# Cardiac Mitochondrial Adaptations Induced by Elevated Lipid Exposure.

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## Abstract

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Apolipoprotein E (ApoE) is necessary for normal lipid metabolism. A deficiency of this protein impedes plasma clearance of both triglycerides and cholesterol. These lipids are consequently broken down through alternate pathways, thus resulting in atypical cellular function. Cardiac mitochondria play a significant role in cellular respiration specifically, considering the extent of oxygen consumption that is required by the heart muscle. Their capacity to utilize oxygen and produce energy may be affected by the buildup of cholesterol and triglycerides, due to a deficiency in ApoE. This study will investigate the influence of ApoE deficiency on energy metabolism in young and older female mice. Whether or not any of these effects are exacerbated by further supplementation of lipids through diet will also be evaluated in male mice. Cardiac mitochondrial oxygen consumption, mitochondrial oxidative phosphorylation (OXPHOS) capacity and protein expression in cardiomyocytes will help in examining these influences. Our findings have the potential to provide important information regarding lipid shunting into non-oxidative pathways, which can ultimately disrupt normal cellular function leading to damaging effects of the heart.



**Keywords**: Apolipoprotein E, mitochondria, cardiac, oxidative capacity, bioenergetics, metabolism

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# Introduction

## Theoretical Context

### *Apolipoprotein E*

Cardiovascular disease (CVD) is and has been the primary cause of death in the United States since 1918 [American Heart Association, 2005]. It is well established that CVD is also a leading universal public health concern as it lies at the root of serious illness, disability and of course death. Central to this condition is chronic inflammation whereby oxidized lipoproteins are deposited on the vascular walls [Ballinger et al., 2002]. Lipoproteins including Apolipoprotein E (ApoE) for example, are soluble molecules that enable the transport of lipids in the plasma. ApoE in particular, is a multifunctional lipoprotein, as it is an important component in the structure of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) [Mahley, 1988]. It also participates in the uptake of triglyceride-rich lipoproteins (TRL) by the liver and has, as such, an ability to suppress the development of atherosclerosis [Raffai, 2012; Lahoz et al., 2001]. ApoEs role in atherosclerotic regression takes effect regardless of controlled cholesterol levels in the blood [Raffai et al., 2005]. Furthermore, ApoE even at suboptimal levels can manage the progression of atherosclerosis despite a confirmed case of hypercholesterolemia [Raffai et al., 2005]. The genetically engineered ApoE deficient (ApoE-/-) mouse, established 20 years ago and still commonly used, spontaneously develops atherogenic lesions and hypercholesterolemia similar to humans [Plump et al., 1992; Piedrahita et al., 2000].

Hypercholesterolemia, a significant risk factor associated with CVD, contributes to high levels of oxidative stress in the vascular environment, thus contributing to endothelial dysfunction and atherogenesis [Harrison et al., 2011; Torzewski et al., 2007]. Accumulation of oxidative stress causes altered metabolism, elevated reactive oxygen species [ROS] production and subsequent mitochondrial damage over time. These factors induce pro-inflammatory

pathways and other key steps in the pathogenesis of CVD [Harrison et al., 2011]. Furthermore, the development of a fatty acid (FA) pool due to ApoE deficiency creates a lipotoxic state whereby oxidative stress is enhanced in the mitochondria, possibly inducing apoptosis of both adipose and nonadipose cells, as seen in various experimental models using different species [Nguyen et al., 2007; de Vries et al., 1997; Van der Vusse et al., 1992].

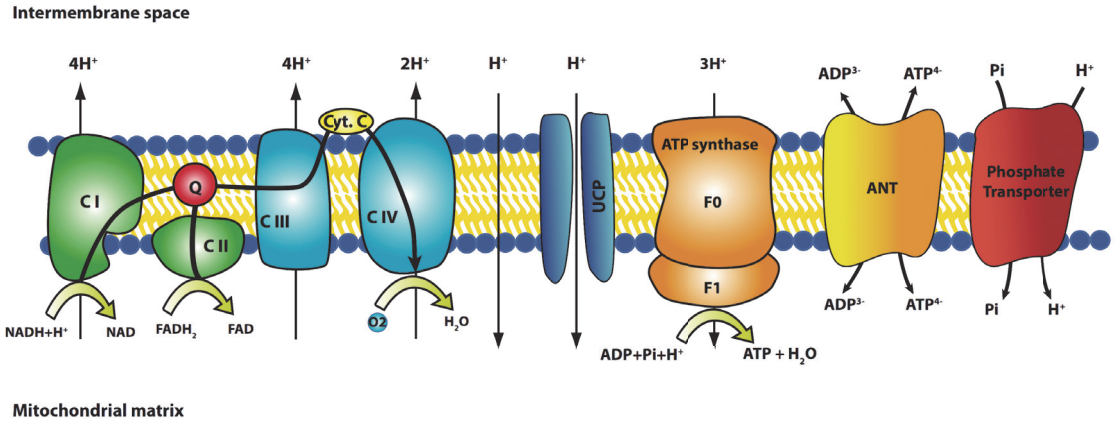
### *Mitochondrial Roles*

Mitochondria may not take up much cellular space yet their exponential significance is often underestimated. They are vital to cellular life as well as its end so, it should come as no surprise that more and more diseases can be linked at least in some way to mitochondrial dysfunction, especially in regards to pathologies of the heart [Duchen, 2004]. Overall, the alteration of mitochondrial capacity can result in major or minor disability and also death. For example, neurodegenerative diseases (e.g. Parkinson's), age-related diseases, diabetes and multi-organ failure in sepsis share a pathophysiological relation to some mitochondrial abnormality. The pure fact that we need such a thing as oxygen to survive and experience such bodily processes as digestion and absorption is to satiate the demands of the mitochondria, and to provide them with enough fuel to carry out all of their important roles [Duchen, 2004]. In fact, 98% of all inhaled oxygen is used by the mitochondria alone [Duchen, 2004]. Therefore, their optimal functioning is critical to the heart as well as the central nervous system and all the other systems of the body where they are located.

Mitochondria are an important source of ROS and when damaged (i.e. by FA accumulation) exhibit decreased oxidative based energy and thus less fuel for cells [Elahi et al., 2009]. In addition, approximately 1-2% of oxygen consumed by the mitochondria is

incompletely reduced to water leading to ROS production; under altered physiological circumstances this could be damaging for the heart as it consumes a great amount of oxygen and is also an important source of ROS [Turrens, 1997]. This is also a fundamental consequence since mitochondria are organelles in which energy or adenosine triphosphate (ATP) production and cellular respiration occur [Stanley et al., 2005; Saks, 2006]. Furthermore, the maintenance of ATP drives all cellular processes in the body especially the heart that, is crucial for a prominent workload organ such as the heart because of its high-energy demands [Goldberg et al., 2012; Giordano, 2005; Barth et al., 1992; Bers, 2001; Huss & Kelly, 2005].

**Figure 1**: Schematic representation of the mitochondrial oxidative phosphorylation system.



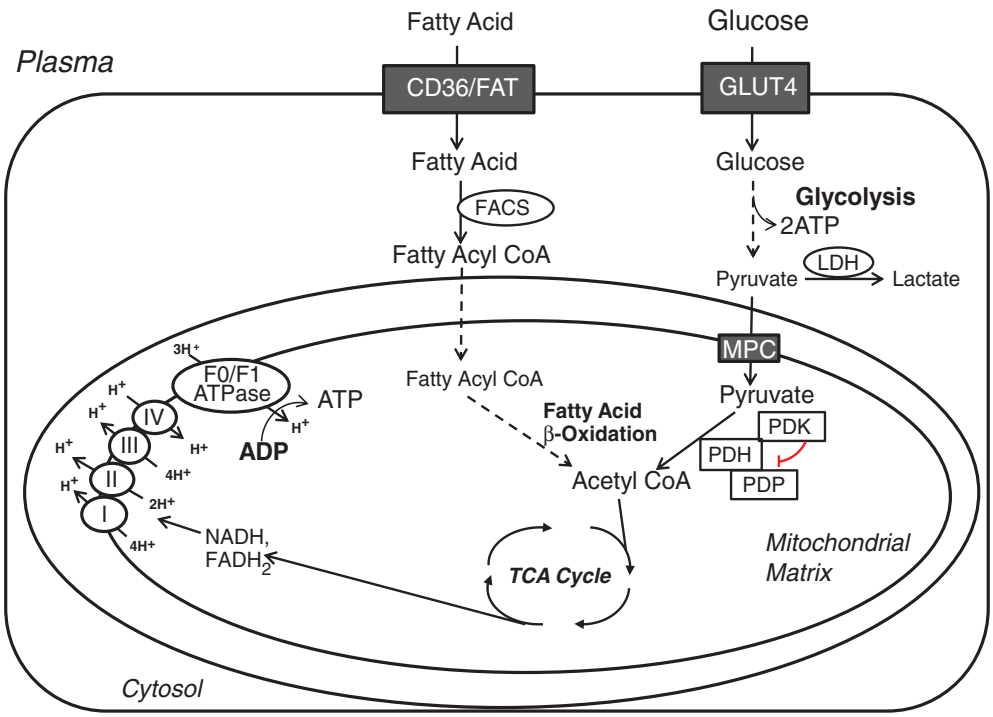

[Gouspillou et al., 2011]

The capacity of the mitochondria relies heavily on substrate availability, specifically lipids and carbohydrates. Their metabolism is based on the utilization of substrates through β-oxidation and the Kreb's cycle or through oxidative phosphorylation (OXPHOS) in general. OXPHOS is the form of aerobic metabolism whereby carbohydrates, lipids and proteins form $CO_2$ and $H_2O$ intermediates to ultimately yield ATP. It begins with the electron entry at either complex I or II [Irwin et al., 2013]. β-oxidation specifically, produces acetyl-CoA from FAs that

enter the Kreb's cycle to be used by the electron transport system (ETS) for electron transporters, reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), in order to generate ATP [Lopaschuk et al., 1994; Fillmore & Lopaschuk, 2013]. A schematic of the ETS is illustrated in Figure 1. The proton motive-force or rather a proton gradient created by the passage of electrons along the inner mitochondrial membrane assists the production of ATP [Duchen, 2004]. Therefore, the ability of the Kreb's cycle to extract electrons and generate $H^+$ from citrate to generate NADH and $H^+$ and $FADH_2$ for subsequent passage to the ETS, is its most imperative function [Jeukendrup & Gleeson, 1969]. Mitochondrial bioenergetics is further summarized schematically in Figure 2.

The Kreb's cycle is the main common aerobic metabolic pathway involving carbohydrates, lipids and proteins [Akram, 2013]. Acetyl-CoA oxidation by this cycle is responsible for two thirds of the sum of ATP production and oxygen consumption [Baldwin & Krebs, 1981]. However, a by-product of mitochondrial respiration is ROS that, in high concentrations risks damaging cell membranes and DNA including mitochondrial DNA (mtDNA) [Duchen, 2004]. Nuclear DNA and mtDNA both encode the enzymes critical to mitochondrial function [Huss & Kelly, 2005]; mtDNA differs from typical DNA making it highly susceptible to oxidative stress and damaging effects from ROS [Sheeran & Pepe, 2006]. The leak of electrons to oxygen occurring between mitochondrial complex II and III but not directly from complex II, may contribute the most to mitochondrial ROS production, a result mirroring a simultaneous increase in mitochondrial potential [Duchen, 2004]. ROS generation can actually increase further if respiration is inhibited after these complexes [2004].

**Figure 2**: OXPHOS in carbohydrate and FA metabolism.




[Fillmore & Lopaschuk, 2013]

The ETS may therefore be thought of as one of the mitochondria's most valuable assets. Complex I (the first enzyme) is the largest of the 5 complexes in the respiratory system and vital to energy production in the cell. It has the ability to extract energy from NADH resulting from lipid and carbohydrate oxidation of which, is then trapped across the mitochondrial inner membrane (potential difference). Electrons pass from complex I to III via coenzyme Q10 (coQ10) meanwhile complex I ships protons from the matrix into the intermembrane space contributing to the proton gradient [Irwin et al., 2013]. Complex II oxidizes $FADH_2$ after which, electrons via coQ10 are once again transferred over to complex III, another point of entry for electrons along with complex I [Irwin et al., 2013; Dudkina et al., 2008]. Complex III undergoes a redox reaction involving two enzymes while exporting more protons into the intermembrane space. Complex IV is the final electron acceptor and proton pump and reduces oxygen to a water molecule by using the electrons it has received [Irwin et al., 2013; Crofts, 2004]. The stored

energy can then fuel ATP synthesis when complex V undergoes a conformational change while exposed to the electrochemical potential and the proton gradient along with the temporary new structure of this complex, is used to convert ADP to ATP by transporting protons back into the matrix [Schultz & Chan, 2001; Irwin et al., 2013].

### *Impact of mitochondrial function on cardiac metabolism*

FAs and glucose are oxidized in the mitochondria and contribute the most to cardiac ATP production [Stanley & Chandler, 2002; Taegtmeyer, 1994]. However, FAs are the preferred substrate (70% of total ATP) making its appropriate uptake from the plasma important for cardiac viability [Bing et al., 1954; Shipp et al., 1961; Wisnecki et al., 1987]. Lipids have furthermore, been found to be necessary for the pure beating of heart cells [Nutrition Reviews, 1966]. Under normal physiological conditions, the rate of uptake of FAs into muscle fibers is deemed proportional to the difference in FA concentrations outside and inside the cell and more importantly, it is determined by their arterial concentrations [Vik-Mo & Mjøs, 1981]. The cardiac source of lipids comes from either free or unesterified FAs (FFAs) or lipoprotein-bound or esterified FAs, the latter being especially significant [Ballard et al., 1960; Goldberg et al., 2012].

FA oxidation is understandably a critical process within the mitochondria of the myocardium. Under fasting aerobic conditions, all of 60-90% of total oxygen consumed contributes to FA oxidation [Vik-Mo & Mjøs, 1981]. An increase in FA oxidation in even the hearts of control mice can cause decreased cardiac efficiency [How et al., 2005]. Cardiac efficiency is determined by the oxygen requirement for a given amount of work conducted by the heart. Therefore, disrupted energy metabolism affects oxidative metabolism from insufficient

oxygen supply and thus impairing cardiac efficiency [Fillmore & Lopaschuk, 2013; Jaswal et al., 2011]. This impaired cardiac efficiency may be a factor in the pathogenesis of heart failure [Fillmore & Lopaschuk, 2013]. In contrast, a decrease in lipid oxidation promotes cardiomyopathy and lipid accumulation in cardiomyocytes [Goldberg et al., 2012]. Some factors that could affect lipid oxidation include: transport of FAs into muscle cells, into the mitochondria and within the bloodstream, as well as their oxidation though β-oxidation and the Kreb's cycle [Vik-Mo & Mjøs, 1981].

Furthermore, a chronic decrease in the heart's ability to oxidize FAs has been shown to contribute to heart failure since this fuel source reflects a crucial ATP generating pathway in the adult heart [Bishop & Altschuld, 1970; Taegtmeyer & Overturf, 1988; Allard et al., 1994; Christe & Rogers; 1994; Massie et al., 1995; Sambandam et al., 2002; Huss & Kelly, 2005]. FAs remaining unoxidized especially by β-oxidation can be diverted towards diacylglycerol, triacylglycerol, ceramide and other toxic lipid productions [Lowell & Shulman, 2005; Roden, 2005; Lopaschuk & Folmes, 2007; Lopaschuk et al., 2010]. It has been shown that decreased β-oxidation in the heart despite elevated FA availability is associated with intramyocardial lipid accumulation [Young et al., 2002]. This may stimulate a disproportionate activity between β-oxidation and the Kreb's cycle or even β-oxidation inhibition in favor of increased glucose oxidation, possibly resulting in incomplete FA oxidation and an overproduction of ROS [Saddik et al., 1993; Koves et al., 2005; Muoio & Koves, 2007].

All mitochondrial pathways are critical for the heart to ensure the acquirement of enough ATP without being exposed to dangerous amounts of circulating FAs nor ROS. These pathways need to respond to the continuous flux in energy demand and substrate availability proportionately and efficiently, considering the heart has a limited substrate storage capacity

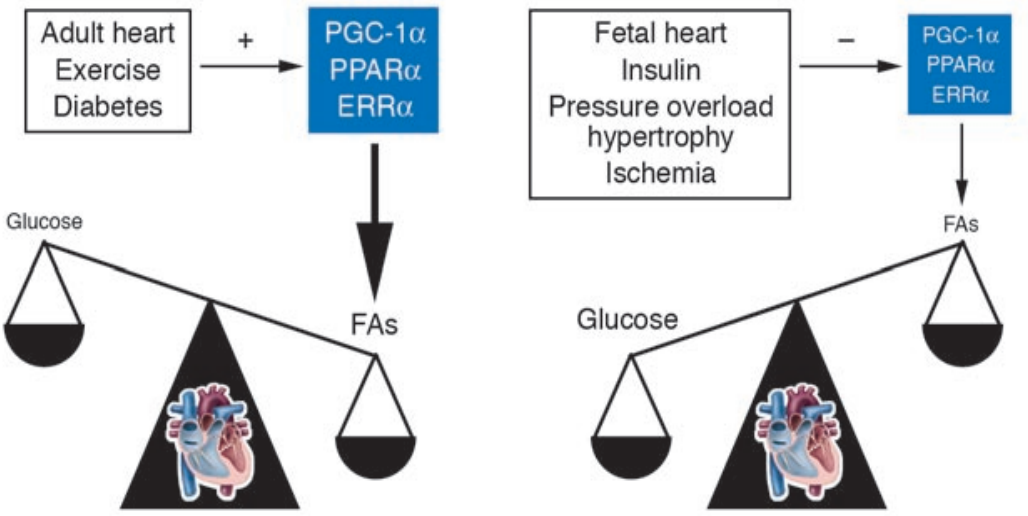
[Huss & Kelly, 2005]. Carbohydrate and FA metabolism are considered regulators of one another otherwise known as the Randle cycle meaning, pyruvate dehydrogenase a key enzyme in carbohydrate metabolism can be inhibited by FA oxidation end products, acetyl-CoA and NADH [Koonen et al., 2005; Jaswal et al., 2011]. In the failing heart, there is an up-regulation in glycolysis while oxidative metabolism within the mitochondria is decreased [Jaswal et al., 2011]. Substrate availability also influences myocardial oxygen consumption ($MVO_2$) in that FA utilization can in fact increase $MVO_2$ since their oxidation has a greater oxygen requirement than that of carbohydrates [Vik-Mo & Mjøs, 1981]. Greater FA availability than what the heart is normally exposed to may have negative implications for the heart muscle. In fact, a vicious cycle affiliated with the myocardial ischemic process may ensue when elevated free FA are left to circulate in the plasma, suggesting their destructive involvement in said cycle [Vik-Mo & Mjøs, 1981].

Cardiac metabolism can shift without delay from lipid to carbohydrate metabolism in order to produce necessary ATP when the heart is exposed to great demands and ischemic conditions, a phenomenon that can be prevented by decreasing FA oxidation through alleviating the oxygen requirement [Goodwin et al., 1998; Lopaschuk et al., 2010]. Uncoupling proteins (UCPs) cause mitochondria to shift from producing ATP to produce heat and in heart failure for instance, elevated levels of FFA in the plasma may activate these UCPs leading to inefficient OXPHOS [Vettor et al., 2002; Murray et al., 2004; Murray et al., 2008]. Atherosclerosis a risk factor of CVD also predisposes the development of heart failure [McGovern et al., 1996; Rosen et al., 2007]. In the failing heart, oxygen and ATP are utilized at a faster rate than they can be replenished and this becomes increasingly so [Ingwall & Weiss, 2004]. There is also an uncoupling of electron transport among the ETS and ATP production (uncoupled oxidative

phosphorylation) and wasteful energy generation yet the amount of oxygen available does not meet the need for energy. A decline in mitochondrial oxidative capacity as seen in the failing heart, may be an adaptive mechanism to compensate for shifts in cardiac mitochondrial physiology [Huss & Kelly, 2005]. A relationship exists among the imbalanced mitochondrial supply and demand along with an up-regulation of the genes responsible for fat metabolism in the pathogenesis of heart failure [Ingwall & Weiss, 2004].

Therefore, gene expression is likely responsible for the shift in metabolic and energy metabolism in failing and normal hearts [Huss & Kelly, 2005]. PPARγ coactivator-1 (PGC-1), specifically PGC-1α and PGC-1β, may play a role in regulating cardiac mitochondrial substrate and energy metabolism (bioenergetics) respectively, as they perform similar roles in other tissues with great oxidative capacity [Huss & Kelly, 2005]. PGC-1α can actually be induced to increase mitochondrial respiratory coupling and FA oxidation by up-regulating the respective enzymes, as found in cultures containing cardiomyocytes while the nuclear receptor PPARα assists in this regulatory transcriptional cascade [Lehman et al., 2000; Huss et al., 2004; Huss &Kelly, 2005]. PPARα likely responds to physiological stimuli affecting FA delivery while estrogen-related receptors (ERRs), another type of nuclear receptor, may stimulate OXPHOS and help direct signaling of transcription within the PGC1-α network [Huss et al., 2004; Huss & Kelly, 2005]. Physiological cues from varying stimuli that affect the body influence gene expression and these receptors to help maintain a dynamic balance in cardiac metabolism which, favors glucose oxidation in the fetal heart and FA oxidation in the adult heart, as seen in Figure 2 [Huss & Kelly, 2005]. Although, there is an obvious preference of fuel type this favored balance is not fixed. A shift from the primary utilization of one energy substrate or the other depends on the sources of stimuli.

**Figure 3**: Shifts in energy substrate utilization affects cardiac metabolism.




[Huss & Kelly, 2005]

On the right hand side we see the substrate shift favoring glucose that occurs in fetal and diseased hearts, such as in ischemic conditions. On the left hand side we see this shift favoring lipid substrates in cases of uncontrolled diabetes or provided with an exercise stimulus and generally in the heart when fatty acids are available

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The heart is a highly oxidative muscle of the body. Any disruption in mitochondrial function can stimulate disturbances in carbohydrate and lipid metabolism and more importantly cause disturbed cardiac function. Heart failure therefore, is thought to be an end result linked to the combination of mitochondrial derived oxidative stress and decreased OXPHOS [Ong & Hausenloy, 2010]. Gradual and progressive dysfunction in mitochondrial respiration is implicated in the basis of heart failure no matter the cause [Huss &Kelly, 2005]. Furthermore, the failing heart exhibits disturbed activity of the ETS complexes, regulation of the Kreb's cycle as well as the supply and proper functioning of NADH [Sheeran & Pepe, 2006]. This is significant seeing as how heart failure despite technological advances remains a main cause of death and disability in the developed world [Sheeran & Pepe, 2006].

*Rationale, objectives and hypotheses*

Cardiovascular disease is a growing international health concern and so are its associated risk factors such as dyslipidemia and hypercholesterolemia. These elements lead to oxidative stress whereby the mitochondria are impaired as seen in hepatic and skeletal muscle tissue in humans, rat and ApoE-/- murine models. The mitochondria are an important research target in regards to cardiovascular health because of their role in generating cellular energy, by means of oxidative phosphorylation, a process that is affected by impaired uptake of cholesterol and lipids from the blood (i.e. ApoE deficiency). The altered role of mitochondria may be potentially implicated in the pathophysiology of heart failure [Sheeran & Pepe, 2006]. The ApoE-/- murine model has been studied extensively and findings using this model can be highly applicable to public health. However, the effects of elevated cholesterol and lipid levels on the function of the mitochondria in the heart, specifically in cardiomyocytes, and whether this alters oxygen consumption in these cells has yet to be investigated.

Despite the possible protective effect of estrogen on cardiomyocytes, CVD is still highly prevalent among females and not solely males, the sex in which this disease is primarily studied. There are sex differences in the development and implications of CVD as well [Wang et al., 2011]. Furthermore, there are important sex differences in lipid profiles, which may contribute to underlying pathological mechanisms in CVD and other conditions. Using a female mouse model, we will examine how the heart muscle is still affected by an altered lipid profile despite their sex. One study by Sothern et al. showed that levels of cholesterol, especially LDL, and triglycerides are significantly different in obese girls and obese boys before and after weight loss [Sothern et al., 2000]. Differences in plasma lipid metabolism and female biased genes may account for these findings and therefore warrants further examination [Zhang et al., 2011]. Thus, it is just as

necessary to investigate implications of impaired cholesterol and other lipid clearance from the plasma resulting from ApoE deficiency, on cardiac physiology and function in the female sex. Clinically, this would lead to a better understanding of the interaction of a pro-atherosclerotic environment and cardiac mitochondrial roles and its potential implication in heart failure. This is especially importantly as CVD including heart failure are becoming more and more prevalent among the female sex especially, in the post-menopause state when there is a significant decrease in their cardioprotective effect [Wang et al., 2011], in which case the knowledge regarding the previously mentioned relationship would prove significantly useful.

I investigated how an altered lipid profile, one that is favorable for the development of atherosclerosis and maintained in an ApoE knockout mouse, modifies energy metabolism otherwise known as the bioenergetics, in cardiomyocytes. Lesions can begin in the vasculature of this model as early as 3 weeks old making it an efficient model to use. The protocol can be easily replicated and applied to future studies, while the instruments used are valid and have been frequently used in numerous other studies [Gnaiger, 2012a]. The implication of the mitochondrial respiratory complexes was investigated using an established protocol that permits the analysis of its oxidative capacity as well as mitochondrial coupling. The results from using said protocol had determined whether disturbed energy metabolism was present.

The primary objective of this study was to examine the effects of impaired clearance of cholesterol and lipids (i.e. elevated in the blood circulation), on promoting shifts in cardiac mitochondrial oxidative capacity. More specifically, I had quantified cardiac mitochondrial oxygen consumption of both a 2-month and an 8-month-old group using an ApoE-/- murine model and compared these values to age-matched controls. I then assessed whether or not the coupling of oxidation and phosphorylation was affected, as it is another indicator of altered

functioning in the respiratory capacity of mitochondria of these same groups. In addition, I determined the implications of ApoE deficiency on substrate and energy metabolism to evaluate if either of these had been disturbed in the experimental groups compared to controls. So the effects of a high lipid-environment over 2 different periods of time had been studied and whether aging had contributed any consequences in said environment. Finally, it was of interest to see how the effects seen so far with high lipid exposure in the blood stream due to Apoe-/-, were amplified by saturating these lipid levels through supplementation of high amounts of fat through the diet.

*Hypotheses*

It was hypothesized that the mitochondria from ApoE-/- cardiomyocytes will exhibit an altered oxidative capacity resulting from shifts in their lipid profile compared to 2-month-old controls. We expected to see increased oxygen consumption in the ApoE deficient groups as compared to controls because of a probable increase in FA oxidation. It was also hypothesized that there would be an initial increase in the coupling of oxidation and phosphorylation in the groups lacking ApoE and not in the control groups. This was thought to likely be a compensation that would remain or increase with prolonged lipid exposure in the blood stream available to the heart in the 8 month-old experimental group compared to 2-month and 8-month-old control groups. Furthermore, we expected to see a shift in substrate metabolism and altered energy metabolism in the ApoE-/- groups as compared to controls, both in the young and older age groups. Finally, we expected to see these same effects only further amplified when the high lipid exposure was saturated with additional high amounts of lipids given through the diet.

# II

# Implications of Apolipoprotein E Deficiency on Cardiac Mitochondrial Oxygen Consumption in a Young Mouse Model

Cynthia Rocha, Celena Scheede-Bergdahl, Thomas Whitesell, Andreas Bergdahl

## Contribution of Authors

Cynthia Rocha: Animal handling, high-resolution respirometry, euthanized animals, surgical extraction of cardiac tissue, saponin-induced permeabilization, statistical analysis, and preparation of manuscript.

Celena Scheede-Bergdahl: Concept development and preparation of manuscript.

Thomas-Whitesell: Protein extraction, probing for VDAC expression, isolation of citrate synthase activity, and statistical analysis.

Andreas Bergdahl: Concept development, preparation of manuscript, immunoblotting, statistical analysis, provided animals, laboratory space and equipment.

# Implications of Apolipoprotein E Deficiency on Cardiac Mitochondrial Oxygen Consumption in a Young Mouse Model

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## ABSTRACT

**Background: Apolipoprotein E (ApoE) is necessary for normal lipid metabolism. Deficiency of this protein hinders plasma clearance of both triglycerides and cholesterol. Lipids are, shunted through alternate metabolic pathways, thus resulting in abnormal mitochondrial function. Given the oxygen consumption required for normal heart function, the mitochondria are of particular importance and the preservation of their optimal function is critical.**

**Methods: Using high-resolution respirometry (HRR) and immunoblotting to quantify mitochondrial oxidative phosphorylation (OXPHOS) capacity in permeabilised cardiomyocytes, the goal of this study was to investigate the effects of ApoE deficiency on energy metabolism in the hearts of young mice.**

**Results: Our results demonstrate a reduced basal respiration (ADP restricted) in the ApoE-/- mice. This is furthermore translated to an increased efficiency of the mitochondria, as expressed by a higher respiratory control ratio in ApoE-/- mice.**

**Conclusions: This indicates that early stages of ApoE deficiency positively affects oxidation and phosphorylation coupling within cardiac mitochondria. Our findings provide important information regarding the early phases of preferential lipid metabolism in cardiomyocytes and can help explain the benefits of short term high fat intake for prevention of development and progression of heart failure.**

### Selected abbreviations

**ACR** - acceptor control ratio
**ApoE** - apolipoprotein E
**ApoE-/-** - apolipoprotein E deficient
**ADP** - adenosine diphosphate
**ATP** - adenosine triphosphate
**CVD** - cardiovascular disease
**ETS** - electron transport system
**$FADH^2$** - flavin adenine dinucleotide
**FA** - fatty acid
**$GM^3$/$GMS^3$** - glutamate, malate state 3 / glutamate, malate, succinate state 3
**LDL** - low-density lipoproteins
**NADH** - nicotinamide adenine dinucleotide
**OXPHOS** - oxidative phosphorylation
**RCR** - respiratory control ratio
**ROS** - reactive oxygen species
**ROX** - residual oxygen consumption
**TRL** - triglyceride-rich lipoproteins
**VDAC** - voltage-dependent anion channel
**VLDL** - very low-density lipoproteins

## INTRODUCTION

Cardiovascular disease (CVD) is a leading public health concern as it lies at the root of serious illness, disability and death.Central to this condition is chronic inflammation whereby oxidised lipoproteins are deposited onto blood vessel walls [1]. Lipoproteins are soluble molecules that enable lipid transport in the plasma. Of these, Apolipoprotein E (ApoE) is an important component in the structure of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) [2].

It also participates in the uptake of triglyceride-rich lipoproteins (TRL) by the liver and has, as such, an ability to suppress the development of atherosclerosis [3-4].The genetically engineered ApoE deficient (ApoE-/-) mouse, established 20 years ago and still commonly used, spontaneously develops elevated cholesterol levels similar to what is seen in humans [5-6].

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### CONFLICTS OF INTEREST

There are no conflicts of interest related to this project.

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Hypercholesterolemia, a significant risk factor for CVD, contributes to high levels of oxidative stress in the vascular environment, resulting in endothelial dysfunction and atherogenesis [7-8]. Accumulation of oxidative stress causes altered metabolism, elevated reactive oxygen species (ROS) production and subsequent mitochondrial damage over time. These factors induce pro-inflammatory pathways and other key steps in the pathogenesis of CVD [7]. Furthermore, the development of a fatty acid (FA) pool due to ApoE deficiency creates a lipotoxic state whereby oxidative stress is enhanced in the mitochondria, possibly inducing apoptosis of both adipose and nonadipose cells, as seen in various experimental models and species [9-11]. Under certain conditions, mitochondria are significant sources of ROS and will, when damaged (i.e. by long-term FA accumulation,) exhibit decreased oxidative based energy and thus less fuel for cells [12]. This is of fundamental importance since the mitochondrion is the organelle in which energy (ATP) production and cellular respiration occur [13-14].

Considering the maintenance of ATP drives all cellular processes, this process is particularly crucial for a high-level workload organ such as the heart due to its high-energy demands [15-19]. The capacity of the mitochondria relies heavily on substrate availability, specifically lipids and carbohydrates. Their contribution to energy metabolism is based on the utilisation of substrates through β-oxidation and the Kreb's cycle. β-oxidation produces acetyl-CoA from FAs that enter the Kreb's cycle, generating electrons that are subsequently transported to the electron transport system (ETS) by NADH and FADH2 in order to maximise ATP production [20]. The Kreb's cycle is the main common aerobic metabolic pathway involving carbohydrates, lipids and proteins [21]. Acetyl-CoA oxidation by this cycle is responsible for two thirds of the sum of ATP production and oxygen consumption [22].

While both glucose and FAs are oxidised in the mitochondria and thus contribute to ATP production in the cardiomyocytes, the latter is the preferred substrate (generating 70% of total ATP) [23-25]. For this reason, the uptake of FA from the plasma is critical for cardiac viability [23-27]. Lipids have also been found to be necessary for the maintenance and re-initiation involved with the pure beating of heart cells, thus further emphasising their importance [28].

Since the heart has limited substrate storage capacity, the uptake of nutrients needs to be finely balanced as the underlying pathways have to respond both proportionately and efficiently to continuous flux in energy demand and substrate availability [19]. Under normal conditions the mitochondria maintains a fine equilibrium between glucose and FAs, however, during physiological stress, the mitochondria shifts towards an increased carbohydrate metabolism. This adds stress to the heart and shunts the lipids into non-oxidative pathways creating more ROS than energy [29]. The same detrimental phenomenon occurs when there is a reduced oxygen supply as a result of the occlusion of the coronary vessels, a major cause of cardiovascular distress [30].

As CVD is a growing international health concern, more focus is placed on its associated risk factors, such as dyslipidemia and hypercholesterolemia. These elements lead to oxidative stress whereby the mitochondria are impaired, as seen in hepatic and skeletal muscle tissue in humans, rat and ApoE-/- murine models. The mitochondria are an important research target in regards to cardiovascular health because of their role in generating cellular energy, by means of oxidative phosphorylation, a process affected by uptake of cholesterol and lipids from the plasma. The subsequently altered role of mitochondria may potentially be implicated in the pathophysiology of heart failure [31].

The ApoE-/- murine model has been studied extensively and findings using this model can be highly applicable to public health; however, the effect of ApoE deficiency on the function of the mitochondria in the heart and whether this alters oxygen consumption in these cells has yet to be investigated.

This study examined how a shortage of ApoE affects the cardiac mitochondrial oxidative capacity. We quantified cardiac mitochondrial oxygen consumption in a 2-month old ApoE-/-murine model in comparison to age-matched controls. The results determined whether disturbed energy metabolism was present by investigation of the oxidative capacity of the mitochondrial respiratory complexes. We hypothesised that the mitochondria from the ApoE-/-cardiac tissue would experience increased respiratory function and energy metabolism as compared to controls.

## MATERIALS AND METHODS

### Animal Care

Two month old female, age-matched, C57Bl/6 (Control) and ApoE-/- (Experimental) mice were obtained from Charles River Breeding Farms (St Constant, Quebec, Canada) and Jackson Laboratories (Bar Harbor, Maine, USA) respectively. The mice were housed as breeding pairs in a thermo-neutral environment (22ºC), on a 12:12 h photoperiod, and were provided standard dry laboratory mouse chow and water ad libitum. The animals used in this study were weaned at 21 days and left to mature under socialising conditions until 2 months of age. All procedures were approved by the Animal Ethics Committee of Concordia University (protocol ID: 2013BERG) and were conducted in accordance with guidelines of the Canadian Council on Animal Care.

The mice were not sex-matched, as all efforts were made to reduce all parameters to a minimum. There are a number of recognized differences between the sexes in terms of tissue organisation, biochemistry as well as response to drugs. As the focus was on investigating the effects of a lack of apolipoprotein E in the heart tissue, a female to female comparison between the ApoE knock-out and age-matched control mice gave a better research design.

### Experimental protocol

The beating heart was removed immediately after euthanasia with CO2 according to the approved animal protocol and split into two different portions. One portion was snap frosen in liquid nitrogen, then stored at -80ºC for biochemical analysis; the other part (the apex) was placed in an ice cold relaxing buffer (BIOPS) and used immediately to measure mitochondrial respiration. The BIOPS contains(in mM): CaK2EGTA 2.77, K2EGTA 7.23, Na2ATP 5.77, MgCl2·6H2O 6.56, Taurine 20, Na2Phosphocreatine 15, Imidazole 20 mM, Dithiothreitol 0.5, MES 50, pH 7.1.

Snap freezing reduces the chance of water present in the sample forming ice crystals during the freezing process, and thus better maintain the integrity of the sample. In the case of tissue, snap freezing slows the action of proteases and nucleases which inhibit degradation of molecules and proteins. The snap frozen hearts can be considered to have an unspoiled tissue integrity very similar to the one used in our Oxygraph experiments.

### Preparation of permeabilised cardiac fibres

The apex was removed and the left ventricle dissected for preparation of permeabilised myofibres. This was done by gentle dissection during which the fiber bundles were separated using sharp forceps. The fibres were then incubated in 3 ml BIOPS buffer containing 50 µg/ml saponin for 30 minutesand subsequently washed in ice-cold buffer (MiR05) for 2 x 10 min. MiR05 contains (mM): EGTA 0.5, MgCl2·6H2O 3.0, K-lactonionate 60, Taurine 20, KH2PO4 10, HEPES 20, Sucrose 110, BSA 1g/l, pH 7.1.

### Mitochondrial respiratory measurements

Measurements of oxygen consumption were performed in MiR05 at 37ºC using a polarographic oxygen sensor (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Approximately 2.0 to 2.5 mg of muscle tissue (wet weight) was place in either chamber in a cross-sectional design. O2 flux was resolved by DatLaband all experiments were carried out in hyperoxygenated levels to avoid O2 diffusion limitations. A sequential substrate addition protocol was used to allow functional dissection of the electron transport system:state 2 respiration (absence of adenylates) was assessed by addition of malate (2 mM) and octanoylcarnitine (1.5 mM), by adding ADP (5 mM) we could reach state 3 respiration for complex I. This was followed by addition of glutamate (10 mM) and succinate (10 mM) achieving maximal coupled state 3 respiration with parallel electron input to complex I and II.

Oligomycin(2 µg/ml) was then added to block complex V and thereafterantimycin A (2.5 µM) to inhibit complex III. Finally ascorbate (2 mM) and TMPD (500 µM) were added to evaluate Complex IV respiration. The acceptor control ratio (ACR) was calculated by dividing the respirationfollowing ADP addition by the values after malate titration. The respiratory control ratio (RCR) was evaluated by dividing the respirations of succinate by those of oligomycin for each of the samples while mitochondrial leak was drawn from the difference between oligomycin and antimycin.

### Protein Extraction, Immunoblotting, and Immunofluorescence

Cell lysates were extracted in lysis buffer containing 250 mMNaCl, 50 mM HEPES (pH 7.5) 10% glycerol, 1% triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM Na4P2O7, 1 mMNaF, 800 µM Na3VO4 and centrifuged at 12,000 × g for 10 min. Supernatant was collected and the lysates were separated on a 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (0.45 µm, 162-0115, Bio-Rad) using 10 mM sodium tetraborate buffer. The membranes were blocked in 5% BSA in TBS-T buffer (10 mm Tris-HCl, pH 7.5, 150 mm NaCl, 0.05% Tween 20) for 1 h at room temperature followed by overnight incubation at 4°C with primary antibodies: voltage-dependent anion channel or VDAC, (1:3000, MSA03 MitoSciences), total OXPHOS (oxidative phosphorylation) rodent antibody cocktail (1:4000, MS604 MitoSciences) and beta-tubulin (1:5000, ab6064 Abcam). The blots were washed, incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse, ab6728; anti-rabbit, ab6721; Abcam) and visualised with a chemiluminescence system (Immun-Star Chemiluminescent; 1705070; Bio-Rad). The bands were analysed using the Image J software.

### Statistics

Summarised data are presented as means ± standard error of mean (SEM) in all figures. Statistical comparisons were done using a two-tailed Student's t-test. For all statistical evaluations, $p < 0.05$ was considered significant.

## RESULTS

### Mitochondrial density

There was no difference (100 ± 6.4 vs. 107.7 ± 10.1) in relative mitochondrial density as showed by the expression of voltage dependent anion channel (VDAC) (*Figure 1*) when comparing control and ApoE-/- mice (n = 8). No changes were detected in protein expression of Complex I to Complex V when normalised with the loading control β-tubulin (*Figure 2*) using the same number of animals.

*Figure 1: No difference in mitochondrial density was detected using immunoblotting with the primary antibody of the voltage dependent anion channel (VDAC),(100 ± 6.4 vs. 107.7 ± 10.1, n = 8 control vs. ApoE-/-), n = 8 control vs. ApoE-/-)*

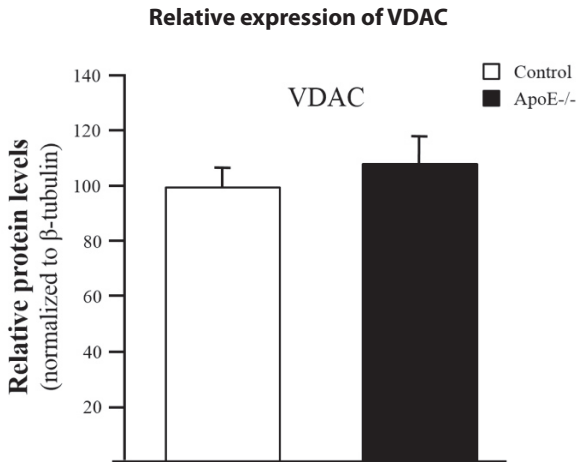
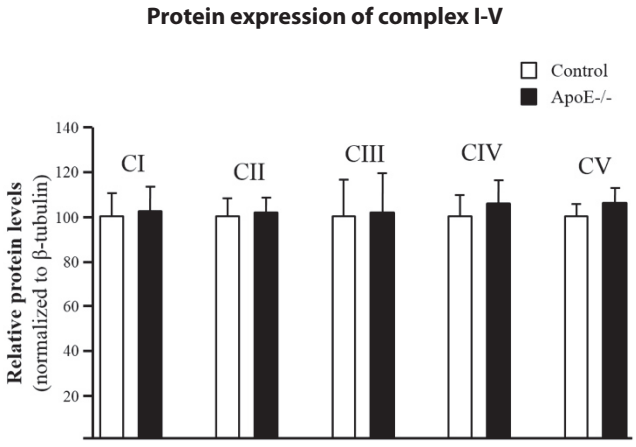



*Figure 2: Immunoblotting of Complex I to Complex V using the total OXPHOS rodent antibody cocktail from MitoSciences (MS604) together with beta-tubulin from Abcam (ab6064) as loading control. No relative changes were detected when running the samples from control and ApoE-/- hearts (n = 8) in parallel.*



## Mitochondrial respiration

To establish oxidative capacities of mitochondria in the cardiac muscles in situ as well as control of the respiratory activity by the principal regulator, ADP, we studied the oxygen consumption rates of saponin-permeabilised fibres. As seen in *Figure 3A*, the hearts from the ApoE-/- mice showed a significantly decreased (20.6 ± 1.4 and 11.0 ± 1.0pmol/s/mg, respectively, p< 0.001)mitochondrial oxidative phosphorylation (OXPHOS) capacity with the Complex I linked substrate malate(basal, ADP-restricted). Residual oxygen consumption (ROX) was similar in both groups (data not shown). There was a non-significant trend showing a potential increase in OXPHOS capacity with Complex II substrates (106.1 ± 9.5 and 130.4 ± 14.4pmol/s/mg, p = 0.18) while the maximal, ADP stimulatedrespiration was similar in both groups(180.0 ± 16.5 and 217.2 ± 35.3 pmol/s/mg). No difference was found in Complex IV OXPHOS capacity between the hearts from control and ApoE-/- mice (data not shown).

*Figure 3:* ***A)*** *Hearts from the ApoE-/- mice showed a significantly (20.6 ± 1.4 and 11.0 ± 1.0 pmol/s/mg, respectively, p< 0.001) lower OXPHOS capacity with Complex I linked substrates. There was a non-significant trend (106.1 ± 9.5 and 130.4 ± 14.4 pmol/s/mg, p = 0.18) showing increase in OXPHOS capacity with Complex II substrates while the maximal respiration was similar in both groups (180.0 ± 16.5 and 217.2 ± 35.3 pmol/s/mg).*
***B)*** *There was an increase in acceptor control ratio (ACR) when comparing the control animals with the ApoE-/-(5.1 ± 0.33 and 11.9 ± 0.84 pmol/s/mg, respectively, p < 0.001). n = 8.*

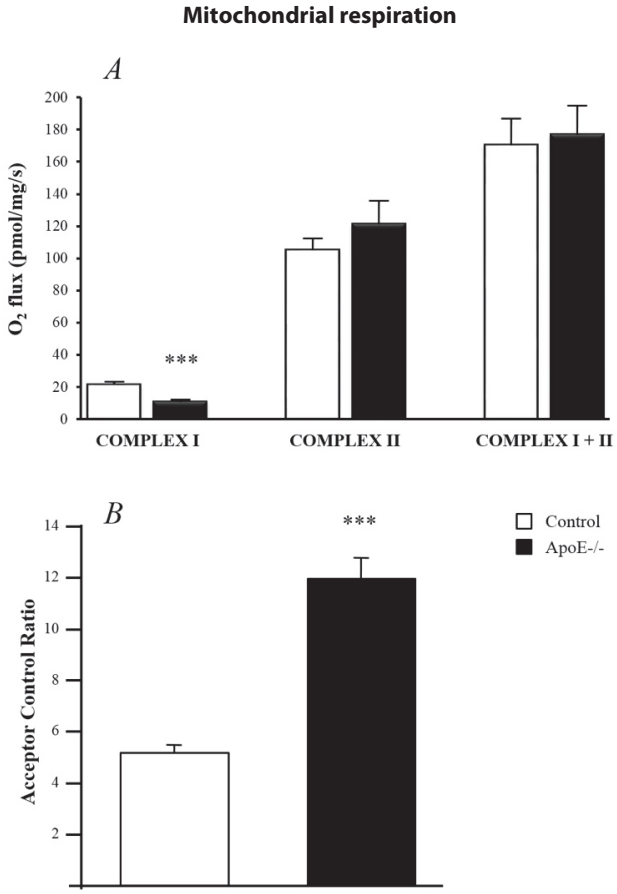



*Figure 3B* shows the acceptor control ratio (ACR, maximal, ADP stimulated respiration divided by basal, ADP restricted respiration), a ratio representing the degree of coupling between oxidation and phosphorylation.

This parameter was significantly higher in ApoE-/- mice than in control tissue (5.1±0.3 and11.9 ± 0.8, respectively, p< 0.001).Mitochondrial leak estimated as antimycin A flux rates subtracted from oligomycin flux rates indicated no change between the two mouse groups (data not shown, control 58.1 ± 4.7 and ApoE-/- 51.9 ± 6.0). No difference was seen in substrate control ratio for succinate (GM3/GMS3) from the control hearts compared to the hearts from the ApoE-/- mice (*Figure 4A*). Lipid OXPHOS capacity shown in *Figure 4B* was significantly higher in hearts from control mice compared to ApoE-/- (0.38 ± 0.03 and 0.28 ± 0.01, respectively, p < 0.01). The respiratory control ratio (RCR) was significantly higher in the ApoE-/- mice (2.3 ± 0.1 and 3.0 ± 0.3, p< 0.05) indicating that the mitochondria have a high capacity for substrate oxidation and ATP turnover and a low proton leak as there was no difference in mitochondrial density.

*Figure 4:* ***A)*** *No difference was detected in substrate control ratio (0.67 ± 0.05 and 0.63 ± 0.03 pmol/s/mg, control vs. ApoE-/-)*
***B)*** *Lipid coupling control ratio showed significant reduction in the ApoE-/- mice (0.38 ± 0.03 and 0.28 ± 0.01 pmol/s/mg, p < 0.01). Data are means ± SE. n = 8*

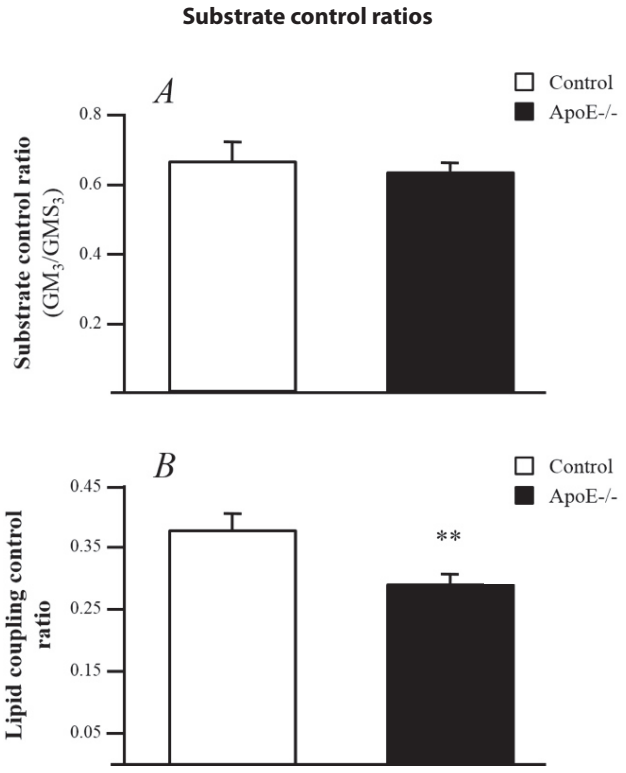
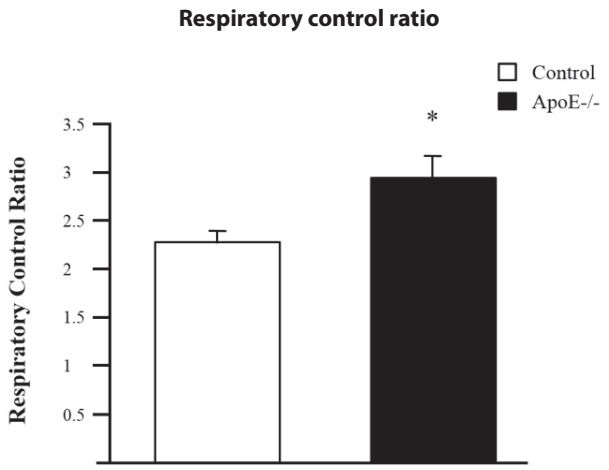


*Figure 5: The respiratory control ratio in the ApoE-/- mice was up regulated indicating a more efficient oxidation of substrates (2.3 ± 0.11 and 3.0 ± 0.29pmol/s/mg, p < 0.05, n = 8)*




## DISCUSSION

The major finding of our study is that the animals with ApoE-/-have a reduced basal mitochondrial respiration and increased respiratory control ratio as a result of an accelerated lipid substrate oxidation. The increase in oxidative capacity found in this study is associated with a significant increase in the coupling between oxidation and phosphorylation in the ApoE-/- mice as suggested by the higher ACR values [32]. This indicates that the cardiac tissue in the ApoE-/- animal has a higheroxidation:phosphorylation ratio. Since basal respiration is affected by both the degree of transmembrane proton leak and the total number of mitochondria, which was unchanged in our study, the low Complex I stimulated/ADP restricted respiration in the ApoE-/- mice is most likely due to a low degree of transmembrane proton leak.The fact that there was no difference in ADP stimulated respiration (Complex II) between control and ApoE-/-,despite there being a lower Complex I respiration, supports the notion that the ApoE-/- animals must have an enhanced coupling between oxidation and phosphorylation. Given that a high plasma concentration of FA has been previously associated with a concurrent alteration in membrane phospholipid composition, these biochemical changes may explain the reduction in the leak across the inner mitochondrial membrane [33].

Due to a lack of ApoE, there is a subsequent increase in serum fatty acid levels and a subsequent shift in substrate metabolism that favors a greater dependency on FA as a primary energy source. This reliance on FA results in an increased myocardial respiration (as seen by an increase in RCR) because more oxygen is required to generate ATP from fatty acids than glucose.

The increased oxygen uptake, however, is not specifically for the generation of ATP since it may also be play a role in fatty acid esterification andreactive oxygen species production[34-35]. Since high plasma lipid levels are usually associated with cellular dysfunction due to toxicity, it would be logical to suppose that the ApoE-/- state would have a negative effect on the mitochondria, with subsequent cardiac damage. Additionally, in prolonged obesity, where there is an elevation of FA, it has been shown that there is a decrease in the myocardial energy transduction efficiency to contractile work [36-37].

These alterations are similar to observations made in experimental animal models of obesity and may be responsible for the impairments in cardiac contractility often seen in obese persons. Our results are consistent with findings that demonstrated that myocardial FA metabolism increases before ventricular contractile function dysfunction[38]. In mitochondrial measurements from obese patients, the RCR was significantly enhanced, which is consistent with our ApoE-/- findings. RCR value analysis is very important as it relates the two most important states of mitochondrial respiration(3 and 4), and has thus become one of the most accepted parameters to describe the mitochondrial coupling [39]. The higher RCR values in the ApoE-/- hearts indicate a better coupled mitochondria, while the RCR valuesin the control animals indicate a degree of uncoupling between oxidativephosphorylation and ETC [40].

Studies in rodents suggest that, in the absence of obesity, the replacement of refined carbohydrates in the diet with fat can attenuate or prevent the development and progression of heart failure. These findingssuggest the novel concept that a diet high infat and low in carbohydrate prevents the development and progression of cardiac dysfunction compared to low fat/high carbohydrate diets [41-42]. The idea of replacing dietary carbohydrate with fat to prevent heart failure may seem counterintuitive, however, it is largely in line with current thinking regarding dietary fat and prevention of CVD[43-44]. The ingestion of a high fat diet leads to elevated concentrations of plasma lipids which is what we see in the ApoE-/- mice. The increased respiration in the ApoE deficient animals could thus be indicative of a similar mechanism.

In addition, our data suggests that the heart is an important sink for plasma FAs, thus preventing excessive and directly harmful delivery of FAs to other tissues, such as in the vasculature, where shifts towards this metabolic source has detrimental effects. The heart, like the liver, is uniquely capable of disposing of FAs because of its high energy requirements and its ability to package and secrete fatty acids as VLDL [45]. Nonetheless, prolonged excessive FA delivery to the heart, as in the liver, could lead to intracellular lipid accumulation and lipotoxicity and/or oxidative stress. Furthermore, a chronic decrease in the heart's ability to oxidise FAs has been shown to contribute to heart failure since this fuel source reflects a crucial ATP generating pathway in the adult heart [19, 46-51]. FAs remaining unoxidised, especially by β-oxidation, can be diverted towards diacylglycerol, triacylglycerol, ceramide and other toxic lipids [38, 52-54]. It has been shown that decreased β-oxidation in the heart despite elevated FA availability is associated with intramyocardial lipid accumulation [55]. This may stimulate a disproportionate activity between β-oxidation and the Kreb's cycle or even β-oxidation inhibition in favour of increased glucose oxidation, possibly resulting in incomplete FA oxidation [56-58].

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# III

# Effects of age-related cardiac redox dysregulation in apolipoprotein E deficient mice

Cynthia Rocha, Gilles Gouspillou, Andreas Bergdahl

## Contribution of Authors

Cynthia Rocha: Animal handling, high-resolution respirometry, euthanized animals, surgical extraction of cardiac tissue, saponin-induced permeabilization, statistical analysis, and preparation of manuscript.

Gilles Gouspillou: Preparation of manuscript, experimental perspective pertaining to uncoupling.

Andreas Bergdahl: Concept development, immunoblotting, statistical analysis, preparation of manuscript, provided laboratory space and equipment, provided animals.

# Effects of age-related cardiac redox dysregulation in apolipoprotein E deficient mice.

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As the mean age of the population increases dramatically so does the prevalence of cardiovascular disease. Despite the advances in research in each respective field, the implications of aging and high lipid exposure on mitochondrial oxidative capacity and respiration have yet to be evaluated. The goal of this study was to evaluate the effects of age-related changes and an altered lipid profile on the functioning of cardiac mitochondria. We hypothesized that continued exposure to a high-lipid environment would diminish the compensatory positive effects on mitochondrial metabolism, previously seen in our younger model. Female apolipoprotein E-deficient (Apoe-/-) mice were randomly assigned to a 2-month-old control, 8-month-old control, 2-month-old ApoE-/- or an 8-month-old ApoE-/- group, all of which were fed a normal chow diet. Left-ventricular cardiac apex tissue was used for immunoblotting and high-resolution respirometry (HRR), to quantify mitochondrial complexes and measure oxidative phosphorylation (OXPHOS) capacity. Our results indicate a significant increase in complex I protein expression in the older groups. We found a significant proton leak between both ApoE-/- groups. Furthermore, HRR revealed increased efficiency of substrate utilization. There was indeed an aging effect but also an experimental lipid effect from the ApoE knockout model as well. Taken together, this follows with the understanding that cardiomyocytes prefer lipid substrates for fuel. The shift in metabolism indicates that despite prolonged exposure to a high-lipid environment and aging-related effects, when combined we still observe mitochondrial adaptations and increased respiratory efficiency, as seen with short-term exposure to elevated lipids. Our findings have important implications in further understanding the relationship between lipids, cardiovascular disease and aging.

**Keywords:** Apolipoprotein E, cardiac mitochondria, oxidative capacity, bioenergetics, metabolism

Cardiovascular disease (CVD) in the elderly is a leading public health concern as it relates to serious illness, disability and death [1, 2]. Central to the pathogenesis is an altered lipid profile whereby oxidized lipoproteins deposit onto the vasculature subsequently resulting in chronic inflammation [3]. Cardiac efficiency has been shown to decrease following increased fatty acid oxidation and oxygen consumption suggestive of uncoupling or altered energy metabolism in cardiac mitochondria when exposed to high lipid content [4]. Apolipoprotein E (ApoE) is vital for its structural role in low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) [5]. ApoE also enables hepatic

uptake of triglyceride-rich lipoproteins (TRL) and may help regulate inflammatory and antioxidant pathways [6, 7]. ApoE deficiency is consequently involved in the pathophysiology of atherosclerosis and dyslipidemia [8].

Fatty acid (FA) accumulation driven by ApoE deficiency induces a lipotoxic state in mitochondria due to oxidative stress [9-11]. Furthermore, a chronic decline in the heart's ability to oxidize FAs has been shown to contribute to heart failure [12-18]. FAs remaining deoxidized can be diverted towards diacylglycerol, triacylglycerol, ceramide and other toxic lipid productions [19-22]. Reduced FA oxidation may provoke disproportionate activity between β-oxidation and the Kreb's cycle or β-oxidation inhibition in favor of increased glucose oxidation, subsequently resulting in incomplete FA oxidation and an overproduction of ROS [23-25].

What makes this shift troublesome is that OXPHOS function has been shown to decrease in aging mitochondria [26]. The age related defects [27] impede enzyme activity in the ETC, particularly in regards to complexes III and IV with no changes reported in complexes I and II [28, 29]. In addition, the apoptosis related to cytochrome c in both right and left ventricles has been found to significantly increase with age [30]. The aging process also lead to an altered lipid profile and impaired resistance to stress [31, 32]. Taken together, the mitochondria become even more susceptible to oxidative stress and ROS production [33] with aging accompanied by altered mitochondrial metabolism in the heart [28, 29, 34, 35]. Such conditions, turn the mitochondria into both a significant source and target of reactive oxygen species (ROS) and contributes to a reduction in ATP [36]. This is especially troublesome in the heart considering its high-energy demands and intense workload [37-40, 18]. The capacity of the mitochondria relies heavily on substrate availability, specifically, preferred lipids but also carbohydrates [41-43].

Under normal physiological conditions, the rate of uptake of FAs by muscle fibers is proportional to the difference in lipid concentrations outside and inside the cell [44]. Given the limited storage of substrates in the heart, there is a dynamic homeostasis between uptake, cardiac responsiveness to substrate availability and the flux in energy demands [18]. Cardiac efficiency is determined by the oxygen requirement for a given workload. Therefore, insufficient oxygen supply will affect oxidative phosphorylation and impair cardiac efficiency, which makes it a primary factor in the pathogenesis of heart failure [45, 46]. In contrast, a decrease in lipid oxidation promotes cardiomyopathy and lipid accumulation in cardiomyocytes [37]. Some factors that could affect lipid oxidation include: transport of FAs into muscle cells, into the mitochondria and within the bloodstream, as well as their oxidation though β-oxidation and the Kreb's cycle [44]. Furthermore, elevated FFAs, which are intrinsic uncoupling protein activators, lead to increased levels of uncoupling protein 3 (UCP3) and thus increased mitochondrial uncoupling [47, 48].

Uncoupling proteins (UCPs) normally moderate the proton leak across the inner mitochondrial membrane in order to regulate mitochondrial oxidant or ROS production [49]. However, they can also cause mitochondria to shift from ATP to heat production and in heart failure for instance, elevated levels of FFA in the plasma may activate these UCPs leading to inefficient OXPHOS [50, 51]. UCP3 in the heart has even been linked with FFA in the plasma of human hearts [48] and perhaps with decreased cardiac efficiency seen in mice [52]. Substrate availability also influences myocardial oxygen consumption ($MVO_2$) in that FA utilization can in fact increase $MVO_2$ since their oxidation has a greater oxygen requirement that that of carbohydrates [44]. Such an increase in MVO2 can reflect mitochondrial uncoupling of ATP production and oxygen consumption [53]. Despite this increase as seen in failing hearts to compensate for a higher ATP demand [54, 55], the greater FA availability can induce oxygen deprivation [11]. In fact, a vicious cycle affiliated with the myocardial ischemic process may ensue when elevated free FA are left to circulate in the plasma, suggesting their destructive involvement in said cycle [44].

The mitochondria are a crucial research target considering their role in aging and since their main function of generating cellular energy is disturbed by excessive uptake of lipids from the plasma [56]. The findings drawn from an ApoE-/- murine model can be highly applicable to public health and in further understanding ApoE's role in CVD development, as well as in expanding treatment options for such mitochondrial related diseases. However, the long-term effect of ApoE deficiency on the function of the cardiac mitochondria and whether this alters their oxygen consumption despite the aging process remains unanswered.

This study examined how ApoE deficiency affects cardiac mitochondrial oxidative capacity. We compared cardiac mitochondrial oxygen consumption and protein expression in a matured 8-month old ApoE-/- murine model to age-matched controls and compared them to 2-month old control and ApoE-/- groups. We hypothesized that the mitochondria from the matured 8-month old ApoE-/- cardiac tissue would experience decreased respiratory function and energy metabolism as compared to controls resulting from a high lipid environment or exposure rather than from a prominent age-related effects.

## Materials and Methods

*Animal Care*

We used 2-month and 8-month-old female C57Bl/6 (Control) and ApoE-/- (Experimental) mice obtained from Charles River Breeding Farms (St Constant, Quebec, Canada) and Jackson Laboratories (Bar Harbor, Maine, USA) respectively. The mice were weaned at 21-25 days, left to mature under socializing conditions in a thermo-neutral environment (22ºC) on a 12:12 h photoperiod and provided with standard dry laboratory mouse chow and water ad libitum. All procedures were approved by the Animal Ethics Committee of Concordia University (protocol ID: #30000259). The protocols were conducted in accordance with guidelines of the Canadian Council on Animal Care.

*Experimental protocol*

The heart was removed following euthanasia with $CO_2$ and immediately split in two separate portions. One part was snap frozen in liquid nitrogen, and later stored at -80ºC for future biochemical analysis while the apex was placed in an ice cold relaxing buffer (BIOPS). It was used instantly to measure mitochondrial respiration. The BIOPS buffer was comprised of the following (in mM): $CaK_2EGTA$ 2.77, $K_2EGTA$ 7.23, $Na_2ATP$ 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, Taurine 20, $Na_2$Phosphocreatine 15, Imidazole 20, Dithiothreitol 0.5, MES 50, pH 7.1

*Preparation of permeabilized cardiac fibers*

Through gentle dissection the fiber bundles from the apex were separated using sharp forceps. The fibers were then incubated on ice in 3 ml BIOPS buffer containing 50 µg/ml saponin for 30 minutes and subsequently washed in ice-cold buffer (MiR05) for 2 x 10 min. MiR05 contains (mM): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3.0, K-lactonionate 60, Taurine 20, $KH_2PO_4$ 10, HEPES 20, Sucrose 110, BSA 1g/l, pH 7.1.

*Mitochondrial respiratory measurements*

Measurements of oxygen consumption were performed in MiR05 at 37ºC using a polarographic oxygen sensor (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Approximately 2.0 to 2.5 mg of muscle tissue (wet weight) was analyzed in a cross-sectional design. $O_2$ flux was resolved by DatLab and all experiments were carried out in hyperoxygenated levels to avoid $O_2$ diffusion limitations. Using a sequential substrate addition protocol we were able to functionally dissection the electron transport system: state 2 respiration (absence of adenylates) was assessed by addition of malate (2 mM) and octanoyl carnitine (1.5 mM), by adding ADP (5 mM) we could reach state 3 respiration for complex I. This was followed by addition of glutamate (10 mM) and succinate (10 mM) achieving maximal coupled state 3 respiration with parallel electron input to complex I and II. Oligomycin (2 µg/ml) was then added to block complex V and thereafter antimycin A (2.5 µM) to inhibit complex III. Finally ascorbate (2 mM) and

TMPD (500 μM) were added to analyze Complex IV respiration. The acceptor control ratio (ACR) was calculated by dividing the respirations of ADP by those of malate for each sample. The respiratory control ratio (RCR) was evaluated by dividing the respirations of succinate by those of oligomycin for each of the samples. The mitochondrial leak was drawn from the difference between oligomycin and antimycin

A respirations of each sample. The lipid coupling control ratio (L/P) was calculated by dividing octanoyl carnitine respirations by those of glutamate per sample. Lastly, the substrate control ratio (SCR) was evaluated by diving glutamate respirations by those of succinate for each of the samples.

*Mitochondrial Uncoupling*

There were 6 different protocols, some of which were variations of one another, used to test for uncoupling as we wanted to be ensure that the results obtained would not be from a potential uncoupling effect. The first protocol began with the addition of oligomycin (2 μg/ml) followed by succinate (10 mM), then FCCP step titrations (1 μl/step) and finally antimycin A (2.5 μM). The second protocol was the same as the first except GDP (10mM) was added in between the addition of succinate and FCCP. The third protocol consisted of the sequential addition of succinate (10 mM), ADP (5 mM) and then glutamate (10 mM) and malate (2 mM) simultaneously. The fourth protocol included the addition of malate (2 mM), octanoyl carnitine (1.5 mM), ADP (5 mM), glutamate (10 mM), oligomycin (2 μg/ml) and then finally multiple additions of GDP (10mM). The fifth protocol began with the addition of malate (2 mM) then octanoyl carnitine (1.5 mM), glutamate (10 mM), oligomycin (2 μg/ml) and again multiple additions of GDP (10mM). The sixth an final protocol consisted of adding malate (2 mM) then octanoyl carnitine (1.5 mM), oligomycin (2 μg/ml) and then multiple additions of GDP (10mM). Uncoupling protein 2 and 3 (UCP2 and UCP3)-mediated uncoupling could hence be investigated through the titrations of oligomycin, GDP and antimycin A.

*Protein Extraction, Immunoblotting, and Immunofluorescence*

Cell lysates were isolated in lysis buffer containing 250 mM NaCl, 50 mM HEPES (pH 7.5) 10% glycerol, 1% triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM $Na_4P_2O_7$, 1 mM NaF, 800 μM $Na_3VO_4$ and centrifuged at 12,000 × *g* for 10 min. The supernatant was collected and separated on a 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (0.45 μm, 162-0115, Bio-Rad) by using a 10 mM sodium tetraborate buffer. The membranes were blocked in 5% BSA in TBS-T buffer (10 mm Tris-HCl, pH 7.5, 150 mm NaCl, 0.05% Tween 20) for a duration of 1 h at room temperature. Afterward, the membranes were left to incubate overnight at 4°C with primary antibodies: total OXPHOS rodent antibody cocktail (1:4000, MS604 MitoSciences) and beta-tubulin (1:5000, ab6064 Abcam). The blots were washed, incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse, ab6728; anti-rabbit, ab6721; Abcam) and visualized with a chemiluminescence system (Immun-Star Chemiluminescent; 1705070; Bio-Rad). The bands were analyzed using the Image J softwareto analyze protein expression of mitochondrial respirator complexes I through V (CI-CV).

*Statistics*

Summarized data are presented as means ± standard error of mean (SEM) in all figures. Statistical comparisons were done using a two-tailed Student's t-test. For all statistical evaluations, $p < 0.05$ was considered significant. The number of samples is represented by n.

## Results

*Relative Expression of Complexes I-V*

Immunoblotting revealed a statistically significant increase in CI between the young control group and the older control group (100 ± 20.2 and 346.8 ± 50.3, respectively, $p<0.03$) as well as in the older ApoE-/- group (100.0 ± 20.2 and 209.4 ±34.6, respectively, $p<0.05$), normalized to the young control group. These trends can be seen in Figure 1.

The other complexes (CII-CV) showed no statistically significant difference in expression. There was also a significant decrease in protein expression between the older control and older ApoE-/- group (346.8 ± 50.3, respectively, $p<0.05$). These results indicate both the presence of an aging and experimentally induced effect. Please note that the values for the control expression has been set to 100% in order to display relative changes. This does not mean however, that the mitochondrial complexes have identical expression.

*Mitochondrial respiration*

By examining oxygen consumption rates of saponin-permeabilized fibres we determined oxidative capacities of mitochondria in the cardiac muscles in situ along with control of the respiratory activity by ADP, the main regulator. The $O_2$ flux showed an increased response in both the older control and ApoE-/- as compared to controls (data not shown). Residual oxygen consumption (ROX) was similar in both groups (data not shown).

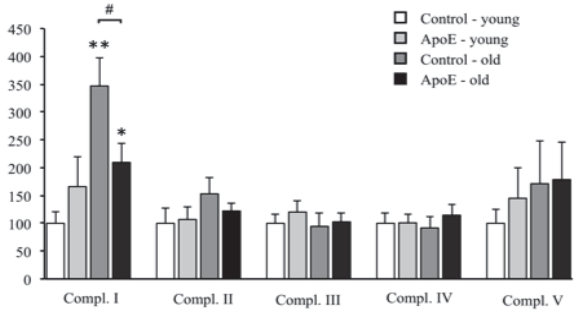


**Figure 1**: Immunoblotting of Complex I to Complex V using total OXPHOS rodent antibody cocktail from MitoSciences (MS604). There is a significant increase between CI of young control and old control (100 ± 20.2 and 346.8 ± 50.3, respectively, $p<0.03$) as well as older ApoE-/- (100.0 ± 20.2 and 209.4 ±34.6, respectively, $p<0.05$) as represented by *. There is also a significant decrease in CI between the old control and the old ApoE group (346.8 ± 50.3, respectively, $p<0.05$) represented by #. The other complexes show no significant increases. The data show both an aging and experimental effect.

Mitochondrial leak, estimated as antimycin A flux rates substracted from oligomycin flux rates, indicated a significant increase in the 8-month-old ApoE-/- group compared to the younger one as seen in Figure 2 (92.6 ± 15.5 vs 46.4 ± 4.72pmol/s/mg, respectively, p<0. 03). Figure 4 shows no significant increase between these two groups in terms of substrate control ratio for succinate (GM3/GMS3) (0.56 ± 0.04 and 0.59 ± 0.03, respectively), neither for the respiratory control ratio (RCR, state 3 over state 4 respiration so succinate divided by oligomycin respiration) as seen in Figure 7 (2.6 ± 0.2 and 2.9 ± 0.4, respectively).

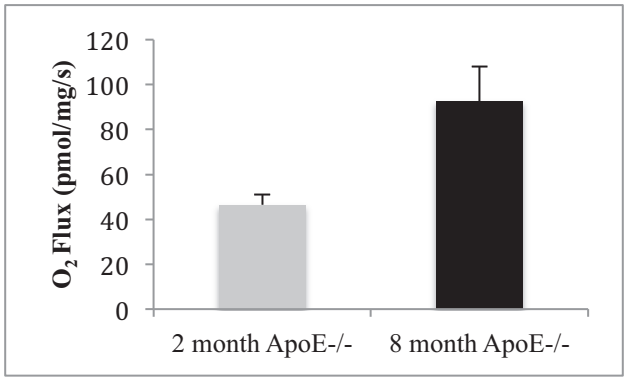


**Figure 2**: Mitochodnrial leak assessed by the difference between oligomycin and anitmycin A average rates of respiration. The data reflect a significantly increased mitochondrial leak in the older ApoE-/- group compared to controls (92.6 ± 15.5 vs 46.4 ± 4.72pmol/s/mg, respectively, $p<0.03$, n=11).

Acceptor control ratio shown in Figure 6 (ACR, maximal, ADP stimulated respiration divided by basal, ADP restricted respiration in other words ADP divided by malate respiration), a ratio representing the degree of coupling between oxidation and phosphorylation showed no significant increases (11.2 ± 0.9 and 13.0 ± 2.23, respectively). There was also no significant difference in the lipid coupling control ratio (L/P) (0.25 ± 0.02 and 0.25 ± 0.02) between these two same groups illustrated in Figure 5. Lipid OXPHOS capacity or the lipid coupling control ratio (L/P) shows the absence of a significant difference between these same two groups. However, there was a significant difference in substrate utilization that being significantly higher in the 8-month ApoE-/- group for the majority of the substrates as seen in Figure 3. Data show a significant increase in the utilization of malate (7.63 ± 1.55 and 12.9 ± 1.11, $p<0.02$), octanoyl carnitine (26.3 ± 5.0 and 42.0pmol/s/mg ± 3.3, $p<0.02$), ADP (86.8 ± 15.5

and 141.6pmol/s/mg ± 14.6, p<0.02), glutamate (104.4 ± 17.0 and 171.4pmol/s/mg ± 17.07, p<0.02), succinate (182.8 ± 27.2 and 289.5pmol/s/mg ± 22.9, p<0.01) by comparing the 2-month ApoE-/- to the 8-month-old ApoE-/- group (n=11). Although oligomycin utilization also significantly increased (64.9 ± 6.5 and 110.7pmol/s/mg ± 15.9, p<0.03) there was no significant change in antimycin A or in ascorbate and TMPD (18.5 ± 3.9 and 18.1pmol/s/mg ± 2.2; 242.0 ± 40.8 and 207.6pmol/s/mg ± 37.4, respectively) between the young and older ApoE-/- groups.

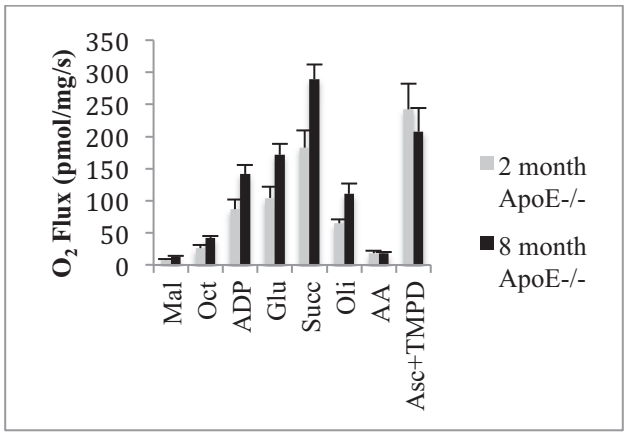


**Figure 3**: Substrate utilization by the mitochondria of permeabilized cardiac myofibers assessed through high-resolution respirometry (n=11). Data show a significant increase in the utilization of malate (7.63 ± 1.55 and 12.9 ± 1.11 , p<0.02), octanoyl carnitine (26.3 ± 5.0 and 42.0pmol/s/mg ± 3.3, p<0.02), ADP (86.8 ± 15.5 and 141.6pmol/s/mg ± 14.6, p<0.02), glutamate (104.4 ± 17.0 and 171.4pmol/s/mg ± 17.07, p<0.02), succinate (182.8 ± 27.2 and 289.5pmol/s/mg ± 22.9, p<0.01) and oligomycin (64.9 ± 6.5 and 110.7pmol/s/mg ± 15.9, p<0.03). Antimycin A and ascorbate+TMPD utilization were not significant (18.5 ± 3.9 and 18.1pmol/s/mg ± 2.2; 242.0 ± 40.8 and 207.6 pmol/s/mg ± 37.4, respectively) between the young and older ApoE-/- groups.

*Uncoupling*

There was no significant uncoupling found using any of the 6 uncoupling protocols (data not shown). The mitochondrial leak observed in this project therefore is not likely attributed to significant mitochondrial uncoupling.

*Aging*

There was an aging effect observed between our 2-month control and 8-month control groups. Despite this aging effect we can still report significant results as previously described.

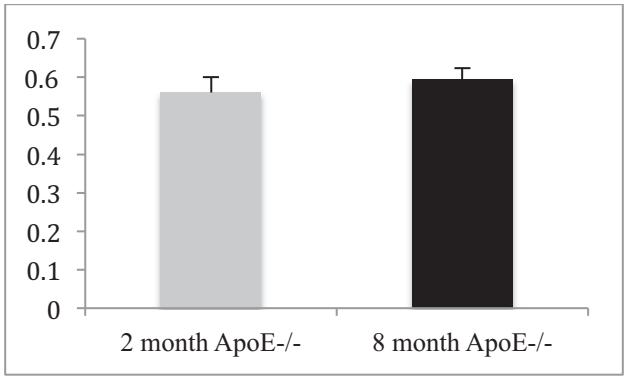


**Figure 4**: Substrate control ratio (SCR) representing how the mitochondria handle different substrates expressed by dividing glutamate by succinate average rates of respiration. Data show a non-significant increase in this ratio from young to the older ApoE-/- group (0.56 ± 0.04 and 0.59 ± 0.03, respectively, n=11).

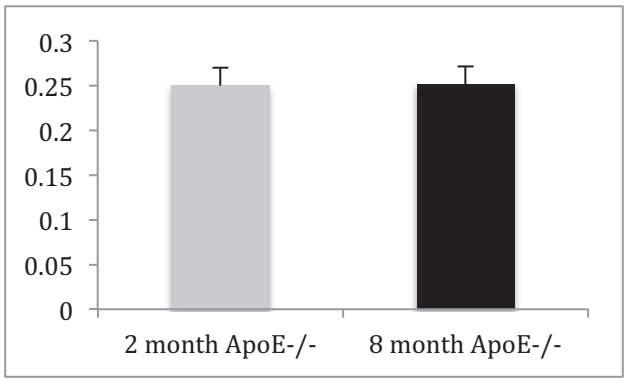


**Figure 5**: Lipid coupling control ratio (L/P) reflecting the efficiency to utilize lipids yet under similar respiratory levels, obatined by dividing octanoyl carnitine by glutamate average rates of respiration. The data show no significant increase in the L/P from the young to the older ApoE-/- group (0.25 ± 0.02 and 0.25 ± 0.02, respectively, n=11).

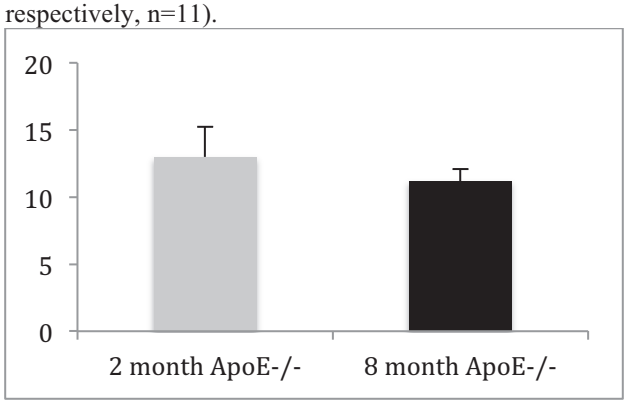


**Figure 6**: The acceptor control ratio (ACR) assesses the relative quality of phosphorylation determined by

dividing ADP by malate average rates of respiration. The data illustrate a non-significant decrease in the ACR of the older ApoE-/- group compared to the younger group (11.2 ± 0.9 and 13.0 ± 2.23, respectively, n=11).

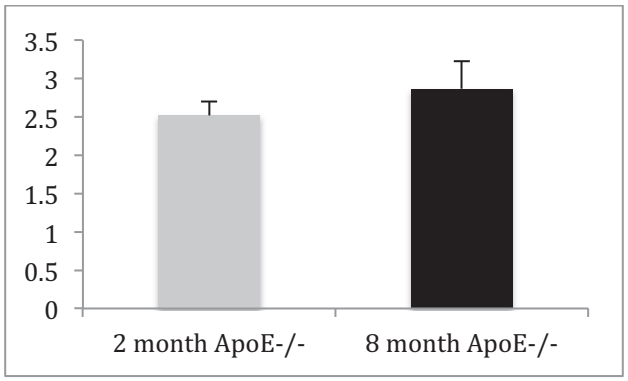


**Figure 7**: The respiratory control ratio (RCR) represents state 3 respiration over state 4 respiration obtained by diving succinate by oligomycin average rates of respiration. The data demonstrate a non-significant increase in the between the young and older ApoE-/- groups (2.6 ± 0.2 and 2.9 ± 0.4, respectively, n=11).

## Discussion

The main finding of this study is that the 8 month control and ApoE deficient group both experienced altered cellular metabolism and mitochondrial protein expression. There may be a shift in lipid levels as it is known that chow fed control mice as used in this study have total plasma cholesterol (PC), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides (TG) levels of around 60, 10, 50 and 65mg/dL respectively [57-60]. In contrast, the ApoE-/- model fed normal show an increase in PC (8-fold), LDL (14-fold), and TG 1.7-fold) with a shift from HDL to very-low-density lipoprotein (VLDL), where most of the PC lipoprotein fractions become rather atherogenic [57-64]. This shift between HDL and VLDL becomes especially prominent with a further increase in these values when these deficient animals are supplemented with a high fatt diet [57, 62, 63, 65]. Other studies have shown that at 8 months of age ApoE-/- mice experience close to 90% occlusion when fed a high fat diet [66], a diet-induced occlusion comparable to humans [67]. It is likely that our results are translatable to a male 8-month old ApoE-/- model considering that it has been shown that as the animals age, the discrepancies between the sexes becomes negligible [68].

As the heart ages there is known to be an excessive generation of oxidative stress thus implicating the mitochondria in the pathogenesis of cardiovascular disease [69, 70] since this stress is detrimental to the heart with a potential to increase the amount of ROS produced. Hence, the paralleled need in research and for clinicians to better understand the effects of aging on cardiac mitochondrial metabolism and bioenergetics. Therefore, this model promotes the study of the interactions between lipid metabolism, cardiac mitochondria and aging. Typically, the cellular effects attributed to aging in turn, promote cardiac aging [71] forming a destructive cycle. Aging induces increased ROS, reduced OXPHOS and mitochondrial dysfunction [72] yet we observed increased mitochondrial respiratory efficiency, which may seem counterintuitive. However, in recent studies mitochondria have proven vital in longevity, stimulating mitochondrial fitness and healthy aging despite age-related cellular changes, for their ability to alter expression of mitochondrial ribosomal protein [73] and their upregulation of cytoprotective activities [72].

Although there was an aging effect between our 2-month control and 8-month control groups we nevertheless saw statistically significant differences between our 2-month ApoE-/- and 8-month ApoE-/- groups, between young and older control groups as well as the older ApoE-/- group. Finally, there was also significance between the older control and older ApoE-/- groups. Furthermore, we found no significant age-related declines in complex III and IV as reported in other studies [34, 28, 29, 74-77]. Meanwhile, complex II only slightly increased as predicted [76-79, 74], seeing as how the proteins in this complex are not encoded for by mitochondrial DNA (mtDNA) [80]. Therefore, previously reported results coincide with our findings. Complex I was the most affected in this study in both aged groups, which is also expected considering 7 of the 13 mtDNA encoded proteins of the respiratory complex are located here and are most affected by aging [81].

Based on the L/P, there was no impairment in lipid oxidation. Voltage-dependent anion channel (VDAC) would have to be performed as well as incorporating cytochrome c to see to determine if mitochondrial density was altered and to further comprehend the basis for the mitochondrial leak effect. One study found ROS generation within the mitochondria during metabolism and was compensated for by means of a proton leak [82]. Uncoupling protein 1 (UCP1) was not found to jeopardize OXPHOS or the production of ATP [82], both of which were not compromised in our study. It is this same UCP1 that contributes to nearly 80% of the proton leak in skeletal muscle mitochondria. Similarly, our observed increase in proton leak cannot be associated with reductions in ATP synthesis, in RCR nor with age-related diminished state 3 respiration, as described in a number of other studies [26]. Energy demands and ROS are no doubt increasing as the cardiac mitochondria metabolize the elevated lipid levels in our study, so perhaps they too generated a proton leak as compensation. VDAC and cytochrome c would also be a useful features as there is a reported decrease in the overall quantity of cardiomyocytes, along with a significant increase in cytochrome c with aging [30]. In addition, VDAC would help in indicating whether any disruptions in equilibrium between mitochondrial biogenesis and mitophagy had occurred with aging and the high lipid availability [83].

**Conflicts of interest**

There are no conflicts of interest related to this project.

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# IV

# Altered Mitochondrial Functioning in Apolipoprotein E-Deficient Mice Induced by a Low-Carbohydrate High-Fat Diet

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## Contribution of Authors

Cynthia Rocha: Animal handling, high-resolution respirometry, euthanized animals, surgical extraction of cardiac tissue, saponin-induced permeabilization, statistical analysis, and preparation of manuscript.

Olivia Koury: Animal handling, immunoblotting, statistical analysis, euthanized animals, surgical extraction of cardiac tissue and preparation of manuscript.

Andreas Bergdahl: Concept development, preparation of manuscript, provided animals, specialized LCHF diet, laboratory space and equipment.

# Altered Mitochondrial Functioning in Apolipoprotein E-Deficient Mice Induced by a Low-Carbohydrate High-Fat Diet

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**Summary:** Low-carbohydrate diets have been growing in popularity in America for decades, proving to be one of the most popular and efficient methods of weight loss. Despite the superficial health benefits this type of diet may contribute in the case insulin resistance, hypertension, and lipid control for example, its implications on mitochondrial oxidative capacity and respiration have yet to be evaluated. The goal of this study was to evaluate the safety of low-carbohydrate high-fat (LCHF) diets by determining whether they foster an environment altering mitochondrial functioning. Male Apolipoprotein E-deficient mice were randomly assigned to either a control diet or low-carbohydrate high-protein diet for 6 weeks. Heart tissue was used for immunoblotting, and high-resolution respirometry (HRR) to quantify mitochondrial complexes and measure oxidative phosphorylation (OXPHOS) capacity. Our results indicate increased expression of all five mitochondrial complexes in the LCHF group compared to the control. Furthermore, HRR revealed increased efficiency of substrate utilization implying improved mitochondrial respiration. These findings correlate well with the understanding that cardiomyocytes prefer lipid as a fuel source, and the shift in metabolism caused by a LCHF diet provides such a favourable environment.

**Keywords:** Apolipoprotein E • cardiac mitochondria• oxidative capacity •bioenergetics• low-carbohydrate high-protein

The recent rise in obesity has been associated with a number of adverse health effects such as heart disease, certain types of cancer, type-2 diabetes mellitus, and respiratory complications [1]. In efforts to reduce associated health risks, overweight and obese patients have been advised to maintain a healthy BMI. One of the most efficient means of weight loss is through dieting, more specifically a low-carbohydrate. This diet has gained much popularity with the American public [2], and advocates claim it 'burns more fat' and leads to quicker weight loss without adverse long-term effects [3]. Macronutrient composition is altered in a low-carbohydrate diet, drastically affecting the cellular metabolism. Although the same energy sources are utilized, majority of the energy is now derived from fatty acids and ketones due to the extreme reduction of carbohydrate. This shifts metabolism from a 'glucocentric' source to an 'adipocentric' energy source [4]. In low-carbohydrate high-fat (LCHF) metabolism, 70% of caloric requirements come from fatty acids via dietary fat or lipolysis, 20% come from ketone bodies acetoacetate and β-hydroxybutyrate, and

10% from glucose via gluconeogenesis or glycogenolysis [5]. Low-carbohydrate diets have been studied extensively in the past decades regarding weight loss, lipid markers, insulin sensitivity, hypertension, and endothelial dysfunction [6-11]. However, no study has addressed a possible link with LCHF diets and heart failure due to cardiac mitochondrial dysfunction.

Despite a general decrease in the number of deaths from cardiovascular disease (CVD) in the United States, congestive heart failure (CHF) remains as prevalent today as it was two decades ago [12]. Although very broadly defined, CHF can be best described as a multifactorial syndrome that renders the myocardium unable to pump blood efficiently to sustain demand [13]. Oxidative stress is now understood to be a key factor in heart failure, and a main reason for oxidative stress is the imbalance and negative impact of reactive oxygen species (ROS) produced by the mitochondria [14-18]. In recent years, studies have linked characteristics of heart failure to defective mitochondrial energetics and abnormal substrate metabolism [19- 21]. There is significant evidence showing mitochondrial dysfunction not only in cardiomyocytes, but also skeletal muscle of heart failure patients [22]. Aside from mitochondrial dysfunction, other problems that may contribute to the noticeable energetic defects are the number of mitochondria, substrate of choice, or oxidative capacity [23]. These facts imply that preserving cardiac mitochondrial functioning can become a future therapeutic aim for CHF [24-26].

Mitochondria are potentially significant sources of ROS especially when damaged (i.e. by long-term FA accumulation,) where they exhibit decreased oxidative based energy and thus less fuel for cells [27]. This is a central concern since the mitochondrion is the organelle in which energy (ATP) production and cellular respiration occur [28, 29]. Considering the maintenance of ATP drives all cellular processes, this process is imperative for a high-level workload organ such as the heart due to its high-energy demands [25, 30-33]. The capacity of the mitochondria relies heavily on substrate availability, notably lipids and carbohydrates. These substrates contribute to energy metabolism depending on their utilization through β-oxidation and the Kreb's cycle. β-oxidation produces acetyl-CoA from FAs that enters the Kreb's cycle, generating electrons that are successively transported to the electron transport system (ETS) by NADH and $FADH_2$ to amplify ATP production [34]. The Kreb's cycle is the focal common aerobic metabolic pathway for carbohydrates, lipids and proteins [35]. Acetyl-CoA oxidation by this cycle is responsible for two thirds of the sum of ATP production and oxygen consumption [36].

Despite glucose and FAs both being oxidized in the mitochondria to ATP production in the cardiomyocytes, the latter is the preferred substrate (generating 70% of total ATP) [37-39]. Therefore, the uptake of FA from the plasma is vital for cardiac viability [21, 37- 40]. Furthermore, lipids have also been deemed necessary for the maintenance and re-initiation involved with the pure beating of heart cells, further highlighting their significance [41]. The heart has limited substrate storage capacity, thus the uptake of nutrients needs to be finely balanced since the underlying pathways have to respond both appropriately and competently to the continuous flux in energy demand and substrate availability [25]. Under normal conditions the mitochondria maintain a fine equilibrium between glucose and FAs, however, during physiological stress, the mitochondria shifts towards an increased carbohydrate metabolism. This shift adds stress to the heart and shunts the lipids into non-oxidative pathways creating more ROS than energy [42]. The activity of respiratory complex I in the mitochondria is especially prone to the effects of oxidative damage [43]. The same detrimental phenomenon occurs when there is a reduced oxygen supply as a result of the occlusion of the coronary vessels, a major cause of cardiovascular distress [44].

The mitochondria are an important health research target considering their role in generating cellular energy, by means of oxidative phosphorylation, a process affected by uptake of cholesterol and lipids from the plasma. The subsequently altered role of mitochondria may potentially be implicated in the

pathophysiology of heart failure [45] and overall cardiovascular health. The apolipoprotein E deficient (ApoE-/-) murine model is a well-established model as it allows for the investigation of atherosclerosis due to its susceptibility to develop lesions rapidly, and similarly to humans [46-48]. ApoE is a 34 kDa glycoprotein made in the liver and brain, responsible for lipoprotein metabolism [48]. It helps to clear circulating cholesterol by mediating the binding of ApoE-containing lipoproteins and LDL receptors [49-52].

This study examined whether ApoE-/- mice exhibit altered cardiac mitochondrial oxidative capacity when exposed to a LCHF environment. We quantified cardiac mitochondrial consumption in a 6-week old ApoE-/- murine model in comparison to age-matched controls. The results determined if alterations in energy metabolism had occurred by examining the oxidative capacity of the mitochondrial respiratory complexes. We hypothesized that the mitochondria from the ApoE-/- cardiac tissue would experience increased respiratory function and disturbed energy metabolism as compared to controls.

**Methods**

*Animal Care*

Apolipoprotein E knockout (ApoE-/-) mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and used for breeding. The resulting litters were weaned and separated based on sex at 21-28 days. Only the tissue from male mice was used for the purpose of this study. The males were housed individually in a thermo-neutral environment (22ºC), on a 12:12 h photoperiod, and randomly assigned to either a control diet (CON) or a low-carbohydrate high-fat (LCHF) diet for 6 weeks. Both groups of mice had access to water and their respective diets ad libitum. All procedures were approved by the Animal Ethics Committee of Concordia University (protocol ID: #30000259) and were conducted in accordance with guidelines of the Canadian Council on Animal Care.

*Diet Specifications*

Both CON and LCHF animal diets were iso-caloric. Table 1 displays the diet specifications in terms of percentage of caloric intake from fat, carbohydrates (CHO) and protein. The CON diet, 5075 Charles River Autoclavable Rodent Diet, reflects a healthy, standard macronutrient distribution. Whereas, the LCHF diet, obtained from Harlan Laboratories (TD.04524) simulates an Atkin's diet used for weight loss and contains 43% from fat, 11% CHO, and 46% protein. The LCHF diet is a modification of TD.88137 (Harlan Laboratories), used for studies on atherosclerosis.

*Experimental protocol*

The beating heart was removed immediately after euthanasia with $CO_2$ according to the approved animal protocol and split into two different portions. One portion was snap frozen in liquid nitrogen, and stored at -80ºC for biochemical analysis; the other portion (the apex) was placed in an ice cold relaxing buffer (BIOPS) and used immediately to measure mitochondrial respiration. The BIOPS contains (in mM): $CaK_2EGTA$ 2.77, $K_2EGTA$ 7.23, $Na_2ATP$ 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, Taurine 20, $Na_2$Phosphocreatine 15, Imidazole 20 mM, Dithiothreitol 0.5, MES 50, pH 7.1

*Preparation of permeabilized cardiac fibers*

The apex was dissected for preparation of permeabilized myofibers. This was done by gentle dissection during which the fiber bundles were separated using sharp forceps. The fibers were then incubated in 3 ml BIOPS buffer containing 50 µg/ml saponin for 30 minutes and subsequently washed in ice-cold buffer (MiR05) for 2 x 10 min. MiR05 contains (mM): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3.0, K-lactonionate 60, Taurine 20, $KH_2PO_4$ 10, HEPES 20, Sucrose 110, BSA 1g/l, pH 7.1.

*Mitochondrial respiratory measurements*

Measurements of oxygen consumption were performed in MiR05 at 37ºC using a polarographic oxygen sensor (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Approximately 2.0 to 2.5 mg of muscle tissue (wet weight) was place in either chamber in a cross-sectional design. $O_2$ flux was resolved by DatLab and all experiments were carried out in hyperoxygenated levels to avoid $O_2$ diffusion limitations. A sequential substrate addition protocol was used to allow functional dissection of the electron transport system:

state 2 respiration (absence of adenylates) was assessed by addition of malate (2 mM) and octanoyl carnitine (1.5 mM), by adding ADP (5 mM) we could reach state 3 respiration for complex I. This was followed by addition of glutamate (10 mM) and succinate (10 mM) achieving maximal coupled state 3 respiration with parallel electron input to complex I and II. Oligomycin (2 μg/ml) was then added to block complex V and thereafter antimycin A (2.5 μM) to inhibit complex III. Finally ascorbate (2 mM) and TMPD (500 μM) were added to evaluate Complex IV respiration. These mitochondrial respiratory measurements were recorded for both the control group and the experimental group fed the LCHP diet.

*Mitochondrial Uncoupling*

There were 6 different protocols, some of which were variations of one another, used to test for the presence of uncoupling as we wanted to ensure that the results obtained would not be from a potential uncoupling effect. A portion of the apex tissue mentioned previously was also studied using high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) to test for uncoupling, running simultaneously along side the mitochondrial respiratory experiments. The first protocol began with the addition of oligomycin (2 μg/ml) followed by succinate (10 mM), then FCCP step titrations (1 μl/step) and finally antimycin A (2.5 μM). The second protocol was the same as the first except GDP (10mM) was added in between the addition of succinate and FCCP. Therefore, uncoupling protein 1 (UCP1)-mediated uncoupling could be studied through the titrations of oligomycin, GDP and antimycin A. The third protocol consisted of the sequential addition of succinate (10 mM), ADP (5 mM) and then glutamate (10 mM) and malate (2 mM) simultaneously. The fourth protocol included the addition of malate (2 mM), octanoyl carnitine (1.5 mM), ADP (5 mM), glutamate (10 mM), oligomycin (2 μg/ml) and then finally multiple additions of GDP (10mM). The fifth protocol began with the addition of malate (2 mM) then octanoyl carnitine (1.5 mM), glutamate (10 mM), oligomycin (2 μg/ml) and again multiple additions of GDP (10mM). The sixth an final protocol consisted of adding malate (2 mM) then octanoyl carnitine (1.5 mM), oligomycin (2 μg/ml) and then multiple additions of GDP (10mM).

*Protein Extraction, Immunoblotting, and Immunofluorescence*

Tissue from the cross-section excised from right above the apex was pulverized/homogenized using liquid nitrogen with ~150μl lysis buffer containing (in mM) 250 NaCl, 50 HEPES (pH 7.5), 10% glycerol, 1% triton X-100, 1.5 $MgCl_2$ 1 EGTA, 10 $Na_4P_2O_7$ NaF, 800 μM $Na_3VO_4$. After 1 h on ice the cell slurry was centrifuged at 13,000 rpm for 10 min, and the supernatant was collected. 10μl of lysate was mixed with 2μl DTT and 2μl sample buffer and loaded on a 12.5% acrylamide-SDS gel followed by a transfer onto a 0.45μm nitrocellulose membrane (162-0115 Bio-Rad) in 10mM sodium tetraborate buffer. Ponceau staining was done as loading control before the membranes were blocked in 3% bovine serum albumin (BSA) in 0.1% Tween in Tris-buffered saline (TBS) (10 mm Tris-HCl, pH 7.5, 150 mm NaCl) for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibody, total OXPHOS rodent antibody cocktail (1:2000, MS604 MitoSciences). The membranes were washed, and incubated with secondary antibody (1:15000, anti-mouse, ab6728 Abcam). Membranes were exposed with ECL chemiluminescence (Immun-Star Chemiluminescent; 1705070; Bio-Rad) and developed bands were analyzed with Image J Software.

*Statistical Analysis*

Summarized data are presented as means ± standard error of the mean (SEM) for immunoblotting, and standard estimate. Statistical comparisons were done using a two-tailed Student's t-test. n represents the sample number. For all statistical evaluations, In the figures presented, *p< 0.05, ** p< 0.01.

**Results**

*Relative Expression of Complexes I-V*

Immunoblotting revealed an increase in all five complexes in the LCHF diet, normalized to the CON. These trends can be seen in Figure 1 however, only CIV showed a statistically significant difference in

expression (CON: 100% ±5.7, LCHP: 122% ±4.8, p< 0.05 n= 6). Therefore, the only significant change in protein expression was in CIV when running samples from CON and LCHF hearts in parallel.

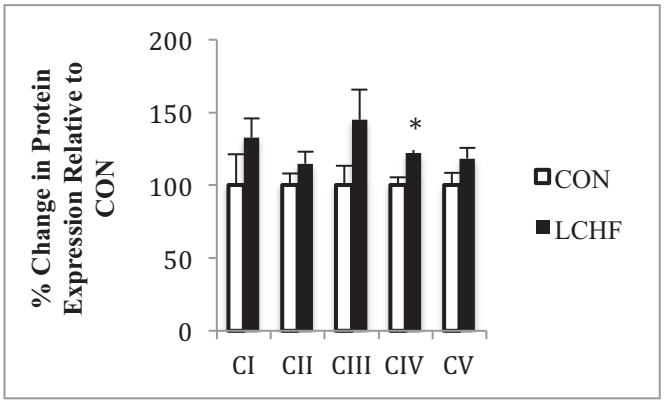


***Figure 1:*** *Immunoblotting of Complex I to Complex V using total OXPHOS rodent antibody cocktail from MitoSciences (MS604). Trends show an increase in all 5 complexes in the LCHP diet relative to the CON diet (CI 100%± 21.4 and 132.7%± 13.1, CII 100%± 8 and 114.8%± 8.5, CIII 100%± 13.3 and 144.9± 20.8, CIV 100%± 5.7 and 122.1%± 5.7 p<0.02, CV 100%± 8.4 and 188.5%± 7.3, respectively, n=6).*

*Mitochondrial respiration*

We examined oxygen consumption rates of saponin-permeabilized fibres to determine oxidative capacities of mitochondria in the cardiac muscles in situ along with control of the respiratory activity by ADP, the principal regulator. Mitochondrial leak, estimated as antimycin A flux rates subtracted from oligomycin flux rates, indicated a significant increase in the LCHF group as compared to the control group as seen in Figure 3 (52.4 ± 16.1 and 102.2 ± 12.1pmol/s/mg, respectively, p<0.03). There was also a significant difference between these two groups in terms of substrate control ratio for succinate (GM3/GMS3) (0.62 ± 0.08 and 0.44 ± 0.02, respectively, p<0.05) as illustrated in Figure 4. Neither the respiratory control ratio (RCR, state 3 over state 4 respiration so succinate divided by oligomycin respiration) (2.2 ± 0.8 and 3.0 ± 0.6 ) or the acceptor control ratio (ACR, maximal, ADP stimulated respiration divided by basal, ADP restricted respiration in other words ADP divided by malate respiration) (8.5 ± 1.4 and 12.0 ± 1.4), respectively, a ratio representing the degree of coupling between oxidation and phosphorylation showed any significant differences as seen in Figures 5 and 6. Therefore there was no indication of a likely more efficient oxidation of substrates, nor a significant change within the OXPHOS process. Lipid OXPHOS capacity shown in Figure 7 shows the absence of a significant difference in the lipid coupling control ratio (L/P) between these two same groups (0.33 ± 0.04 and 0.26 ± 0.03). However, there was a significant difference in substrate utilization, as seen in Figure 2. Data show a significant increase in the utilization of octanoyl carnitine (28.3 ± 2.8 and 38.4pmol/s/mg ± 2.9, p<0.03), ADP (75.0 ± 5.6 and 140.0pmol/s/mg ±25.1, p<0.03), glutamate (87.2 ± 5.1 and 161.8pmol/s/mg ± 25.9, p<0.02), succinate (155.5 ± 27.0 and 362.8pmol/s/mg ± 47.9, p<0.01), oligomycin (74.8 ± 14.8 and 122.1pmol/s/mg ± 13.5, p<0.04) and ascorbate+TMPD (212.5 ± 24.8 and 292.9pmol/s/mg ± 19.5, p<0.03) by the LCHP group compared to controls (n=6). Malate and antimycin A utilization were not significant (9.7 ± 1.2 and 11.6pmol/s/mg ± 1.34; 22.4 ± 4.3 and 20.0 pmol/s/mg ± 3.5, respectively). The $O_2$ flux showed an increased response in the LCHF group as the substrates were titrated as compared to controls (data not shown). Residual oxygen consumption (ROX) was similar in both groups (data not shown).

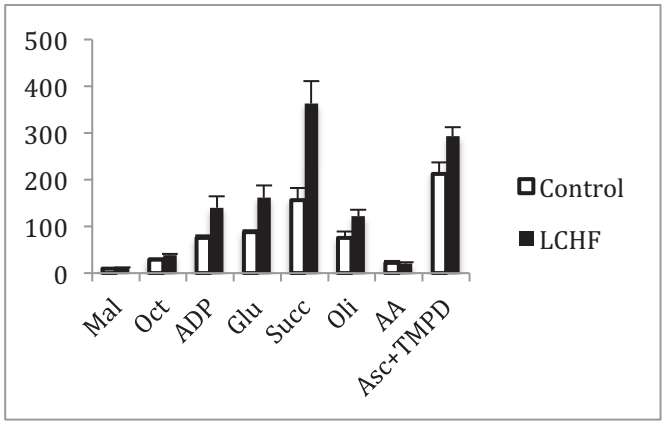


***Figure 2****: Substrate utilization by the mitochondria of permeabilized cardiac myofibers assessed through high-resolution respirometry. Data show a significant increase in the utilization of octanoyl carnitine (28.3 ± 2.8 and 38.4pmol/s/mg ± 2.9, p<0.03), ADP (75.0 ± 5.6 and 140.0pmol/s/mg ±25.1, p<0.03), glutamate (87.2 ± 5.1 and 161.8pmol/s/mg ± 25.9, p<0.02), succinate (155.5 ± 27.0 and 362.8pmol/s/mg ± 47.9, p<0.01), oligomycin (74.8 ± 14.8 and 122.1pmol/s/mg ± 13.5, p<0.04) and ascorbate+TMPD (212.5 ± 24.8 and 292.9pmol/s/mg ± 19.5, p<0.03) by the LCHF group compared to controls (n=6). Malate and antimycin A utilization were not significant (9.7 ± 1.2 and 11.6pmol/s/mg ± 1.34; 22.4 ± 4.3 and 20.0 pmol/s/mg ± 3.5, respectively).*

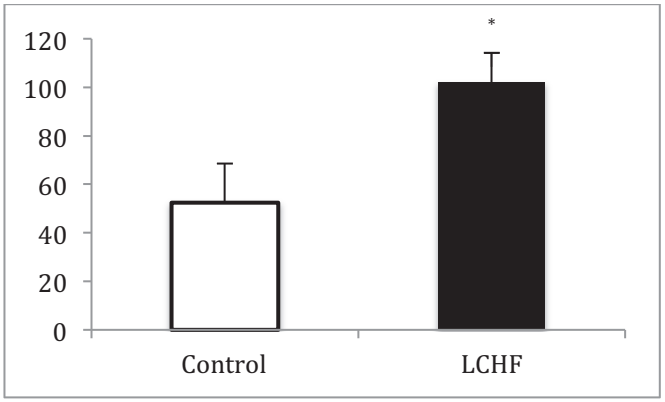


***Figure 3**:Mitochodnrial leak assessed by the difference between oligomycin and anitmycin a average rates of respiration. The data reflect a significantly increased mitochondrial leak in the LCHF group compared to controls (102.2 ± 12.1 vs 52.4 ± 16.1pmol/s/mg, respectively, p<0.03, n=6).*

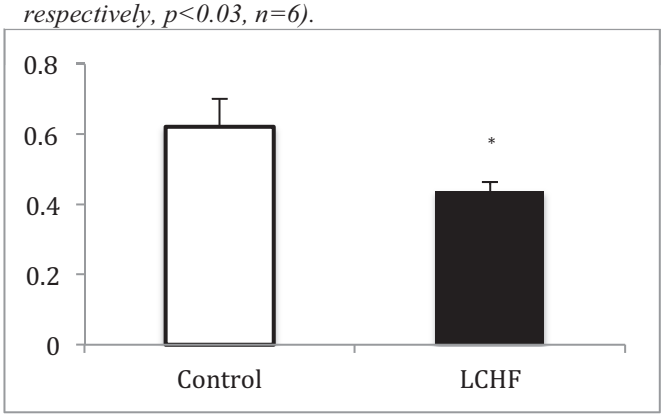


***Figure 4**: Substrate control ratio (SCR) representing how the mitochondria handle different substrates expressed by dividing glutamate by succinate average rates of respiration. Data show a significant decrease in this ratio from control to the LCHF group (0.62 ± 0.08 and 0.44 ± 0.02, respectively, p<0.05, n=6).*

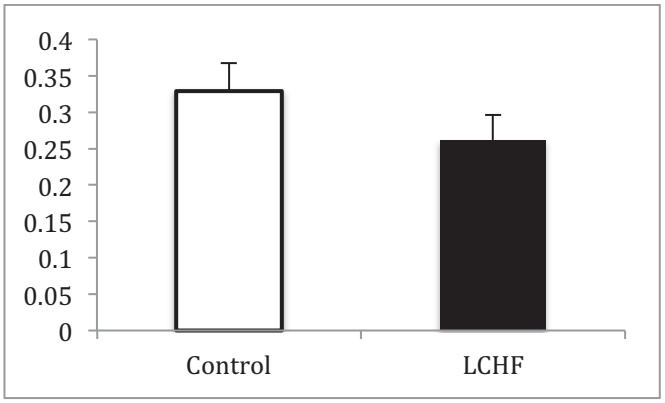


***Figure 5**: Lipid coupling control ratio (L/P) reflecting the efficiency to utilize lipids yet under similar respiratory levels, obtained by dividing octanoyl carnitine by glutamate average rates of respiration. The data show a non-significant decrease in the L/P from the control to the LCHF group (0.33 ± 0.04 and 0.26 ± 0.03, respectively, n=6).*

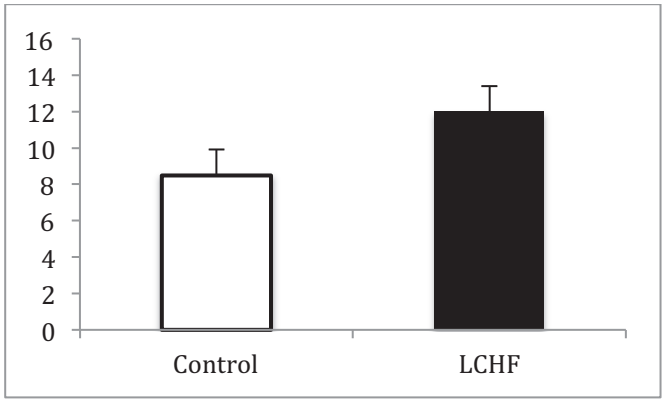


***Figure 6**: The acceptor control ratio (ACR) assesses the relative quality of phosphorylation determined by dividing ADP by malate average rates of respiration. The data illustrate a non-significant increase in the ACR of the LCHF group compared to the control group (12.0 ± 1.4 and 8.5 ± 1.4, respectively, n=6).*

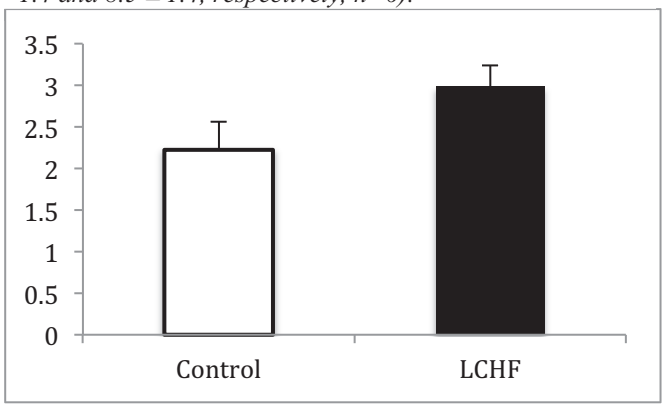


***Figure 7**: The respiratory control ratio (RCR) represents state 3 respiration over state 4 respiration obtained by diving succinate by oligomycin average rates of respiration. The data demonstrate a non-significant increase in the RCR of the control group in comparison to the LCHF group (2.2 ± 0.3 and 3.0 ± 0.3, respectively, n=6).*

**Discussion**

The main finding of this study is that animals assigned to a LCHF diet experience a drastic shift in cellular metabolism due to altered macronutrient distribution, favoring an increase in the efficiency of mitochondrial respiration. Taken together, the significant decrease in SCR and increase substrate utilization, specifically of ADP, indicate an increase in the efficiency of the mitochondria to metabolize different substrates. This observation was paralleled by protein expression using immunoblotting, as complexes 1 to 5 were all increased

in the LCHF diet compared to the CON, with a significant increase in complex 4. The relatively high lipid content in the LCHP diet is most probably what impacts the mitochondria in this study rather than the elevated protein content, considering the two main fuel sources for cardiac mitochondria are carbohydrates and lipids. The low-carbohydrate content shifts mitochondrial metabolism in the heart towards its preferred fuel, lipids [53]. Due to the lack of ApoE in the CON and LCHF groups, there is a baseline increase in serum fatty acid levels, which is only supplemented with additional lipid substrate availability in the LCHF group due to the altered macronutrient composition. This may lead to an even more prominent substrate metabolism shift as previously mentioned, that would promote a greater dependency on fatty acids as the source of energy as seen in previous studies [54].

One possible hypothesis to explain our results would be that it may be in fact cytochrome c that is affected by the high lipid environment induced by the LCHF diet and not directly complex IV. The extremely basic and minute hemoprotein, cytochrome c, is plentiful in the mitochondrial intermembrane space. Its main two roles are transporting electrons in mitochondrial respiration and by being released into the cytosol from the mitochondria, it subsequently activates the apoptotic pathway [55-58]. The upregulation of complex IV may be a compensation for how the relatively high lipid content in this diet affects cytochrome c. The second possible hypothesis that could contribute to better interpreting our results would be an upregulation in β-oxidation responding to the elevated fatty acid availability. Complex I and II are less important in creating a proton gradient however, β-oxidation likely provides an extra stimulus somewhere between complexes I or II and complex IV promoting the extra surge of shuttled electrons into complex IV. Cytochrome c's role would also be somewhat implicated in this potential explanation as well.

Fatty acid metabolism has a higher oxygen cost as compared to carbohydrate metabolism which could lead to ischemic-like conditions when the mitochondria experience a prolonged exposure to a high lipid environment. Studies have shown that ischemia leads to a loss of cytochrome c (59). Therefore in the LCHF group, there may have been a change in the presence of cytochrome c thus in turn affecting complex IV and the electron leak from the cardiac mitochondria, altering overall mitochondrial respiration.

All in all, these speculations are in accordance with our findings since there was an increase in substrate utilization yet a decrease in SCR, implying a decrease in complex I. ADP being significantly increased in the LCHF group along with the significantly increased expression of complex IV could translate into an increased electron flow somewhere in the path towards complex IV, a critical component in removing electrons from the system. This is typically evaluated through effects on cytochrome c.

The significant increase in mitochondrial leak despite no presence of an uncoupling effect, nor a change in the degree of coupling between oxidation and phosphorylation, as seen with the L/P ratio, would have to be further evaluated for thorough understanding of this effect. In addition, variations in mitochondrial density should be assessed with expression of voltage-dependent anion channel (VDAC) using immunoblotting. To further evaluate the hypothetical interpretations of our data, we could monitor changes when using cytochrome c during high-resolution respirometry titrations using the Oxygraph in supplemental experiments, to confirm whether it is indeed affected by this high lipid environment.

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**Conflicts of interest**

There are no conflicts of interest related to this project.

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# V

# Closing Remarks

## Closing Remarks

The aim of this thesis was to investigate whether elevated plasma lipid levels and age impact energetic demands of the heart and substrate metabolism. These alterations could be critical in affecting cardiac performance. Any important findings regarding modified bioenergetics of the heart holds immense promise, considering the clinical applications to the growing numbers of people afflicted with CVD every day. A greater understanding of the influence and mechanism of altered oxidative capacity of the heart is significant, since the heart consumes the most oxygen in the body.

Using females was unique yet highly applicable, considering the non-sex discriminating pathogenesis of CVD and its comorbidities and sex differences in lipid profiles. The interpretation of our results will contribute to understanding the overall influence of ApoE's disrupted role in lipid metabolism on cardiac mitochondrial oxidative capacity. Our findings also further reinforce the relevance of this model even today.

Both a short-term and prolonged exposure to a high-lipid environment proved to affect the mitochondria in a potentially beneficial manner, regardless of age-related effects in the latter. Even when the cardiac mitochondria were supplemented with additional lipids through the LCHF diet, saturating their plasma lipid levels, there were compensatory adaptations in metabolism and bioenergetics that ensued, which may prove to be advantageous. Therefore, this thesis will hopefully contribute to removing the stigma surrounding lipids and the belief that all they cause is harm. All fats however, are not created equal. There is a caveat in that processed fats will continue to be harmful for overall health but the appropriate types of fat abundantly present among other nutrient,s do promote benefits in cellular respiration and metabolism as shown in the cardiac mitochondria. It would thus potentially harmful to oxidative tissues such as

the heart to eliminate fats as is done in most forms of dieting. Finally, just because an over abundance of lipids proves to be advantageous and induces improved functioning in the heart, we would have to examine the effects on any of the surrounding tissues. It is likely that there is delicate balance in the amount of lipids that can be beneficial to both the heart and other parts of the body. However, this thesis would support the efficacy of lipids in protecting the mitochondrial function within the heart.

If given the opportunity, it would be useful to conduct additional experiments to see if chronic lipid exposure in even older animals would diminish the alterations observed in mitochondrial respiration. Probing for VDAC expression and adding cytochrome c during HRR or may provide a more comprehensive view of altered mitochondrial metabolism. I would like to use the same techniques along with mass spectrometry to see how others vital oxygen-dependent organs, such as the brain and liver, would be affected by cardiac mitochondrial adaptations to high lipid levels and aging. New information can expand our knowledge of physiological processes in the heart, how they may become susceptible to pathogenesis or destructive pathways and suggest possible future research targets for prospective treatments. This thesis further supports the potential use of lipids in cardiac rehabilitation.

# VI

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