointments will occur at one of the two addresses listed above, on weeks 2/4/6/8, and will last approximately 30 minutes per session. Individual, virtual appointments will occur on weeks 1/3/5/7/9 and will last approximately 20 minutes. The initial and last visits (weeks 0/10) will be approximately 45 minutes due to Resting Metabolic Assessments. This is a total of 5 hours and 10 minutes over a duration of 10 weeks. You will also be responsible for obtaining the aforementioned lab draws prior to week 0/6/10 at North Memorial Lab in Maple Grove, MN.

**PAYMENTS:** There are no costs associated with participating in this study.

**PARTICIPANT'S RIGHTS:** If you have read this form and decide to participate in this study, please understand your participation is voluntary and you have the right to withdraw your consent or discontinue participation at any time without penalty or loss of benefits to which you are otherwise entitled. If the decision is made to withdraw from the study, please provide a written request for withdrawal and mail to: *Bethany Tangen, 13700 Reimer Drive N., Ste. 220, Maple Grove, MN 55311*.

**CONFIDENTIALITY:** Your research data will be used for the purposes of this study only. Copies of your medical and clinical measures will be stored in a locked file at Bethel University and will only be accessed by the previously named investigators and research staff. If you decide to withdraw from the study, your confidential medical data and clinical measures will be destroyed within 24 hours of receipt of the written request to withdraw from the study.

Data collection and reporting for the purposes of this study will remain confidential and your identity will not be identifiable. Upon completion of the study, you have 8 weeks to obtain a copy of your clinical data measures before they will be destroyed as per confidentiality mandates.

**SIGNIFICANT RESEARCH FINDINGS:** Significant new findings developed during the course of research may occur, due to your willingness to continue participation. Any significant new findings will be provided to you.

**PARTICIPATION GUIDELINES:** There are circumstances that may arise under which your participation may be terminated without your consent.

- *Failure to follow the instructions of the protocol director and study staff*
- *The protocol director decides that your continued participation could be harmful to you*
- *Pregnancy*
- *You require treatment not allowed in the study*
- *The study is cancelled*
- *Other administrative reasons*
- *Unanticipated circumstances*

Please consult with your primary health care provider before embarking on this or any dietary change. Should you have any questions/concerns or you experience a research related injury, please contact the Bethel University IRB board at (651) 638-6901.

*FOR QUESTIONS ABOUT THE STUDY, ANSWERS TO PERTINENT QUESTIONS ABOUT THE RESEARCH, PARTICIPANT RIGHTS, OR RESEARCH-RELATED PROBLEMS, CONTACT: Bethany K Tangen @* (218) 452-0818

*A copy of this consent form is available upon request.*

# Authorization To Use Your Health Information For Research Purposes

Because information about you and your health is personal and private, it generally cannot be used in research studies without your written authorization. If you sign this form, it will provide the needed authorization. The form is intended to inform you regarding how your health information will be used or disclosed in the study. Your information will only be used in accordance with this authorization form and the informed consent form as required or allowed by law. *Please read carefully before signing.*

**What is the purpose of this research study and how will my health information be utilized in the study?**

The purpose of this research study is to measure the clinical effects of a ketogenic diet and/or hypoxic/hypercapnic breathing measures of metabolic health including: triglycerides, lactate dehydrogenase (LDH), hemoglobin A1c (HgA1c) and gas exchange via Resting Metabolic Assessment in patients with Type 2 Diabetes Mellitus or prediabetes as previously diagnosed by their primary care physician and determined by HgA1c.

**Do I have to sign this authorization form?**

You do not have to sign this authorization form. But if you do not sign the form, you will not be able to participate in this research study.

**If I sign, can I revoke it or withdraw from the research later?**

If you decide to participate, you are free to withdraw your authorization regarding the use and disclosure of your health information (and to discontinue participation in the study) at any time. After any revocation, your health information will no longer be used or disclosed in the study. If you wish to revoke your authorization for research use or disclosure of your health information in this study, you must provide it in writing to: *Bethany K. Tangen, 13700 Reimer Drive N., Ste. 220, Maple Grove, MN 55311.*

**What Personal Information Will Be Obtained, Used or Disclosed?**

Your health information related to this study, may be used or disclosed in connection with this research study, including, but not limited to, age, gender, date of birth, lab values: fasting triglycerides, lactate dehydrogenase (LDH), HgA1c (hemoglobin A1c), fasting insulin, Resting Metabolic Assessment data.

**Who May Use or Disclose the Information?**

The following parties are authorized to use and/or disclose your health information in connection with this research study:

- The Protocol Director: Kelly Gibas LPCC, CFMP, DBH, Bethel University
- Principle Researchers:
    - Bethany K. Tangen, Bethel University Physician Assistant Program
    - William C. Grillo, Bethel University Physician Assistant Program
    - Joshua W. Pinson, Bethel University Physician Assistant Program
- Research Staff:

- Wallace Boeve, EdD, PA-C; Program Director, Physician Assistant Program
  Bethel University
- Julie A. Gomer LPCC, CFMP, DBH; Bristlecone Health, Inc.

**When will my authorization expire?**
Your authorization for the use and/or disclosure of your health information will end on August 1, 2020 or when the research project ends, whichever is earlier.

**Will access to my medical record be limited during the study?**
To maintain the integrity of this research study, you may not have access to any health information developed as part of this study until the study is completed.

_____ _____
***Signature of Adult Participant*** *Date*

_____ _____
***Signature of Legally Authorized Representative (LAR)*** *Date*
*(e.g., parent, guardian or conservator)*
*Relationship to Participant:* _____

_____ _____
***Signature of Investigator(s)*** *Date*
*(Bethany K. Tangen, William C. Grillo or Joshua W. Pinson)*

_____ _____
***Signature of Witness*** *Date*

## Appendix C- IRB approval

February 17, 2020

Bethany Tangen

Bethel University

St. Paul, MN 55112

Re: Project FA-29-19 Mitigating Mitochondrial Dysfunction in the Insulin Resistant Phenotype: Nutritional Ketosis and Intermittent Hypoxia

Dear Bethany,

On February 17, 2020, the Bethel University Institutional Review Board completed the review of your proposed study and approved the above referenced study. One condition is to add a statement on page 5 of your Informed Consent Form that "deciding not to participate or withdrawing at any time will not affect your relationship with Bethel University." Most informed consent forms are one to two pages in length while your informed consent form has a lot of esoteric words and is five pages in length. I mention this in case you and your team choose to shorten your informed consent form.

Please note that this approval is limited to the project as described on the most recent Human Subjects Review Form documentation, including email correspondence. Please be reminded that it is the responsibility of the investigator(s) to bring to the attention of the IRB Committee any proposed changes in the project or activity plans, and to report to the IRB Committee any unanticipated problems that may affect the welfare of human subjects. The approval is valid until February 17, 2021.

Sincerely,

Craig Paulson, Ph.D.

Chairperson, Bethel University Level One IRB Committee

## Appendix D- Instructions for Logging Data

**Intervention Group 1: Ketogenic Diet Only**

You are being supplied with an Abbott Precision Xtra Meter, lancets, and ketone test strips. Please use the skills taught in the educational session to do a point of care fingerstick measurement between 4-8PM daily, before you eat your dinner meal. Record the measurement daily in the Google Document provided for you at the educational session. Your name will not be assigned to the Google Document; rather you will be assigned a number in order to protect your privacy. Only the investigators of the study will have access to the Google Document. If you have any questions or concerns please email bethany-tangen@bethel.edu. Please expect a 24 hour response.

**Intervention Group 2: Respiratory Training Device Only**

_____You are being supplied with an Expand-a-lung Breather Respiratory Training Device. Please use the skills taught in the educational session to utilize the Respiratory Training Device appropriately, for 10 minutes daily. It is normal to feel uncomfortable when starting use of the Respiratory Rraining Device; the discomfort arises from retraining of your breathing, and will decrease and resolve with regular use. Although it is very unlikely to experience side effects, if you believe the discomfort is escalating, you may terminate the RTD session. Please record the amount of time which you used the breather daily in the Google Document provided for you at the educational session. Your name will not be assigned to the Google Document; rather you will be assigned a number in order to protect your privacy. Only the investigators of the study will have access to the Google Document. If you have any questions or concerns please email bethany-tangen@bethel.edu. Please expect a 24 hour response.

**Intervention Group 3: Ketogenic Diet and Respiratory Training Device**

You are being supplied with an Abbott Precision Xtra Meter, lancets, and ketone test strips. Please use the skills taught in the educational session to do a point of care fingerstick measurement between 4-8PM daily, before you eat your dinner meal. You are also being supplied with an Expand-a-lung Breather Respiratory Training Device. Please use the skills taught in the educational session to utilize the Respiratory Training Device appropriately, for 10 minutes daily. It is normal to feel uncomfortable when starting use of the Respiratory Training Device; the discomfort arises from retraining of your breathing, and will decrease and resolve with regular use. Although it is very unlikely to experience side effects, if you believe the discomfort is escalating, you may terminate the RTD session. Each day, please record both the amount of time for which you used the breather that day, and your daily ketone measurement, in the Google Document provided for you at the educational session. Your name will not be assigned to the Google Document; rather you will be assigned a number in order to protect your privacy. Only the investigators of the study will have access to the Google Document. If you have any questions or concerns please email bethany-tangen@bethel.edu. Please expect a 24 hour response.

## Appendix E- Agreement to Work with Organizations

Bethany Tangen <bethany-tangen@bethel.edu>

# Bristlecone Health permission waiver

1 message

**Julie Gomer** <jgomer@bristleconemedical.com> Tue, Jan 7, 2020 at 12:31 PM To: Bethany Tangen <bethany-tangen@bethel.edu>

On behalf of Bristlecone Health, Inc., I give permission for Bethel University Physician Assistant students Bethany K Tangen, William C Grillo and Joshua W. Pinson, to utilize the Bristlecone Healthy Inc. clinic and resources to conduct their Master's Research study.

Sincerely,

***Dr. Julie A. Gomer, CFMP, LPCC***

BRISTLECONE MEDICAL, INC.

***Doctor of Clinical Behavior Sciences Certified Functional Medicine Practitioner Licensed Professional Clinical Counselor***13700 Reimer Drive North

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## Appendix F - Survey

**Survey**

Please respond to the following on a scale of 1-10, with 1 being none and 10 being extreme:

1. How confident are you with the pathophysiology of mitochondrial dysfunction in the cell danger response?
2. Has learning cellular pathology enhanced your understanding of disease?
3. How confident are you in comprehending research regarding the cell danger response?
4. How confident are you in applying research findings regarding the cell danger response?
5. How interested would you be in learning more about cellular pathology in your post baccalaureate program?
6. How likely are you to allow current research on cellular pathology to impact your practice or profession?