THE ROLE OF INDIVIDUAL n-6 POLYUNSATURATED FATTY ACIDS ON REGULATING CARDIOVASCULAR REACTIVITY

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE COLLEGE OF GRADUATE STUDIES
(Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Okanagan)

September 2019

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Abstract

Linoleic acid (LA), the major dietary n-6 polyunsaturated fatty acid (PUFA) is known to aggravate cardiovascular diseases (CVD) by inducing mitochondrial oxidative damage. Along with mitochondrial damage, CVD patients often have higher LA in blood plasma, as a result of increased dietary LA or its defective removal. We hypothesized that improving mitochondrial oxidative stress can accelerate LA removal, and lower vascular reactivity. Globally blocking LA bioconversion lowered mitochondrial redox potential, and increased the expression of pro-hypertensive adrenergic receptor α-1a in cardiomyocytes (Aim 1) in vitro. Using heterozygous elongase 5 (Elov5) mice with impaired removal of LA, a higher LA accumulation as well as an increased aortic pressure was identified in Elov5+/− mice, compared to Elov5+/+ mice using Pulsed-wave Doppler (Aim 2). Interestingly, limiting LA intake could not overcome the genetic deficiency of Elov5 and demonstrated similar aortic pressures in mice fed with olive oil. This indicates a crucial role of Elov5 in maintaining LA concentrations in vivo, as well as vascular reactivity. In addition, Elov5+/− mice had lower glutathione (GSH) regulating genes in the heart. GSH is a master antioxidant and can alter mitochondrial oxidative damage. Indeed, addition of GSH ethyl ester lowered oxidative stress and improved LA removal in cardiomyocytes in vitro.

Next, we used EndoG+/− (endonuclease G) mice, lacking a mitochondrial endonuclease which cleaves mtDNA under oxidative stress. EndoG+/− protected against GSH loss in heart under high n-6 PUFA diet. Such effects were confirmed in vitro when over-expression of EndoG damaged mitochondrial function in fibroblasts treated with LA (Aim 3). In conclusion, accumulation of LA either by dietary or genetic approach potentiates vascular reactivity. We also identified two targets that can potentially lower LA accumulation and vascular and cardiac reactivity, i.e. a)
Elov15 and b) GSH, both of which can protect the mitochondria and ensure LA removal to prevent against CVD.

Keywords: PUFA, LA bioconversion, CVD, Elov15, heart, GSH
Lay Summary:

Cardiovascular Disease (CVD) is a chronic inflammatory disease which is affected by long-term eating habit such as food choices and daily calorie intake. There is no drug to cure this type of diseases. The best strategy is to prevent the development of CVD. Numerous studies showed the connection between high n-6 PUFA and the risk of CVD. However, the mechanisms that govern this dietary n-6 PUFA derived cardiac toxicity have not been established. My thesis provides clues as to why n-6 PUFA can accumulate in the heart and provides potential ways to mitigate such an occurrence. They are:

1. It is n-6 PUFA bio-converting efficiency that matters to avoid further CVD development.
2. Protecting mitochondrial oxidative damage is the pre-condition of keeping n-6 PUFA bioconversion at a higher rate.

I hope that these findings are further investigated to provide means for potential drug development, therefore preventing n-6 PUFA toxicity.
Preface:

Chapter 2 and 3:

Omega-6 PUFA bioconversion regulates alpha-1-adrenoceptor expression in mice
Jiayu Ye, Amy Botta, John Winkler, Sukhinder Kaur Cheema, Ismail Laher, Sanjoy Ghosh (In prep)

C57/Bl6 mice feeding and aortic contractile measurements were completed by Amy Botta, Sukhinder Kaur Cheema and Ismail Laher. ex vivo macrophages data were collected by John Winkler. These data are not shown in this thesis.

Chapter 4:

Gpx1 deficiency decreases cardiac Elovl5 by inhibiting LA bioconversion under high n-6 PUFA diet.

Jiayu Ye, Lisa Renaud, Amy Botta, Sanjoy Ghosh (In prep)

Gpx1+/- mice feeding and heart echocardiography were completed by Lisa Renaud. C57/Bl6 control mice data were completed by Amy Botta. These data are not shown in this thesis.

All data displayed in this PhD thesis are completed by Jiayu Ye. All animal experiments conducted in this thesis were reviewed and approved by the UBC animal care committee.
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<tbody>
<tr>
<td>13-HODE</td>
<td>13-Hydroxyoctadecadienoic acid</td>
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<td>9-HODE</td>
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<td>Ac-DEVD-AMC</td>
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<td>Adr</td>
<td>adrenergic receptors</td>
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<td>α-linolenic acid</td>
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<td>ALDH2</td>
<td>aldehyde dehydrogenase 2</td>
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<td>DCF-DA</td>
<td>dichloro dihydro fluorescein diacetate</td>
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<td>Dia. BP</td>
<td>diastolic blood pressure</td>
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<td>end distole volume</td>
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<td>FS</td>
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<td>GC</td>
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<td>Gclc</td>
<td>glutamate—cysteine ligase</td>
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<td>Gclm</td>
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<td>glutathione peroxidase</td>
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<td>glutathione</td>
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<td>glutathione reductase</td>
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<td>IFN-γ</td>
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<td>IL-13</td>
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<td>interleukin 15</td>
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<td>IL-2</td>
<td>interleukin 2</td>
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<td>IVSd</td>
<td>interventricular septal end diastole</td>
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<td>interventricular septal end systole</td>
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<td>5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide</td>
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<td>KC</td>
<td>keratinocyte chemoattractant</td>
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<td>LC-PUFA</td>
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<tr>
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<td>LED</td>
<td>LA+EPA+DHA</td>
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<tr>
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<td>left ventricular mass</td>
</tr>
<tr>
<td>LVIDd</td>
<td>left ventricular internal diameter end diastole</td>
</tr>
<tr>
<td>LVIDs</td>
<td>left ventricular internal diameter end systole</td>
</tr>
<tr>
<td>LVOT</td>
<td>left ventricular outflow tract</td>
</tr>
<tr>
<td>LVOT DIST</td>
<td>LVOT distance</td>
</tr>
<tr>
<td>LVOT VTI</td>
<td>left ventricular outflow tract velocity time integral</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
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<td>methonal</td>
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<td>mitochondrial membrane potential</td>
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<td>MUFA</td>
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<td>NAC</td>
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<tr>
<td>ND5</td>
<td>mitochondrial encoded NADH: ubiquinone oxidoreductase core subunit 5</td>
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<td>peroxisome proliferator-activated receptor-β</td>
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<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
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<td>PWd</td>
<td>posterior wall thickness diastole</td>
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<td>PW-Doppler</td>
<td>pulsed-wave Doppler</td>
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<tr>
<td>PWs</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>α1-adr</td>
<td>α1-adrenergic receptors</td>
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Acknowledgements

First I sincerely thank China Scholarship Council (CSC) that has paid off all my living expenses in the last 4 years, which allowed me to focus on my research. Also, thanks to the faculties and staff in Biology Department of UBC Okanagan, especially Barb Lucente that offers me continuous TAship and University Graduate Fellowship, to cover all my tuition fees. Without these financial supports, I cannot have the achievement I have made today.

Secondly, I would like to extend my special thanks to all my colleagues who helped me to improve English, conduct experiments, and most importantly, to be friends with me. Previous lab mate Dr. Amy Botta who taught me to use gas chromatography, which was the building blocks of all my experiment. Also thanks for John Winkler, who discussed data or research with me without reservation. I’m highly appreciated his time which really means a lot to my research. Thanks to Lisa Renaud for being my friend, as well as helping me to do actual lab work. Without her, I cannot complete my lab work so that this thesis is not able to be seen. A special thanks to Natasha Haskey who helped me the correct my thesis language chapter by chapter. I also want to thank other colleagues helped me in the last four years including Sandeep Gill, Mitchell Figura, Svetlana Smitchouk, Candice Quin and Jacqueline Barnett. Most importantly, thanks to all my committee members Dr. Deanna Gibson, Dr. Andis Klegeris, Dr. Frederic Menard, and Dr. Bruce Mathieson, who gave me valuable advice and guidance, supported me to grow up and finally offered me the degree. Lastly, I want to thank my supervisor Dr. Sanjoy Ghosh. I’m really appreciated the research environment he created in the lab, of which I can have my own research ideas at all times. This is substantial to establish my critical thinking ability, to be able to think myself, to question evidence or sometime even him.
I absolutely understand this is so necessary for me to become an independent researcher in the future.

Most importantly, I have to express my heartfelt thanks to my parents and my boyfriend Xukun Wang. They encourage me, support me, listen to me and trust me at all times. As an IT person had no interests in Biology, Xukun had to listen to my presentations so many times, so that he is able to even list PUFA and linoleic acid bioconversion now. As an international student, our life is more difficult than domestic student. Xukun is my only company at the most of time. I’m really appreciated all his efforts and company. Without you, I do not know if I can survive this far.
Chapter 1: Introduction

Heart disease is the leading cause of death in Canada, resulting in a huge economic burden (Genest, McPherson et al. 2009). Although heart disease is thought to be preventable, there are still approximately 1.6 million Canadians who are at high risk of having a heart attack (Finegold, Asaria et al. 2013; Benjamin, Blaha et al. 2017). The Heart and Stroke Foundation of Canada and the American Heart Association (AHA) have announced proactive strategies to prevent heart disease, which include promoting exercise (Hammonds, Gathright et al. 2016), quitting smoking (Hackshaw, Morris et al. 2018) and following dietary guidelines (Sacks, Lichtenstein et al. 2017; Satija, Bhupathiraju et al. 2017).

In terms of diet, a balance of protein, fat and carbohydrates (macronutrients) are equally important to achieve a healthy lifestyle. The macronutrient composition of our diet has experienced a significant change since the beginnings of human civilization because of the connection between lifestyle and food production. With the modernization of agriculture and animal husbandry, energy dense processed food and animal products now dominate the Western diet (Cordain, Eaton et al. 2005), which contains both high sugar and fat. Interestingly, of all dietary fats we consume, AHA recommend to reduce the intake of saturated fatty acids, in order to protect heart health (Sacks, Lichtenstein et al. 2017).

1.1 Epidemiology of heart diseases in Canada

By 2013, 2.4 million adults over the age of 20 were suffering from heart diseases with 24% of whom had previous history of heart diseases in Canada. This number keeps rising too, with about 158,700 adults newly diagnosed with heart diseases every year.
Although the incidence of heart diseases keeps rising, the annual growth rate in diagnosis showed a decreasing trend since 2000. Women had lower incidence and mortality rates of heart diseases, compared to men (Canada 2018).

Beyond diet, the risk of heart diseases is greatly impacted by social factors, including family income, lifestyle and access to health care. One report demonstrated a correlation between household income and incidence of heart disease (Lee, Chiu et al. 2009). From 1994 to 2005, the incidences of heart diseases experienced a 6% raise in 1.29 million upper-income Canadian families. However, lower middle income families showed an increase of 37% (Lee, Chiu et al. 2009). Lifestyle between the lower middle income population and upper income families varies such as smoking incidence, which is much higher in lower middle income families (Janz 2015). Smoking has long been listed as one of the leading causes of heart disease through increasing cardiac oxidative stress (Ambrose and Barua 2004). Cellular oxidative stress interrupts the regulation of blood pressure, alters lipid metabolism and provokes low grade inflammatory responses, which further promote the development of chronic diseases such as diabetes, obesity and cardiovascular diseases (CVD) (Scherer and Hill 2016). All these metabolic disorders are interconnected and can develop into other complications if the pre-existing disease state is not well managed.

1.2 Lipid hypothesis

In 1955, Dr. Ancel Keys hypothesized that dietary saturated fats elevate circulatory cholesterol which leads to CVD. Later in 1958, he launched the remarkable ‘Seven Countries Study’ which fundamentally established the ‘Lipid Hypothesis’ theory (Keys 1980). This study was a 50-year follow-up study involving 12,763 individuals from Japan, Austria, Italy,
England, Canada and US. This was the first time dietary patterns were linked to heart diseases. It concluded that total amount of dietary fat did not show positive correlation to coronary heart diseases, whereas saturated fats was highly associated with the incidence of coronary heart diseases. The ‘Lipid Hypothesis’ encouraged numerous researchers to follow up on this theory, leading to the notorious demonized reputation saturated fats has today. In addition, nutrition authorities like Dietitians of Canada continue to recommend consuming more unsaturated fats than saturated fats, to prevent heart diseases (Vessby, Uusitupa et al. 2001; Müller, Lindman et al. 2003; Canada 2017). The principal idea behind the ‘Lipid Hypothesis’ is that saturated fats increase cholesterol in the blood serum (Mattson and Grundy 1985; Group 1994). The cholesterol level, or low density lipoprotein cholesterol (LDL-C) in particular, is believed to be the biomarker of CVD and coronary artery diseases (Gordon, Castelli et al. 1977; Mensink, Zock et al. 2003).

However, limiting saturated fat consumption has failed to reduce the prevalence of CVD and as such the cholesterol focused ‘Lipid Hypothesis’ had created a heated debate in the last decade (Ravnskov 2008; Benjamin, Muntner et al. 2019). In fact, A. Keys carefully selected these seven countries to make this conclusion, whereas 22 countries participated in this study (Australia, Austria, France, Italy, Netherland, Switzerland, Japan, US, Canada, Greece, Ceylon, Chile, Denmark, France, German. Fed. Rep., Ireland, Israel, Mexico, New Zealand, Norway, Portugal, Sweden, and UK). Yerushalmy and Hilleboe reanalyzed the original data which included 22 countries in 1957. They drew the conclusion that the correlation between fatty acid consumption and heart diseases was very weak. Instead, protein composition might play a bigger role in affecting the incidence of heart diseases (Yerushalmy and Hilleboe 1957). A 25-year follow up study of Multiple Risk Factor
Intervention Trials (MRFIT) limited cholesterol intake in 6428 middle aged men who didn’t previously have pre-existing heart problems (Stamler, Wentworth et al. 1986; Stamler and Neaton 2008). Researchers were not able to find a link between dietary cholesterol level and risk of CVD (Stamler and Neaton 2008). In reality, cholesterol rich foods, such as egg and dairy products, have very minimal effects on cholesterol levels in blood serum (Blesso and Fernandez 2018). One report with 178 men assigned in different diet groups also rejected the principal of ‘Lipid Hypothesis’. A high saturated fat diet (15% of total energy) did not alter LDL-C or cholesterol levels in blood serum, when compared to a low saturated fat diet (7-9% of total energy) (Krauss, Blanche et al. 2006). Thirty more cohort studies had also contradicted the detrimental effect of saturated fats on accelerating the development of CVD (Ravnskov 1998; Ravnskov 2008; Siri-Tarino, Sun et al. 2010). Opposite to the traditional ‘Lipid Hypothesis’, replacing saturated fats by ‘beneficial’ n-6 PUFA (specially LA) showed a 22% higher heart disease mortality rate (Ramsden, Zamora et al. 2016).

1.3 Dietary fatty acids

Fats are one of three macronutrients that provide energy. Dietary fats are chemically divided into saturated fatty acids (Bertin, Pons et al. 1996), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Of all dietary fat, SFA are considered the primary causative factor contributing to heart disease. Despite a nationwide restriction of SFA consumption, it has failed to alleviate the incidence of heart diseases over the years (Krebs-Smith, Reedy et al. 2010). SFA have no double bonds in the their carbon chain, MUFA have only one double bond and PUFA have two or more double bonds (Gunstone 2012) (Figure 1.1).
SFA, or solid fats, are solid at room temperature, which become liquid once melting temperature is reached. Dietary SFA sources include dairy products, fatty red meat such as pork and beef, and some tropical vegetable oils (e.g. coconut oil) (Hu, Stampfer et al. 1999). The length of the carbon chain varies from C4:0 to C24:0, and the carbon is saturated with hydrogen. The most common SFA are C16:0 (palmitic acid) and C18:0 (stearic acid), which are primarily found in animal fat (Valsta, Tapanainen et al. 2005). C12:0 and C14:0 are also prevalent dietary SFA, which can be largely found in dairy products and tropical oils such as coconut oil (Shamsia 2009).

MUFA are generally considered to be “healthy” fatty acids which are high in the popular Mediterranean diet (Wahrburg, Kratz et al. 2002; Huang and Sumpio 2008). Olive oil is the primary cooking oil in the Mediterranean diet, and oleic acid (C18:1n9) is the most abundant MUFA in olive oil.

PUFA are divided into n-6 PUFA and n-3 PUFA based on the position of the first double bond in their carbon chains. n-6 PUFA has the first double bond located at the sixth carbon away from the methyl end (Gunstone 2012). The most common dietary source of n-6 PUFA in the North American diet includes corn oil, safflower and sunflower oil. These primarily contain linoleic acid (LA, C18:2n6), the parent of n-6 PUFA (Singh 2005). On the other hand, the first double bond of the n-3 PUFA starts from the third carbon away from the methyl end of the fatty acid (Gunstone 2012). The most well-known n-3 PUFA are eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA) which are commonly found in fatty fish such as salmon (Singh 2005).
Figure 1.1: Chemical structures and representatives of dietary fatty acids.

1.4 Current Canadian diets

The advice of the Heart and Stroke Foundation of Canada and the Dietitians of Canada to lower SFA intake, has resulted in the population replacing SFA with unsaturated fat (Wong, Botta et al. 2015). Of unsaturated fats, the consumption of n-6 PUFA has increased notably. Statistics Canada (Catalogue No.21) also announced that total dietary fat intakes increased 18% between 1976-2005, with 54% increase of PUFA and 18% increase of MUFA; however, the total fat intake has plateaued. In 2015, the Canadian Community Health Survey reported that 32.2% of energy intake was derived from fats, which was relatively the same as in 2004 (31.3%) (Canada 2017).

Researchers have suggested that the substitution of n-6 PUFA for SFA lowered the risk of ischemic stroke (Iso, Sato et al. 2002). The World Health Organization (WHO) also reported that replacing SFAs with n-6 PUFA attenuated Coronary Heart Disease (CHD) incidence (Organization 2008). However, many clinical and fundamental researchers later reported that this high n-6:n-3 PUFA ratio should be considered as an indicator of chronic
heart diseases (Simopoulos 2008). This opposes to the traditional idea that ‘PUFA is beneficial’. In order to reduce the n-6 to n-3 PUFA ratio, authorities such as the American Dietetic Association and Dietitians of Canada both recommended that individuals should take n-3 PUFA rich foods daily like fish or fish oil supplements (Kris-Etherton, Innis et al. 2007).

The primary source of n-6 PUFA in the diet is vegetable oils and meat. Soybean oil and canola oil are the two largest cooking oils (Canada 2012), which both have high smoke point (232°C and 204°C). So they are the most popular oils used in cooking in terms of their heat tolerance. In addition, corn is third largest grain crop in Canada, which is largely used for livestock feeding (Marie-Andrée Hamel 2015). Grain feeding and traditional grass feeding have distinct impacts on nutrient accumulation in the tissue of the animal. In general, grass-fed animals have a lower quantity of total fatty acids compared to other non-grass fed animals (Van Elswyk and McNeill 2014). LA in grain-fed cow (29.3mg/100g) is two-fold higher than that of a grass-fed cow (14.7mg/g). On the contrary, α-linolenic acid (ALA, n-3 PUFA) is much lower in a grain-fed cow (3.4mg/100g), compared to 10.6mg/100g of ALA in grass fed cow (Warren, Scollan et al. 2008). The dietary n-6 to n-3 PUFA ratio of grain fed cows is 10:1 to 12:1, compared to 1:1 to 2:1 of a grass fed (Van Elswyk and McNeill 2014). This feeding process contributes to the seemingly unavoidable high n-6 PUFA intake in Canadian diet. LA is the most abundant dietary n-6 PUFA in North America, which contributes 7-10% of daily energy intake (Whelan and Fritsche 2013).
1.5 The controversial viewpoint of dietary n-6 PUFA

In terms of diet, current thought leans towards excessive meat consumption as a major factor behind the risk and the development of CVD in Western world (Willett 2012), However, this line of thinking does not agree with traditional diets of hunter gather societies (Cordain, Eaton et al. 2002). The pattern of hunter-gather diets predominates of animal-based food (65% energy), while plant-based food only occupies 35% of the total energy (Cordain, Eaton et al. 2002). On closer inspection, hunter gather societies had higher fat and protein consumptions, compared to the current dietary guidelines. In addition, there is evidence that consumption of this animal dominated diets result in a relatively lower cholesterol and blood lipids levels (Biss, Ho et al. 1971; Bang, Dyerberg et al. 1980). Therefore, animal protein or fat intake may not be the risk factor that primes for the incidence of CVD. Instead, the overabundance of n-6 PUFA could be to blame. The current dietary n-6:n-3 PUFA ratio among Canadians ranges from 10:1 to 20:1 (Molendi-Coste, Legry et al. 2011), whereas it was close to 1:1 in Paleolithic hunter gatherers times. Researchers analyzed the macronutrients composition in wild animals and also frozen mammoths (41,000-34,000 years BP), bisons (8200-9300 years BP) and horses (4600-4400 years BP) (Guil-Guerrero, Tikhonov et al. 2014). They concluded from the analyses that the dietary ratio of n-6 to n-3 PUFA was 0.79:1 (Simopoulos 2016), LA to ALA was 0.7. In particular, daily total LA intake was 8.84g, and ALA was 12.60g (Eaton, Eaton et al. 1998).

Worldwide food guidelines encourage the population to replace dietary SFA with unsaturated fat in order to protect against cardio-metabolic diseases. This results in excessive intake of n-6 PUFA consumption. A systemic review of multiple meta-analysis studies
claimed the cardio-protective effect of PUFA on patients pre-existing coronary heart diseases. This one-year follow-up study showed that high PUFA (14.9% of total energy) resulted in reduced incidence of CHD, compared to patients with low PUFA diet (5% of total energy) (Mozaffarian, Micha et al. 2010). Another four to ten-year follow-up study also stated that substituting PUFA, primarily n-6 PUFA, for SFA, was conversely associated with the risk of CHD (Jakobsen, O’Reilly et al. 2009). The conclusion of all these studies was that n-6 PUFA was shown to reduce LDL-C level, resulting in its’ protective effect on developing heart diseases (Fernandez and West 2005).

In contrast, more and more evidence has questioned the cardiac benefits of n-6 PUFA as well as the ‘Lipid Hypothesis’ (Ravnskov 2008). Multiple randomized control trails (Turpeinen, Pekkarinen et al. 1979; Ramsden, Zamora et al. 2016) cohort studies (Posner, Cobb et al. 1991; Pietinen, Ascherio et al. 1997; Leosdottir, Nilsson et al. 2007), and prospective studies (McGEE, REED et al. 1984; Kushi, Lew et al. 1985; Fehily, Yarnell et al. 1993) have found no detrimental effects of SFA intake on heart diseases. On the contrary, n-6 PUFA has been shown to increase the risk of chronic inflammatory diseases, including CVD, inflammatory bowel diseases and diabetes (Willett 2007; Ghosh, Molcan et al. 2013; Ramsden, Zamora et al. 2013). As mentioned in 1.4, current Canadian diet has an extremely imbalanced n-6 to n-3 PUFA ratio, which has been blamed to be one of the causative factors inducing chronic inflammatory diseases. As shown in Figure 1.2 (section 1.5), n-6 and n-3 PUFA share the same set of bioconversion enzymes. This indicates that these two species of PUFA compete for the active site of those enzymes, or in other words, enzymes compete for substrates. Delta-6 desaturase, the first enzyme in the PUFA bioconversion pathway, has a higher affinity to α-linolenic acid (parent n-3 PUFA) which should lead to the higher
production of EPA and DHA in theory (Senadheera, Turchini et al. 2011). However, the amount of LA and ALA is not close to the same order of magnitude in our diet. Therefore, n-6 PUFA bioconversion dominates, so that large amount of arachidonic acid (main product of n-6 PUFA) are produced (section 1.5). arachidonic acid (ARA) is the precursor of pro-inflammatory eicosanoids such as prostaglandin series II and anti-inflammatory epoxides (EETs) (Shih 2017). On the contrary, EPA and DHA are considered as the precursors of anti-inflammatory eicosanoids such as prostaglandin series I and resolvins. That being said, ARA is predominantly considered to be the pro-inflammatory mediator which promotes the progression of heart diseases (Simopoulos 2016). EPA and DHA, on the other hand, are the substrates for producing anti-inflammatory signals. In terms of opposite inflammatory responses, ARA has now become the focus of why a high n-6 to n-3 PUFA ratio in our diet may be priming CVD.

1.6 PUFA metabolism

The formation of long chain n-6 PUFA (arachidonic acid, C20:4n-6) from LA (C18:2n-6) requires key enzymes (desaturase and elongase). Desaturation is carried out with two desaturases: delta-5-desaturase (D5D) and delta-6-desaturase (D6D), and forms one double-bond in the carbon chain (Matsuzaka, Shimano et al. 2002). Elongation is conducted with two elongases: elongase 5 (Elov15) and elongase 2 (Elov12) and introduces two carbons into the carbon chain (Jump 2009). D6D, encoded by fatty acid desaturase 2 (FADS2) is the membrane-bound desaturase involved in two steps of the n-6 PUFA biosynthesis. The first step is a rate-limiting step, converting the LA (C18:2n-6) to γ-linolenic acid (GLA, C18:3n-6) (Nakamura and Nara 2004) (Figure 1.2). Next, GLA is elongated by the Elov15, producing
dihomo-γ-linolenic acid (DGLA, C20: 3n-6). D5D desaturates DGLA to synthesize ARA (C20:4n-6), the main product of n-6 PUFA metabolism. Blocking D5D and D6D impedes the generation of downstream products of n-6 PUFA (Williard, Nwankwo et al. 2001). D6D null mice (FADS2−/−) showed impaired male reproduction and led to growth retardation by displaying decreased body weight growth rate. However, this can be mitigated through supplementation with ARA, which is a key component of all mammalian cell membranes (Stroud, Nara et al. 2009).

n-3 PUFA metabolism is similar to n-6 PUFA, as it requires both desaturases and elongases (Figure 1.2). The pathway starts with the n-3 PUFA, ALA. The major end products of n-3 PUFA are EPA and DHA (Brenna, Salem Jr et al. 2009), which are well known to have anti-inflammatory effects such as increasing the production of anti-inflammatory eicosanoids (Calder 2006) and decosanoids (Lewis and Bailes 2011). High levels of LA can compete with ALA in the PUFA pathway, limiting the production of EPA and DHA from ALA (Portolesi, Powell et al. 2007). Therefore, maintaining the optimal ratio of LA to ALA is crucial to balance the production of ARA and EPA/DHA.

In addition to the main pathway for biosynthesis of the n-3 and n-6 PUFA, LA and ALA can be elongated to C20:3n3 and C20:2n6 by Elovl5 (Robertson, Guihéneuf et al. 2013), however the biological significance of these two fatty acids is not known.
1.6.1 Desaturases

D5D (encoded by FADS1) and D6D (encoded by FADS2) are essential for PUFA desaturation. FADS1 and FADS2 are located head to head in chromosome 11 (He, Zhang et al. 2018), therefore they are referred to as FADS1-FADS2 cluster (FADS cluster). The polymorphism of FADS cluster is associated with lipid composition, oxidative stress, and metabolic diseases (Al-Hilal, AlSaleh et al. 2012). Single nucleotide polymorphisms (SNPs) of FADS cluster markedly change the fatty acid composition of membranes. Minor allele haplotype (T-C-del) is associated with a significantly higher DGLA accumulation but
dramatically reduces the ARA level in both erythrocyte (whole cell) and phospholipids of membranes when compared to major allele haplotype (C-T-T) (Rzehak, Heinrich et al. 2008). The alteration of DGLA and ARA levels dictate the activity of the FADS cluster slowing down the process of desaturation from DGLA to ARA. In addition, the minor and major alleles of FADS1 (i.e., rs147547) and FADS2 (i.e., rs147576) SNPs influence the level of total cholesterol, LDL-C, high density lipoprotein (HDL-C) and triglycerides (Al-Hilal, AISaleh et al. 2012), which are biomarkers commonly measured in patients with metabolic diseases. For example, the major allele of SNP rs174576 (T) reduces the risk of cardiovascular diseases (CVD) and type II diabetes by elevating the activity of two desaturases and increasing HDL-C (Huang, Chang et al. 2015). On the other hand, FADS2 deficient mice have an exaggerated inflammatory response, as a result of intestinal ulceration and CVD (Malerba, Schaeffer et al. 2008; Stroud, Nara et al. 2009). Although researchers had these observation, the mechanisms behind is still unknown.

The 18 SNPs in the FADS cluster has been discovered through the examination of PUFA composition in phospholipids (Schaeffer, Gohlke et al. 2006). The most well studied haplotypes of FADS cluster are FADS-A and FADS-D. FADS-A (T) is considered to be a ‘wildtype’ and FADS-D (G) is the derived SNPs (Mathias, Pani et al. 2014). FADS-D metabolizes the parent PUFA (LA and ALA) to LC-PUFA at a higher rate. The FADS-D haplotype is dominant in Africans (<1%) (Mathias, Fu et al. 2012), then Asians, and with the lowest frequency in Americans (Ameur, Enroth et al. 2012). This is partially due to the ethnic differences and traditional diet patterns. Brenna et. al (2016) demonstrated that South Asians and Africans have better selective FADS2 genotype, compared to the US and European populations (Kothapalli, Ye et al. 2016), so are able to convert PUFA more
efficiently. In general, Western populations consume more red meat, whereas Asians traditionally prefer a more plant-based diet. It is known that long chain PUFA (≥ C20, LC-PUFA) like ARA, EPA and DHA accumulate in animal-based products, whereas LA, GLA and ALA are mainly derived from plant sources (Abedi and Sahari 2014). From an evolutionary perspective, individuals who normally consume food containing higher amounts of PUFA like LA, naturally adopt the dominant FADS SNPs that are more efficient for PUFA bioconversion (Schaeffer, Gohlke et al. 2006). This accelerates the removal of upstream PUFA and the production of LC-PUFA, which are essential to support biological functions.

Beyond the genetic variation, desaturase activity also impacts biological functions. The activity of desaturases can be affected by chronic and acute inflammation, disease state and diet. D5D inhibition is shown to be linked with the initiation of the systemic inflammatory response (Gromovsky, Schugar et al. 2017). The FADS2−/− mouse model results in male infertility, colitis and brain dysfunction (Stroud, Nara et al. 2009). In addition, gender, behaviour, and environment also regulate the activity of desaturases (Abdelmagid, Clarke et al. 2015).

1.6.2 Elongase

Elongases are a group of enzymes that elongate the fatty acid carbon chain by adding two extra carbons. There are two elongases involved in PUFA metabolism: Elovl5 and Elovl2. Elovl5 elongates C18 to C20 and C20 to C22, whereas Elovl2 is responsible for C20 to C22, and C22 to C24 elongation (Figure 1.2) (Jump 2009). Elovl5 has the highest affinity for C18 PUFA, whereas Elovl2 favours C22 (Gregory, Gibson et al. 2011). Due to the
distinct requirement of biological metabolism, expression of these two elongases is tissue and time dependent. Liver has the highest expression of Elovl5 and Elovl2, whereas the kidney and lung still present with abundant Elovl5 but not Elovl2 (Wang, Botolin et al. 2005). There is no Elovl2 expression in the heart but cardiac Elovl5 is detectable (Wang, Botolin et al. 2005). Hepatic Elovl5 activity is low in the fetal stage of rats but increases significantly (5-20 fold) 10 days postpartum. Out of all the elongase isoforms, Elovl5 is the only one highly expressed after birth in rats (Wang, Botolin et al. 2005).

Unlike FADS, the scientific literature on Elovl5 polymorphisms is lacking. Over expression of Elovl5 decreases plasma and liver triglycerides (Wang, Torres-Gonzalez et al. 2008; Ishiyama, Taguchi et al. 2010) through increased TAG catabolism (Tripathy, Lytle et al. 2014). Similarly, high fat diets induce obesity and decrease hepatic Elovl5 activity which further blocks peroxisome proliferator-activated receptor-β (PPAR-β) expression (Tripathy, Lytle et al. 2014). PPAR-β had a less understood function but recently has been found to accelerate lipid metabolism and protect against metabolic disorders through promoting the activity of β-oxidation (Palomer, Barroso et al. 2016; Rachid, Silva-Veiga et al. 2018).

Opposite to TAG catabolism, lipogenesis is globally regulated by sterol regulatory element-binding protein 1 (Srebp-1), a nucleus transcription factor (Figure 1.3). Two Srebp1 binding sites, sterol regulatory elements (SREs) directly target the enhancer of Elovl5, therefore increasing Elovl5 expression (Shikama, Shinozaki et al. 2015). Two SRE targets were confirmed in human Elovl5 gene transcription levels, with one located in Elovl5 exon 1 and the other one in the -10kb Elovl5 upstream region (Shikama, Shinozaki et al. 2015). However, over expression of Elovl5 silenced Srebp1 expression by increasing long-chain
PUFA production such as ARA, EPA and DHA. This accumulation signalled the inhibition of lipogenesis which decreased Srebp1 expression (Yahagi, Shimano et al. 1999; Takeuchi, Yahagi et al. 2010). Deletion of Elovl5 promotes Srebp1 expression which leads to fatty liver (Moon, Hammer et al. 2009).

**Figure 1.3: Regulation of Srebp1 on n-6 PUFA bioconversion.** Srebp1: sterol regulatory element-binding protein 1; SRE: sterol regulatory elements; ME: malic enzyme; G6PD: glucose 6-phosphate dehydrogenase; 6PDG: phosphoglucoaonate dehydrogenase; LA: linoleic acid, c18:2n6 ARA: arachidonic acid, c20:4n6.

### 1.7 PUFA and CVD

#### 1.7.1 Discovery of essential fatty acids

‘Essential fatty acids’ (EFAs) cannot be biosynthesized in mammals due to the absence of Δ12-desaturase and Δ15-desaturase (Shils and Shike 2006). Therefore, EFAs must be obtained from the diet (Innis 1991). In 1929, Burr first discovered that natural dietary fats were necessary for growth and development by providing a fat-free diet to rats. He concluded ALA and LA were necessary for early stage growth and biological functional maintenance (Burr and Burr 1929; Burr, Burr et al. 1932).
1.7.2 PUFA metabolism and CVD

Long chain PUFA produce mediators that regulate inflammatory responses (Calder 2006). ARA, the major end product of long-chain n-6 PUFA biosynthesis, is the precursor of pro-inflammatory eicosanoids such as prostaglandin E$_2$ (PGE$_2$). PGE$_2$ protects against gut epithelial cell damage and promotes intestinal repair by restoring intestinal barrier (Blikslager, Roberts et al. 1997). On the other hand, EPA and DHA (n-3 PUFA) produce anti-inflammatory mediators like protectins and resolvins. Protectins and resolvins have the ability to resolve chronic inflammation in obesity, type II diabetes, colitis and CVD (Zhang and Spite 2012). Resolvins also inhibit the biological actions of PGE$_2$, neutrophil transmigration and cytokine production (Tian, Lu et al. 2009). Exaggerated inflammation accelerates the development of chronic diseases. On the contrary, silenced inflammatory responses are not able to defend against pathogens and foreign particles. Therefore, inflammation is the self-defence system, which needs to be carefully balanced to maintain at a healthy state. However, independent of ARA and its pro-inflammatory eicosanoids, LA itself is able to increase the risk of CVD.

1.7.4 LA and CVD

LA is mainly found in vegetable oils including corn, sunflower, soybean and safflower oils in the Western world (Meyer, Mann et al. 2003). If the diet is lacking in LA, atopic dermatitis develops as it is required to support the skin barrier and protect against water loss (Yen, Dai et al. 2008). Due to the negative reputation of SFA, the demand for vegetable oils such as LA is continuously increasing. In clinical studies, LA was shown to decrease the concentration of circulating cholesterol by lowering LDL-C in plasma without affecting
HDL-C level (Ramprasath, Jones et al. 2012). Therefore, LA is considered to lower the risk of development of CVD.

Despite the cardio-protective role of LA, excess LA consumption in the current Western diet drives the imbalance of the n-6 to n-3 PUFA ratio. Researchers have been focusing on the deleterious effects of the PUFA ratio and its risk for developing CVD and its complications. This conclusion was based on the linear bioconversion of LA to ARA (Li, Ng et al. 1998) where excessive LA should be able to produce higher level of ARA and inhibited n-3 PUFA bioconversion. This further disrupted the balance between ARA and long chain n-3 PUFA (EPA and DHA) impairing the homeostasis of the inflammatory responses (Chilton, Murphy et al. 2014). A systemic review by Rett et al (2011) rejected the ‘hypothesized’ linear relationship between dietary LA and the production of ARA in erythrocytes and phospholipids in blood serum. The ARA level did not change with dietary LA level ranging from -40% to 600% (Rett and Whelan 2011).

The role of LA, independent of ARA, on heart health remains under debate and conflicting results are reported in meta-analyses (de Goede, Geleijnse et al. 2012). Cohort studies in the Netherlands with 20,000 people who were free of CVD (de Goede, Geleijnse et al. 2012) and meta-analyses of multiple studies with 310,620 participants (Farvid, Ding et al. 2014) both claimed that the correlation between dietary LA and heart disease were negative. Of note was that these studies were completed before the onset of cardiometabolic diseases. However, Sydney Diet Heart Study (SDHS), the randomized controlled dietary trail with 458 patients of either CVD or CHD, demonstrated the opposite outcome (Ramsden, Zamora et al. 2013). This study included a dietary intervention with an extra 15% safflower oil (high LA)
LA supplementation exacerbated the development of the existing disease state by increasing mortality rate in both CVD and CHD (Ramsden, Zamora et al. 2013). In other disease states, such as type II diabetes, consuming sunflower oil (high LA) further increased plasma LDL-C, which aggravated the current disturbed metabolic state (Madigan, Ryan et al. 2000). Higher intake of LA also was shown to aggravate the metabolic disorders in obese and diabetes rodent models, therefore accelerating the development of pre-existing diseases (Lee, Yu et al. 2019).

1.8 Mitochondrial health, fat and CVD

1.8.1 Fatty acid metabolism and mitochondrial health in the heart

The heart is the central organ that supplies blood to the rest of the body through myocyte contraction and relaxation, therefore the heart muscle requires a continuous and significant supply of energy and oxygen. The heart prefers to use fatty acids over glucose as the energy source (Berg, Tymoczko et al. 2002). Fatty acids are the most concentrated energy source, with 9 kilocalories (kcal) of energy per unit (gram) compared to glucose (4kcal/g). Fatty acids are stored in cells as triglycerides and are catabolised through β-oxidation (Grynberg and Demaison 1996). Mitochondria and peroxisomes preferentially degrade fatty acids with peroxisomes catalyzing very long chain fatty acids and mitochondria catabolise the remainder of the fatty acids (Reddy and Hashimoto 2001). Mitochondrial activity is important in maintaining cardiac energy supply. If there is heart pathology, the heart metabolic mode switches to a glucose dominant energy source due to the dysfunction of the mitochondrion (van Bilsen, van Nieuwenhoven et al. 2008). This leads to the loss of
the mitochondrial integrity, increases oxidative stress and failure of β-oxidation catabolism (Chistiakov, Shkurat et al. 2018).

In terms of the chemical structure of LA, mitochondrial dysfunction induces oxidative stress which can attack the double bonds of LA creating the oxidative metabolites 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE). These metabolites stimulate the efflux of cholesterol, increase the production of oxidative LDL-C (DiNicolantonio and O’Keefe 2018) and promote the risk of heart diseases and the progression of existing cardiac diseases.

1.8.2 Oxidative stress

Oxidative stress is the result of an imbalance in antioxidants and free radicals. Reactive oxygen species (ROS) is a group of free radicals with both protective and detrimental effects in living organisms. They are essential for many metabolic reactions such as activating the immune system to protect against pathogens (Yang, Bazhin et al. 2013), controlling cell signalling and regulating cellular proliferation (Mittler 2017). An excess in ROS production causes mitochondrial oxidative damage resulting in cardiometabolic disorders including CVD, obesity and type II diabetes (Niemann, Rohrbach et al. 2017). Maintaining the homeostasis between ROS and antioxidants is crucial for regulating biological metabolism.

Antioxidants are a group of molecules that help to scavenge excessive ROS in the system. Three first-line defence antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx) (Ighodaro and Akinloye 2017). SOD reduces superoxide to hydrogen peroxide (H$_2$O$_2$) through the Fenton reaction (Ighodaro and
Akinloye 2017). CAT and Gpx can both convert H$_2$O$_2$ to H$_2$O but CAT is absent in mitochondrion (Bai, Rodriguez et al. 1999). Due to the characteristics of the respiration chain, mitochondrion is the major organelle that produces endogenous ROS. Heart muscle cells contain the highest number of mitochondria in the whole body because of the high demand of energy (Sarniak, Lipińska et al. 2016). Maintaining the homeostasis between ROS and antioxidants, especially Gpx, is crucial for heart health. Gpx clears out ROS by oxidizing glutathione (GSH) to glutathione disulfide (GSSG), which then can be converted back to GSH through glutathione reductase (Gsr) (Figure 1.2). GSH is the most efficient internal antioxidant which is synthesized by cysteine, glycine and glutamine through glutamate—cysteine ligase (Gclc) and glutamate-cysteine ligase (Gclm) (Figure 1.2). GSH reduces harmful H$_2$O$_2$ to water while oxidizing itself to GSSG. The GSH:GSSG ratio is widely used as an indicator of oxidative status, with 100:1 as the standard for resting healthy cells (Zitka, Skalickova et al. 2012). There are four Gpx isoforms discovered so far: Gpx1, Gpx2, Gpx3 and Gpx4. Gpx1 is considered to be the primary subtype that is associated with CVD (Tabet and Touyz 2007). Gpx1$^{-/-}$ mice have accelerated progression of cardiac hypertrophy and dysfunction resulting in collagen deposition (Ardanaz, Yang et al. 2010). Also, mice lacking Gpx1 have accelerated development of existing atherosclerosis as a result of the severe oxidative damage (Torzewski, Ochsenhirt et al. 2007).
1.9 LA and oxidative Stress

Many studies have reported that LA boosts ROS levels in the chronic inflammatory state. A cohort study showed that a high LA diet (11.5%) increased oxidative stress and led to damaged endothelial function (Turpeinen, Basu et al. 1998). Similarly, endothelial cells treated with LA increased oxidative stress and mediated tumor necrosis factor (TNF) directed apoptosis (Toborek, Blanc et al. 1997). In addition, the historical opinion of LA on inducing atherosclerosis was that LA triggered the cascade of existing TNF-α (Toborek, Barger et al. 1996) and increased elastase-like protein (Toborek and Hennig 1993), which worsened the endothelial function and increased the development of the pre-existing atherosclerosis. The elevated elastase-like proteins potentially increased the risk of artery stiffness, high blood pressure and cardiac hypertrophy (Wagenseil and Mecham 2012). The cause of all LA-derived detrimental effects on the heart was believed to be the imbalance between ROS and antioxidants. Supplements of antioxidants, such as vitamin E have been shown to attenuate LA induced artery stiffness (Toborek and Hennig 1993). To improve the LA caused heart
damage, accelerating n-6 PUFA bioconversion while increasing the level of antioxidants may be an efficient therapy for patients who have heart disease. However, the link between PUFA metabolism and antioxidants in mammals remains unknown.

1.10 Hypothesis and aims

The Western diet is high in n-6 PUFA, LA in particular, creating an imbalance in the ratio of n-6 to n-3 PUFA (Simopoulos 2009). Numerous studies have demonstrated the adverse effects of excessive n-6 PUFA on promoting the risk of heart diseases, which are mostly attributed to the detrimental effects to the overproduction of ARA (Li, Ng et al. 1998). Although dietary guidelines advocate for increasing dietary n-3 PUFA consumption to lower the n-6 to n-3 PUFA ratio, patients at high risk of metabolic diseases still develop metabolic disorders with LA, independent of n-3 PUFA. Given that our food supply is rich in n-6 PUFA, the only way to reduce n-6 PUFA’s effects on the body (including the heart) is to promote its removal. We hypothesized that LA is the primary n-6 PUFA responsible for cardiac reactivity in excess and clearing it would reduce cardiac reactivity.

To examine the hypothesis, the following three aims were designed:

The first aim was to understand if accumulation of LA, by regulating its’ bioconversion, induces higher alpha-adrenergic responses (Chapter 2).

To investigate this aim, the following experiments were conducted:
1. Determine if chemically or genetically blocking LA bioconversion (FADS2 inhibitor) affected cell viability and expression of adrenergic receptors in NIH/3T3 and differentiated H9c2 or mouse aorta

2. Determine if globally blocking n-6 PUFA bioconversion (Srebp1 siRNA) affected cell viability and expression of adrenergic receptors in differentiated H9c2

The second-aim for this thesis was to evaluate if Elvol5 deficiency causes elevated vascular contractility under a high n-6 PUFA diet (Chapter 3).

To investigate this aim, the following experiments were conducted:

1. Determine if blocking LA bioconversion in Elvol5+/− mice affected aortic blood pressure and heart function

2. Determine if three different high fat diets affected expression of adrenergic receptors and local inflammatory responses in the Elvol5+/− mice aorta.

The third aim for this thesis (Chapter 4) was to understand if supplementing with the antioxidant, GSH, accelerates LA metabolism while simultaneously improving LA-induced heart dysfunction.

To investigate this aim, the following experiments were conducted:

1. Determine the role of additional GSH on in NIH/3T3 and differentiated H9c2.

2. Determine the effect of Gpx1 on regulating PUFA bioconversion of the heart in Gpx1+/− mice fed a high n-6 PUFA diet.
3. Determine the protective role of healthy mitochondria on improving heart function, n-6 PUFA bioconversion and expression of GSH associated genes.

1.11 Experimental models

1.10.1 in vitro study

Permanent cardiac cellular constituents include smooth muscle cells (SMCs), fibroblasts, myocytes, and endothelial cells. Fibroblasts are the most abundant non-myocyte cell type found in the mammalian left ventricle, with a scattered distribution (Nag 1980; Ivey and Tallquist 2016). Fibroblasts are responsible for healing, heart matrix maintenance (Zhou and Pu 2016) and signalling cardiomyocytes (Martin and Blaxall 2012). Stimulated fibroblasts are able to cause muscle stiffness and cardiac hypertrophy by promoting fibrosis (Martin and Blaxall 2012). To understand the toxicity of n-6 PUFA to the heart, fibroblasts (NIH/3T3) and cardiomyocytes (H9c2) were used in this thesis. H9c2 is myoblast, which can be further differentiated into cardiomyocytes. It is known that this H9c2 doesn’t have equal contractility to the primary cardiomyocytes since H9c2 cells lost the ability to beat. However, this in vitro model can be used to study the molecular and cellular processes (Zhou, 2016).

1.10.2 ex vivo study

Although NIH/3T3 and H9c2 are able to represent cardiac metabolism at a cellular level, in vitro work cannot accurately reflect a physiological outcome of an organism. Isolated aortas from C57/Bl6 and Elovl5^{+/−} mice were used in this thesis, to address the protein-gene negative feedback loop and the n-6 PUFA bioconversion efficiency.
1.10.3 in vivo study

In vitro and ex vivo studies provided further insight to support our hypotheses. In order to investigate the role of a high n-6 PUFA diet on heart health, the following genetically modified mice models were used: \( \text{Elov}l5^{+/+} \), \( \text{Gpx}1^{+/+} \), \( \text{Endo}G^{+/+} \). \( \text{Elov}l5 \) is the gene that bioconverts LA to GLA. Lack of \( \text{Elov}l5 \) accumulates LA and decreases the production of downstream n-6 PUFA (Figure 1.2). \( \text{Gpx}1 \) is the primary Gpx expressed in the heart, which oxidizes GSH to GSSG, and at the same time scavenging \( \text{H}_2\text{O}_2 \). \( \text{Endo}G \) is mitochondrial protein which directs caspase independent apoptotic pathway. Each model has been described in detail in designated chapters: \( \text{Elov}l5^{+/+} \) (p63), \( \text{Gpx}1^{+/+} \) (p21) and \( \text{Endo}G^{+/+} \) (p87).

Briefly as described in 1.6.2, \( \text{Elov}l5 \) accelerates fatty acid catabolism. A lack of \( \text{Elov}l5 \) induces fatty acid deposition, which results in fatty liver (1.6.2, chapter 3 introduction). \( \text{Elov}l5^{+/+} \) mice were used in this study to separate the upstream and downstream effects of n-6 PUFA, which further helped investigate the role of LA on heart health.

The \( \text{Gpx}1^{+/+} \) mouse model was used to verify the benefits of GSH on reversing LA induced heart problems. The model information is provided in 1.8.2 and 4.1.

\( \text{Endo}G \) (endonuclease G) is encoded by mtDNA, which is located in the inner membrane of mitochondria (chapter 4). The \( \text{Endo}G^{+/+} \) mitochondrial mouse model was selected to study the effect of mitochondrial health on modulating LA-derived heart toxicity. Studies relating to \( \text{Endo}G \) mostly focused on its’ caspase-independent apoptosis (Li, Luo et al. 2001; David, Sasaki et al. 2005). The insight of \( \text{Endo}G \) and heart function is lacking in the scientific literature.
Chapter 2: Blocking LA bioconversion increased adrenergic receptors expression in fibroblasts and cardiomyocytes.

2.1 Background:

National dietary guidelines recommend replacing saturated fats (Bertin, Pons et al.) with vegetable oils, such as canola, soybean and safflower oil which are rich in n-6 PUFA (Zambiazi, Przybylski et al. 2007). As a result of these national dietary guidelines, the current Western diet has excess n-6 PUFA (Eckel, Jakicic et al. 2014). Classically, SFA is a promoter for heart disease (Bertin, Pons et al. 1996), which has been disputed in recent years (Malhotra 2013).

Linoleic acid (LA) is the most available dietary n-6 PUFA which contributes to ~7% of total energy intake in USA (Blasbalg, Hibbeln et al. 2011). Although LA is an essential fatty acid which can only be obtained from food, only 0.5-2% of LA is required to maintain biological functions such as cell signalling, protein configuration, membrane fluidity and integrity (Hallahan and Garland 2005). A number of studies have demonstrated the detrimental effects of a Western diet rich in LA on developing metabolic diseases, such as obesity (Simopoulos 2016) and cardiovascular diseases (Reaven, Grasse et al. 1994; DiNicolantonio and O’Keefe 2018). Another animal study indicated that high LA diet (10kcal%) induced obesity in C57/Bl6 mice, compared to a diet containing lower LA (1.4kcal%) (Deol, Fahrmann et al. 2017). In addition, replacing SFA with LA did not display beneficial effects on heart health of people with or without history of heart diseases (Ramsden, Zamora et al. 2013; Maki, Eren et al. 2018). All these results indicated a more harmful consequence of LA on heart health, compared to the SFA-rich animal fats.
As described in 1.6, dietary LA can be converted to ARA through desaturation and elongation. ARA is a potent pro-inflammatory mediator since it primes the inflammatory responses by producing pro-inflammatory eicosanoids. It has been claimed that ARA is the cause of chronic inflammatory diseases that were induced by high n-6 PUFA diet (Patterson, Wall et al. 2012). A cohort study indicated that serum ARA level was positively correlated with high blood pressure in healthy children between the ages of 2-9 (Wolters, Pala et al. 2016). ARA, instead of dietary LA, has long been thought to be the main cause of activated chronic inflammatory responses, in the context of high n-6 PUFA diet (Patterson, Wall et al. 2012; Chilton, Murphy et al. 2014). This conclusion is established on the theoretical LA to ARA bioconversion. However, this ‘textbook’ linear bioconversion of LA does not necessarily represent ARA production in reality (Rett and Whelan 2011). Rett et.al has showed that ARA level in blood plasma was the same with dietary LA level increasing up to 600%, compared to normal LA intake.

Evidence indicated that large amounts of dietary LA did not significantly affect the circulatory ARA concentration (Rett and Whelan 2011). Hence, a high LA input may not be equal to a high ARA output per se. As an example, patients with various metabolic disorders from various studies represented higher LA, but lower ARA level in the blood serum, compared to healthy individuals (Seigneur, Freyburger et al. 1994; Simon, Hodgkins et al. 1995; Novgorodtseva, Karaman et al. 2011). Another meta-analysis demonstrated that dietary intervention with LA (extra 5% w/w) increased mortality of patients with heart disease (Ramsden, Zamora et al. 2013). On the other hand, general PUFA intervention (extra 5% w/w) with a mixture of n-6 and n-3 fatty acids, not limiting to LA, did not affect the mortality rate of heart diseases patients (Ramsden, Zamora et al. 2013). Thus, it is crucial to
re-evaluate the influence of n-6 PUFA, LA itself in particular, on the etiology of heart diseases.

Current strategies to prevent heart disease include controlling blood pressure and limiting low-density lipoprotein cholesterol (LDL-C) (Association 2015). To better assist patients, the American heart association and Heart and Stroke Foundation of Canada advocate the ‘Dietary Approaches to Stopping Hypertension’ (DASH) diet (Stewart, Manmathan et al. 2017; foundation N/A). DASH recommends replacing SFA with unsaturated fats, as well as reducing sugar and sodium intake. This results in an imbalanced n-6 PUFA to n-3 PUFA ratio of current Western diet, by encouraging excessive LA intake.

Our lab has shown that excessive LA induced heart muscle stiffness, resulting in dampened cardiac relaxation in mice (Beam, Botta et al. 2015). This insufficient muscle relaxation decreased the stroke volume, leading to diastolic heart failure (Leite-Moreira 2006). In general, heart failure usually occurs as a result of impaired myocardium and aortic contractility. Adrenergic receptors (adr) (Geleijnse, Giltay et al. 2002) are a group of G-protein coupled receptors responsible for vascular contractility. They are divided into α1-adr and β1-adr. α1-adr mediates vasoconstriction, whereas β1-adr controls vasodilation of blood vessels. Three types of α1-adr (α1a-adr, α1b-adr and α1d-adr) distribute differently depending on type of tissues (Kong, Taylor et al. 1994). α1a-adr primarily expresses in the heart, whereas α1d-adr dominates in arteries and aorta. Patients with heart failure had a 40% increase of α1a-adr expression, accompanied by a significantly reduced β1-adr expression (Jensen, Swigart et al. 2009). Elevated blood pressure or arterial hypertension is known to be positively linked with increased α1d-adr level (Michel, Brodde et al. 1990). In a rodent
model, where the α1d-adr had been knocked out (α1d-adr<sup>−/−</sup> mouse), the mice had a lower systolic and mean arterial blood pressure (MAP). The α1d-adr<sup>−/−</sup> mouse also had decreased sensitivity to phenylephrine, a α1a-adr agonist (Tanoue, Nasa et al. 2002). This indicated the direct regulation of α1d-adr on arterial blood pressure, independent of other α1-adr isoforms.

Our lab also observed an increased α1-adr expression in mice fed with a high n-6 PUFA diet (19% w/w), which was attenuated by supplementing with fish oil (1% w/w) (data unpublished). The role of n-6 PUFA and additional n-3 PUFA on α1-adr expression in cardiac muscle and arteries are still unclear. In this chapter, two major cardiac cell lines (fibroblasts and cardiomyocytes) were used to examine the effect of LA on regulating α1-adr and its’ potential impact on cardiovascular reactivity. The influence of additional n-3 PUFA on ameliorating high n-6 PUFA induced adr activity was also explored.
2.2 Materials and method

2.2.1 Cell models

Mouse-derived fibroblast cells NIH/3T3 were purchased from American Type Culture Collection (ATCC, Manassas, VA, Catalog no. CRL-1685). NIH/3T3 cells were cultured in DMEM-low glucose media supplemented with 2 mM sodium pyruvate, 4 mM gluta-GRO, 10% FBS, and 2% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. Cells were passaged at 70–80% confluency, and media was replaced every 3–4 days. H9c2 (CRL-1446) is the myoblast cell line originated from embryonic rat heart tissue. H9c2 is widely used as in vitro model for skeletal and myocardial muscle (Sardão, Oliveira et al. 2007). They are cultured under 10% fetal bovine serum (FBS), 2% pen/strep low glucose DMEM media with 37°C 5% CO₂ in air atmosphere. H9c2 cell is myoblast, which can be further differentiated into cardiomyocytes cell. To do this, they were differentiated by reducing FBS to 1% under plain low-glucose DMEM media, as well as adding 0.1 uM retinoic acid for 7 days (Hescheler, Meyer et al. 1991). Media was changed daily.

2.2.2 Fatty acid preparation

For all fatty acid (FA) treated cells, 100 mM stock FAs (BSA, OA, LA, ARA) were made for storage. Different FAs were dissolved in 70% ethanol (made from 100% anhydrous ethyl alcohol and sterile cell culture water). To make 5 mM FA stock, 3% FA-free, low-endotoxin bovine serum albumin (BSA) solution was prepared in PBS. Then 3% BSA and 100 mM stock FAs solutions were reheated to 37°C in a water bath (Botta 2017). 100 mM FAs stock was diluted in prepared 3% BSA solution to make 5 mM FAs. Since fatty acid is not soluble in water-phase medium, it has to be conjugated with BSA. Conjugation of these 5
mM FAs stock solution was completed at 37°C for 3-4 hours with stirring. The control solution was 5 mM BSA only. Finally, all the 5 mM FAs were sterile filtered and stored in -80 °C. Fatty acid dose response experiment was tested on NIH/3T3 cells, with the concentration of 0.025 mM and 0.1 mM. For the rest of experiments, cells were incubated with the 0.1 mM fatty acids for 18 to 24 hours before proceeding to cell assays.

2.2.3 DH5α competent cells preparation

The first super optimal broth (Dinh, Drummond et al.) (Dinh, Drummond et al.) growth medium was prepared as shown in Table 2.1. 10 ul glycerol DH5α stock (E. coli. strain) was added into 10 ml of SOB in a sterile 100 ml flask and incubated for 18 hours at 37°C and 250 rpm (Hanahan, Jessee et al. 1991). One sterile 500 ml flasks containing pre-warmed 100 ml SOB was mixed with 50 ul overnight cultured DH5α. The bacteria mixture was incubated at 37°C and 250 rpm until the cultures reached an OD600 of 0.5-0.7. Once it was ready, cultures were placed on ice for 15 minutes, and were divided into two pre-chilled 50ml centrifuge tubes. Cultures were centrifuged at 4°C, 5000 rpm for 10 minutes. Supernatant was discarded, followed by adding 50 ml 10% pre-chilled glycerol to each 50 ml tube. Next, a pipette ensured complete cell suspension, and was re-centrifuged at 4°C, 5000 rpm for 10 minutes. The supernatant was discarded and resuspended in 10 ml 10% glycerol. Cells were aliquoted into 0.5 ml tubes, with 50 ul of culture per tube.
Table 2.1. SOB medium composition

<table>
<thead>
<tr>
<th>Components</th>
<th>Mass</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>20g</td>
<td>2%</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5g</td>
<td>0.5%</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5844g</td>
<td>10mM</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1864g</td>
<td>2.5mM</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>0.95211g</td>
<td>10mM</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>1.2037g</td>
<td>10mM</td>
</tr>
</tbody>
</table>

2.2.4 Electroporation

To amplify FADS2 siRNA cocktail, 2 ul of mixed FADS2 siRNA was transferred into 50 ul DH5α competent cells, and gently mixed (Hanahan, Jessee et al. 1991). The mixture was transferred to pre-chilled electroporation cuvettes. Kimwipes were used to remove the excess liquid outside of the cuvette, and placed it into electroporator. Cells were applied with 1.8kV, 5ms for transformation. If arcing occurred, cells were discarded. 950ul of pre-warmed SOB medium was added into the cuvette and mixed gently. The mixture was immediately transferred into 5ml culture tubes and incubated at 37°C, 101 rpm for one hour. Cultured recombinant bacteria were centrifuged at room temperature, 1000 rpm for 10 minutes. 800-900 ul supernatant was discarded, and the remainder was resuspended into a pellet. 30-50 ul culture was transferred into each plate with 100 ug/ul ampicillin and incubated for 24 hours. Ampicillin plate was used here as the selective medium, which indicated successful plasmid transformation. Then plasmid was extracted with QIAprep Spin Miniprep Kit (Qiagen, Germany)
2.2.5 D6D inhibitor treatment

Fibroblasts were seeded in 48-well plates. Treatments started when cells reached 90% confluency in 48-well plates. Cells were treated with various concentration of SC-26196, a D6D inhibitor (Santa Cruz, sc-26196, Catalog no. CAS 218136-59-5) (Obukowicz, Welsch et al. 1998). 25 uM was chosen to be the optimal dose for the following experiment. The duration of the incubation (6 hours) was referred by the published article (Obukowicz, Welsch et al. 1998), and then exposed to various 0.1mM fatty acids for 18 hours.

2.2.6 Cell transfection

H9c2 and NIH/3T3 cells were seeded in 48-well plates. Cell transfection started when cells reached 90% confluency (H9c2 cells requires additional differentiation as listed in 2.2.1). 1nM insulin was added to differentiated H9c2 before the transfection. Knockdown Srebp-1c and FADS2 were achieved by using corresponding siRNA (srebp1c-siRNA, Assay Bio Tech, catalog no.A8425) and DharmaFECT 1 transfection reagent (ThermoScientific, catalog no. T-2001-01). Scrambled RNA (Santa Cruz, catalog no. Sc-37007) was used as the control. The transfection was performed following manufacture’s protocol (ThermoScientific, catalog no. T-2001-01). Cell transfection reagent increases cell permeability, which would accumulate more antibiotic in cells and cause cell toxicity (Kiefer, Clement et al. 2004). Therefore, transfection mix was prepared with 1.5 μl of DharmaFECT 1, was added to 150 μl serum free/antibiotic free media per well 250 ng plasmid (siRNA or scramble vector). This mix was incubated at 37 °C for at least 30minutes to ensure the proper dissolution. After addition of the transfection solution, cells were incubated for a minimum of six hours at 37 °C under 5% CO₂. Then 150 μl low glucose media, containing 2% penicillin streptomycin
and 20% FBS were added to each well. Cells were then incubated for 48 hours to ensure gene knockdown. 0.1mM various fatty acids were dropped into cells and incubated for another 24 hours. Cells were collected for analysis at 72 hours post-transfection. Gene silencing was confirmed by qPCR technique and the result was shown in Appendix D.

2.2.7 Cell culture assays

Neutral red assay

Neutral red assay is also known as the cytotoxicity assay, which determines cell viability by evaluating lysosomal activity (Repetto, Del Peso et al. 2008). This is a unique cell viability assay which is independent of mitochondrial health. 40 mg neutral red dye was dissolved in 10 ml PBS, to make 40 ug/ml stock solution. Neutral red medium was made by diluting the stock solution 100 times with cell culture medium and incubated overnight at the 37 °C. In brief, cell culture medium was removed the next day, with two PBS washes. Then 100 ul neutral red medium was added into each well, followed by two hours of incubation. After the incubation, neutral red medium was replaced with neutral red destain solution (Table 2.2), with two PBS washes in between. Next, the plate was placed in a shaker at room temperature for 10minutes. The OD of neutral red extract was measured at 540 nm with GloMax®-Multi Detection System (Promega, WI, USA).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Deionized water</th>
<th>100% ethanol</th>
<th>Glacial acetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volume</strong></td>
<td>10 ml</td>
<td>10 ml</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>
**ROS Assay**

Dichloro dihydro fluorescein diacetate (H2DCF-DA) is a fluorogenic dye that detects hydroxyl, peroxyl and other ROS activity within the cell (Foldbjerg, Olesen et al. 2009). Working solutions of H2DCF-DA (*Cayman Chemical Co., MI, USA*) were made to 10 μM DCF-DA in 1X PBS with calcium and magnesium. After plating and treatment, cells were washed twice in 1X PBS with calcium and magnesium followed by incubation at 37°C and 5% CO2 for 30 minutes with the working DCF-DA solution. Cells were washed twice again with PBS in the dark. Fluorescence was then read at 490 nm excitation and 510/570 nm emission by the GloMax®-Multi Detection System (*Promega, MI, USA*).

**JC-1 Assay**

Mitochondria support the basic cellular functions by producing ATP and maintaining its proton gradient (Brand and Nicholls 2011). Mitochondrial membrane potential (MMP) helps to evaluate the health of cells, by showing the mitochondrial function (Martinez-Pastor, Johannisson et al. 2004). Here, MMP was measured using 5,5,6,6-tetrachloro-1,1-3,3-tetraethylbenzimidazolcarbocyanine iodide (JC-1, *Cayman Chemical Co., CAS no. 3520-43-2*), which is a dye that accumulates mostly as red aggregates in the mitochondria of healthy cells but preferentially forms green monomers in the cytosol of apoptotic and necrotic cells. Plated and treated H9c2 and NIH/3T3 cells were treated with 1mM (trifluoromethoxy)phenylhydrazone (FCCP, *Cayman Chemical Co., MI, USA*) to serve as a negative control for apoptotic cells. All cells were washed with 1X PBS with calcium and magnesium. Working solutions of JC-1 (100 μM in PBS) were added to each well. Incubation occurred for 30 minutes, followed by removal of the dye by washing twice with PBS. Red fluorescence was
then read at 525 nm excitation and 580/640 nm emission and green fluorescence at 490 nm excitation and 510/570 nm emission, using GloMax®-Multi Detection System (Promega, MI, USA).

Resazurin Assay

Resazurin (Biotum Inc., CA, USA, cat. no. 10054) is a dye that is converted to the fluorescent resofurin when it is reduced in the mitochondria (Strehl and Adler 2012). It is therefore a good measure of mitochondrial redox potential and an indicator of cell viability. A working stock of resazurin was made and used according to manufacturer’s instructions. Fluorescence was measured using and the Glomax reader at 525 nm excitation and 580/640 nm emission.

GSH detection assay

o-Phthalaldehyde (OPA) is a fluorescent probe that can be used for the detection of both oxidized (GSSG) and reduced (GSH) glutathione (Senft, Dalton et al. 2000). Cells are first washed with PBS to remove traces of thiols present in the media and then as OPA fluoresces in the presence of all thiols, two separate reactions are performed. The first reaction is to detect all background thiols, by the addition of N-ethylmaleimide a potent inhibitor of glutathione reductase, which removes all GSH so only background thiols are detected. The second reaction determines GSH plus background thiols then, the first reaction is subtracted from the second to obtain a reading of only GSH. Fluorescence was then determined at 365 nm excitation and 410/460 nm emission using the Glomax reader as before.
**2.2.8 mRNA extraction**

Once cells were harvested, 200 ul QIAzol (Qiagen, cat. no. 79306) was added into each well. mRNA extraction was performed following manufacture’s protocol.

**2.2.9 Quantification of mRNA by quantitative PCR**

PCR mRNA levels were quantified using the real time PCR ΔΔCT method (Botta 2017). cDNA was synthesized with iScript™ cDNA synthesis kit (Biorad, cat. no. 1708891) followed by quantitative PCR on CFX96 real time PCR system (iCycler1000, Bio-Rad, CA, USA) using Ssofast Evagreen Supermix (Bio-Rad, cat. no. 1725201). Primer sequences of analyzed genes are provided in Table 2.3. The reference gene used was 18s ribosomal RNA. Each quantitative PCR plate contained no template controls to check for DNA contamination. Exponential expression was transformed to obtain ‘expression’ by using Biorad system. The equations are listed as below:

\[ \Delta Cq = Cq (target) - Cq (reference) \]

\[ \Delta Cq expression = 2^{-\Delta Cq} \]

Then ΔCq expression was normalized to experimental control to obtain ΔΔCq.
### Table 2.3. qPCR primer list

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward (5’-3’)</strong></td>
<td><strong>Reverse (5’-3’)</strong></td>
</tr>
<tr>
<td>18s (H9c2)</td>
<td>TTGATTAAGTCCCTGCCCTTTGT</td>
</tr>
<tr>
<td>18s (NIH/3T3)</td>
<td>CGGCTACCACATCCAAGGAA</td>
</tr>
<tr>
<td>Adra-1a</td>
<td>GCGGTGGACGTCTTATGCT</td>
</tr>
<tr>
<td>Adra-1b</td>
<td>CCTGTCATGTACTGCGCGA</td>
</tr>
<tr>
<td>Adra-1d</td>
<td>TGGTATCTGTGGGACCAGCTA</td>
</tr>
<tr>
<td>Adra-β1</td>
<td>CTACAACGACATCAAGCTGCT</td>
</tr>
<tr>
<td>FADS1</td>
<td>ACGCGCTACTTTACTTGGGA</td>
</tr>
<tr>
<td>FADS2</td>
<td>CACTATGCCACGTCAACAC</td>
</tr>
<tr>
<td>Srebp1-c</td>
<td>CTGGGACATCGAAACAGCAG</td>
</tr>
</tbody>
</table>

### 2.2.10 Aorta culture

The C57/Bl6 mice aorta was isolated and fat tissues were cleaned by using a dissection microscope. The aorta was then chopped into small pieces, separated and transferred into a 24-well plate. 500 ul pre-oxygenated DMEM high glucose medium was added into each well (Feeley, Westbrook et al. 2014). The aorta was incubated for overnight at 37 °C in a humidified atmosphere of 5% CO2. 25 uM FADS2 inhibitor was added into the medium and incubated for six hours. After incubating with FADS2 inhibitor, the aortas were exposed to various 0.1 mM fatty acids for 24 hours in DMEM-high glucose media supplemented with 2 mM sodium pyruvate, 4 mM gluta-GRO, 10% FBS, and 2% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO2. This method was modified by myself based on the existing protocol (Feeley, Westbrook et al. 2014).
2.2.11 Gas chromatography

Gas chromatographic analysis of total fatty acids in cells was analyzed using gas chromatography (GC, TRACE™ 1300, Bio-Rad) with a flame ionization detector (Keys, Mienotti et al.). Fatty acids were extracted by using a combined extraction and methylation protocol. In summary, 200μl QIAzol and 50ul chloroform was added to cells. The cells were centrifuged for 15 minutes at 13,000 rpm. Supernatant was collected, following by adding 1.2 ml of hexane and 1.2 ml of BF₃/MeOH (Sigma-Aldrich, Cat. no. B1127). Samples were heated at 80 °C for 1.5-2 hours. Next, 2 ml of water was added to the samples and centrifuged at 1400rpm for 2 minutes, and the top hexane layer was removed. Fatty acid methyl esters were analyzed on a Trace 1300 gas using a Supelco Famewax column (30 m × 0.32 mm inner diameter × 0.5 μm). Peak areas and retention times were then calculated using Chromeleon 7 software (Thermo Scientific) and compared against standards (Sigma-Aldrich, MI, USA). Values were expressed as percent of total fatty acid derived from the area of a single fatty acid peak/total area of peaks.

2.2.12 Statistical analysis

All data in the chapter were presented as mean ± SEM. t-test, one-way and two-way ANOVA were applied in this chapter. The statistical significant value was set as p<0.05. Values may have p less than 0.01 or 0.001. Since the goal was not to see the degree of increase or decrease, p<0.01 and p<0.001 were not categorized. Each figure has detailed statistical analysis included in result section before each figure (‘Section Statistical Analysis’).
2.3 Results:

2.3.1 Chemically blocking FADS2 failed to alter adr expression in NIH/3T3 cells.

By examining the fatty acid dose response on NIH/3T3, 0.1 mM fatty acids were found to be the optimal concentration, which exhibited different effects between BSA and LA treatments (Figure 2.1A). Cell viability was shown to be the same in cells incubated with BSA between control and FADS2 inhibitor treated groups. However, cells exposed to LA under FADS2 inhibitor treatment had reduced cell viability, compared to control (Figure2.1B, 2.1C). This indicated the negative effect of LA accumulation on cell viability.

Cell damage, or decreased cell viability, is closely associated with oxidative stress. GSH is one of the primary antioxidants to attenuate oxidative stress, by scavenging free radicals. Our lab previously showed that excess LA induced cardiac toxicity, accompanied by decreased GSH level in mice (unpublished data). However, in this in vitro study, GSH levels stayed the same of all groups (Figure2.1D). Fatty acid profile showed a complete blockage of LA bioconversion pathway under FADS2 inhibitor (sc-26196) treatment. Inhibition of FADS2 enzyme increased LA accumulation, and decreased the production of downstream ARA (C20:4n6) (Figure2.1E). This indicated high efficacy of the sc-29196 on specifically inhibiting D6D activity (D6D is encoded by FADS2) (Obukowicz, Welsch et al. 1998).

However, LA accumulation did not alter adra-1a and adrb-1 in NIH/3T3 (Figure 2.1F, 2.1H), which was opposite to the observation in C57/BL6 mice (p31). Besides, compared to BSA treated cells in control group, Srebp1c expression increased in LA treated cells (Figure 2.1I), but not in cells pre-incubated with FADS2 inhibitor.
Section statistical analysis: Figure 2.1E was analyzed by applying three sets of t-test: Control vs FADS2 inhibitor. \(*p<0.05\) vs Control. The rest subfigures were analyzed by applying two-way ANOVA with post-hoc Tukey test. \(*p<0.05\) vs LA Control. \(^{#}p<0.05\) vs BSA 0.1mM; \(^{a}p<0.05\) vs BSA Inhibitor; \(^{+}p<0.05\) vs BSA Control. n=3-9.
Figure 2.1: Chemically blocking FADS2 failed to alter adra expression in NIH/3T3 cells. Cells were treated with or without FADS2 inhibitor for 6 h, following by 18 h incubation of 0.1 mM fatty acids. A) cell viability with 0.025 mM and 0.1 mM BSA or LA incubation. B) cell mitochondrial reductase activity (resazurin assay), under the treatment of control or FADS2 inhibitor. C) cell lysosomal integrity test (neutral red assay). D) GSH assay. E) Major n-6 PUFA profile in cells exposed to LA. Major adra gene expressions: adra1a (F), adra1b (G) and adrb1b (H). I) Srebp1c gene expression. FADS2: fatty acid desaturase 2; BSA: bovine serum albumin; LA: linoleic acid; GLA: γ-linolenic acid; ARA: arachidonic acid; GSH: glutathione; adra1a: adrenergic receptor α-1a; adra1b: adrenergic receptor α-1b; adrb1: adrenergic receptors β-1; Srebp1c: sterol regulatory element-binding protein 1c.

2.3.2 Genetically blocking FADS2 increased adra-1a expression in NIH/3T3 cells.

Considering of different regulatory mechanisms at gene and protein levels, FADS2 inhibitor may display negative feedback effect on gene expression of FADS2. Therefore, siRNA technique was used to specifically knockdown FADS2 gene (data was shown in Appendix D). Contrary to chemically inhibited FADS2, adra-1a increased in FADS2 knockdown cells that were exposed to LA (Figure 2.2A). On the contrary, compared to
control cells treated with BSA, adra-1b decreased in control cells incubated with LA, and FADS2 inhibited cells with both BSA and LA treatments (Figure 2.2B). The pro-relaxation receptor, adrβ-1 increased in LA incubated cells that were lacking FADS2. Although different adr expressions were detected between control and FADS2 inhibited fibroblasts, their contractility is very limited in nature, due to their physiological and biological functions. Therefore, in the next section, cardiomyocytes (differentiated H9c2) were used to illustrate the responses of adr on LA accumulation.

Section statistical analysis: Figure 2.2 were analyzed by applying two-way ANOVA with post-hoc Tukey test. ∗p<0.05 vs BSA Control; ∼p<0.05 vs LA Control; †p<0.05 vs BSA FADS2 siRNA. n=4-6.
Figure 2.2: Genetically blocking FADS2 increased adra-1a expression in NIH/3T3 cells. Gene expressions of major adra-1 and adr-β1 in H9c2 cells. Cells were transfected with or without FADS2 siRNA for 48 h and followed by 24 h 0.1 mM BSA or LA incubation. A) adra1a. B) adra1b. C) adra1d. D) adrβ1. FADS2 siRNA increased adra-1a expression following BSA and LA treatment. FADS2: fatty acid desaturase 2; BSA: bovine serum albumin; LA: linoleic acid; adra1a: adrenergic receptor α-1a; adra1b: adrenergic receptor α-1b; adrb1: adrenergic receptors β-1.

2.3.3 Blocking FADS2 failed to alter adr expression in differentiated H9c2 cells

Contrary to fibroblasts, heart muscle cells (differentiated H9c2 cells) have higher mitochondrial number and are not able to divide (Claycomb 1992). Therefore, their tolerance to xenobiotics might be different. As per to FADS2 inhibitor dose response study, no differences were shown in lysosomal integrity between the dose of 0 uM and 25 uM (Figure 2.3A). However, 25 uM FADS2 inhibitor significantly decreased mitochondrial reductase activity in cells exposed to LA, but not BSA (Figure 2.3B). Therefore, 25 uM was selected.
as the optimal dose of FADS2 inhibitor, and this dose was applied to the rest of the experiments in this chapter. Cellular n-6 PUFA profile, with or without FADS2 inhibitor, was displayed in Appendix A. Similar to fibroblasts, LA was accumulated in cells with inhibited FADS2 (Figure 2.3C). In addition, gene expressions of adra-1 and adrb-1 statistically remained the same (Figure 2.3D-2.3 G). Contrary to fibroblast, gene expression of Srebp1c did not change in all groups (Figure 2.3H).

Section statistical analysis: Figure 2.3C was analyzed by applying three sets of t-test (LA, GLA and ARA): Control vs FADS2 inhibitor. *p<0.05 vs Control. The rest subfigures were analyzed by applying two-way ANOVA with post-hoc Tukey test. *p<0.05 vs Control LA. #p<0.05 vs FADS2 Inhibitor BSA. n=4-7.
Figure 2.3: Blocking FADS2 failed to alter adr expression in differentiated H9c2 cells. A) H9c2 cell viability (neutral red assay) test. Cells were incubated with FADS2 inhibitor at 0 uM and 25 uM, following by 0.1 mM BSA or LA treatment. B) H9c2 cell viability test (resazurin assay). Cells were incubated with 25 uM FADS2 inhibitor, following by 0.1 mM BSA or LA treatment. C) Major n-6 PUFA of LA treated H9c2 was measured by gas chromatography. Gene expressions of adra1a (D), adra1b (E), adra1d (F) and adrb1(G) in H9c2 cells. H) Srebplc gene expression in H9c2 cells. FADS2: fatty acid desaturase 2; BSA: bovine serum albumin; LA: linoleic acid; GLA: γ-linolenic acid; ARA: arachidonic acid; GSH: glutathione; adra1a: adrenergic receptor α-1a; adra1b: adrenergic receptor α-1b; adra1d: adrenergic receptor α-1d; adrb1: adrenergic receptors β-1.

Genetically silencing FADS2 was also conducted in differentiated H9c2 cells. Gene knockdown was confirmed as shown in Appendix B. A non-significant decreasing trend of cell viability was shown in FADS2 siRNA transfected H9c2, following LA incubation (Figure 2.4A). Total fatty acid distribution was shown in Appendix B. In terms of the entire n-6 PUFA profile, FADS2 siRNA was shown to block ARA production, but not GLA (Figure 2.4B). Opposing to chemically inhibited FADS2, adra1a expression was similar in control and FADS2 siRNA groups (Figure 2.4C). However, compared to control cells that were treated with BSA, expression of adra1b decreased in all other groups (Figure 2.4D). Pro-relaxing adrenergic receptor adrb-1 was similar in all groups (Figure 2.4E).
Section statistical analysis: Figure 2.4B was analyzed by applying three sets of t-test (LA, GLA and ARA): Control vs FADS2 siRNA. *p<0.05 vs Control. Cross comparison is not interested in this study. The rest subfigures were analyzed by applying two-way ANOVA with post-hoc Tukey test. *p<0.05 vs Control BSA. n=3-5.
Figure 2.4: Blocking FADS2 failed to alter adr expression in differentiated H9c2 cells. Cells were transfected with FADS2-siRNA for 48 hours, followed by 24 hours’ incubation with 0.1 mM BSA or LA. A) H9c2 cell viability test (resazurin assay). B) Major n-6 PUFA profile. Data were normalized to total PUFA. C) Gene expression of major adra1a; D) adra1b and E) adrb1. FADS2: fatty acid desaturase 2; BSA: bovine serum albumin; LA: linoleic acid; GLA: γ-linolenic acid; ARA: arachidonic acid; GSH: glutathione; adra1a: adrenergic receptor α-1a; adra1b: adrenergic receptor α-1b; adrb1: adrenergic receptors β-1.
2.3.4 FADS2 inhibitor increased Elovl5 and FADS2 gene expression.

To verify gene-protein negative feedback loop, *ex vivo* aorta was used to evaluate the effect of 25μM FADS2 inhibitor (sc-26196) on its’ gene response. FADS2 inhibitor did block LA bioconversion by increasing the accumulation of LA in both aorta culture (Figure 2.5A) and medium (Figure 2.5B). Full fatty acid profile can be found in Appendix F. However, chemically blocking FADS2 not only activated gene expression of FADS2 (Figure 2.5D), but also increased the level of Elovl5 (Figure 2.5E), the enzyme bioconverting GLA to DGLA. Therefore, chemically blocking FADS2 was not appropriate to study the gene expression of adr, in the context of LA accumulation.

*Section statistical analysis:* Figure 2.5 was analyzed by applying t-test. Figure 2.5A and 2.5B were analyzed by using three sets of t-test: Control LA vs FADS2 inhibitor LA; Control GLA vs FADS2 inhibitor GLA; Control DGLA vs FADS2 inhibitor DGLA; Control ARA vs FADS2 inhibitor ARA. *p<0.05 vs Control. n=4-8.*
Figure 2.5: FADS2 inhibitor increased Elovl5 and FADS2 gene expression. Major n-6 PUFA were measured by gas chromatography in *ex vivo* aorta culture (A) and medium (B). Gene expression of n-6 PUFA bioconversion enzymes: (C) FADS1; (D) FADS2, (E) Elovl5. Data were normalized to Control. FADS1: fatty acid desaturase1; FADS2: fatty acid desaturase 2; Elovl5: elongase 5; BSA: bolvine serum albumin; LA: linoleic acid; GLA: γ-linolenic acid; DGLA: dihomo-γ-linolenic acid; ARA: arachidonic acid; GSH: glutathione; adra1a: adrenergic receptor α-1a; adra1b: adrenergic receptor α-1b; adrb1: adrenergic receptors β-1.
2.3.5 Srebp1c controlled LA bioconversion by regulating FADS2.

As described earlier, FADS2 controls the bioconversion from LA to GLA (Azrad, Turgeon et al. 2013). However, silencing FADS2 did not block the production of GLA in cardiomyocytes, instead, the ARA production was dramatically inhibited (Figure 2.4C). Therefore, to better study the role of LA on adr gene expression in heart, a stronger element was targeted, to achieve the more complete blockage of LA to GLA bioconversion. Srebp-1 is a transcription factor that affects n-6 PUFA bioconversion by regulating all four bioconversion enzymes (Horton, Goldstein et al. 2002). Cardiomyocytes were transfected with Srebp1 siRNA (Appendix C), followed by 0.1 mM BSA or LA incubation. Similar to FADS2-inhibitor treated cardiomyocytes, silenced Srebf1 blocked the entire LA bioconversion in cardiomyocytes, from LA to ARA. Conversion of LA to GLA bioconversion was profoundly inhibited (Figure 2.6C). FADS2 expression in Srebp1c deficient cardiomyocytes exposed to LA was abolished (Figure 2.6B), however FADS1 gene expression remained the same (Figure 2.6A). In addition, Srebp1c-siRNA led to increased gene expression of adra-1a (Figure 2.6D), without affecting other adr (Figure 2.6E-2.6G).

In conclusion, Srebp-1c inhibited the β1-adr expression by increasing FADS2 expression, leading to accelerated LA removal.

Section statistical analysis: Figure 2.6B was analyzed by applying three sets of t-test (LA, GLA and ARA): Control vs FADS2 siRNA. *p<0.05 vs Control. n=3. The rest subfigures were analyzed by applying two-way ANOVA with post-hoc Tukey test. #p<0.05 vs Control LA, +p<0.05 vs Srebp1-siRNA BSA. n=3-5.
Figure 2.6: Srebp1c controlled LA bioconversion by regulating FADS2 A) FADS1 and B) FADS2 gene expressions in cardiomyocytes treated with or without Srebp1c-siRNA, following BSA or LA incubation. C) Major n-6 PUFA were measured by gas chromatography in cardiomyocytes. Gene expression of major adr: adra1a (D), adra1b (E), adra1d (F) and adrb1 (G). FADS1: fatty acid desaturase 1; FADS2: fatty acid desaturase 2; BSA: bovine serum albumin; LA: linoleic acid; GLA: γ-linolenic acid; ARA: arachidonic acid; adra1a: adrenergic receptor α-1a; adra1b: adrenergic receptor α-1b; adra1d: adrenergic receptor α-1d; adrb1: adrenergic receptors β-1; Srebp1c: sterol regulatory element-binding protein 1c.
2.3.6 Additional EPA and DHA promoted LA bioconversion

Health agencies, government, and research institutes suggest that fish oil is heart protective over the last 2 decades, due to the presence of the fatty acids EPA and DHA. Our preliminary data showed that additional fish oil was able to alleviate the high vascular reactivity stimulated by high n-6 PUFA diet (data not published). To study the possible beneficial mechanism of additional EPA+DHA, *ex vivo* aorta was isolated and treated with either 0.1 mM LA or 0.1 mM LA+EPA+DHA (Krejša, Franklin et al. 2010). Aorta culture exposed to LED increased the production of DGLA (*Figure 2.7A*), with increased gene expressions of FADS1 and Elovl5 (*Figure 2.7B, 2.7D*). This indicated that supplementation of EPA+DHA promoted LA removal by accelerating PUFA bioconverting efficiency. Besides, many studies have demonstrated that fish oil promoted anti-inflammatory responses, as a result of reducing oxidative stress (Barbosa, Cecchini et al. 2003; Kim, Kim et al. 2006; Duda, O’shea et al. 2008). Gpx and SOD are two major antioxidant enzymes that are able to scavenge free radicals, therefore improving oxidative stress. In this study, *ex vivo* aorta culture incubated with LED was shown to increase the expression of Gpx1 (*Figure 2.7G*) but not SOD (*Figure 2.7E, 2.7F*), compared to LA treated groups. Therefore, additional EPA and DHA may potentially attenuate high n-6 PUFA induced cardiac toxicity by accelerating LA bioconversion, as well as improving oxidative stress.

*Section statistical analysis:* Figure2.7 was analyzed by applying t-test. *p*<0.05 vs LA. n=4-6. Figure 2.7A was analyzed by using four sets (LA, GLA, DGLA, ARA) of t-test: Control vs LA.
Figure 2.7: Additional EPA and DHA promoted LA bioconversion Isolated aorta culture was incubated with either 0.1 mM LA or 0.1 mM LED for 24 hours. A) n-6 PUFA distribution in aorta culture. n-6 PUFA bioconversion genes’ expression: FADS1 (B), FADS2 (C), Elov5 (D). Three major antioxidants genes’ expression: SOD1 (E), SOD2 (F), Gpx1 (G). FADS1: fatty acid desaturase 1; FADS2: fatty acid desaturase 2; Elov5: elongase 5; LA: linoleic acid; GLA: γ-linolenic acid; DGLA: dihomo-γ-linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LED: LA+EPA+DHA; SOD1: superoxide dismutase 1; SOD2: superoxide dismutase 2; Gpx1: glutathione peroxidase 1.
2.4 Discussion

Our lab previously discovered that high corn oil (20% w/w, CO, high n-6 PUFA) diet activated vascular contractility, compared to low fat (5% w/w) and high corn oil diet with additional fish oil (19% w/w corn oil+ 1% w/w fish oil). Mice fed with high CO displayed higher sensitivity to phenylephrine, a specific adrα-1a agonist. This high CO diet also increased expression of pro-contracting adrenergic receptors. However, the mechanism behind this observation was elusive. This chapter tried to disclose the mechanism of how the LA-rich diet stimulated vascular reactivity, and how the addition of fish oil reversed this hyper-reactivity.

As mentioned, LA is the primary n-6 PUFA in corn oil, which can be bioconverted into longer chain n-6 PUFA like ARA but at a relatively low rate, especially in heart and brain (Gao, Kiesewetter et al. 2010). Understanding the individual role of LA, on regulating adrenergic responses is crucial to identify the mechanism.

Fibroblasts and cardiomyocytes, two major cell types in heart, have their own unique physiology. Due to the negative feedback loop of gene and protein (Krishna, Andersson et al. 2006), chemically blocking FADS2 was confirmed to be an inappropriate approach to evaluate the expression of adrenergic receptors at gene level (Figure 2.5). Genetically blocking FADS2 increased adr expression in fibroblasts incubated with BSA and LA (Figure 2.2). However, this increased adr response was not observed in cardiomyocytes (Figure 2.4). It is known that both NIH/3T3 and differentiated H9c2 have contractility, which is regulated by adr (Saeed, Parmentier et al. 2004). As described earlier, heart muscle holds the most mitochondrial number of the entire body for its energy needs (Pascual and Coleman 2016).
The inconsistent adr response (Figure 2.2 and Figure 2.4) may indicate different sensitivity of fatty acid in fibroblasts and cardiomyocytes. In the other word, inhibiting FADS2 was not strong enough to accumulate LA. Therefore, the response of adr on LA level cannot be detected. In order to see the link between LA and adr gene expression in cardiomyocytes, a more potent regulator was used to further accumulate LA.

Srebp1 is a transcription factor that regulates all fatty acid metabolism, from adding acetyl-CoA to β-oxidation (Horton, Goldstein et al. 2002). These two crucial steps both occur in mitochondria (Kastaniotis, Autio et al. 2017). It is well known that Srebp1 promotes the production of cholesterol and SFA (Horton, Goldstein et al. 2002). In recent years, researchers have begun to explore the interaction between PUFA metabolism and Srebp1 (Takeuchi, Yahagi et al. 2010). Due to the high n-6 to n-3 PUFA ratio found in the diet of Western populations, LA highly accumulates in body system, which traditionally only happens to individuals with higher vegetable consumption. Studies showed that a diet rich in LA increased FADS2 activity (Xu, Zheng et al. 2018). High LA accumulation promoted Srebp1 expression (Geay, Culi et al. 2010). Similarly, Dong et al reported that Srebp1 regulated PUFA bioconversion by targeting FADS2 promoter directly (Dong, Tan et al. 2017). Taking all these together, a LA-rich environment potentially increases Srebp1, therefore increasing FADS2 expression. Although chemically blocking FADS2 failed to study adr responses at gene level (Figure 2.4), Srebp1 expression gave us hints that there was different sensitivity of fatty acids between the two cell types. Compared to BSA, Srebp1c expression increased in the fibroblasts treated with LA (Figure 2.1), however, this observation was not seen in cardiomyocytes (Figure 2.3). This again supported that cardiomyocytes have higher tolerance to fatty acids, compared to fibroblasts. The unique
feature of heart determines its’ high mitochondria density, which provides a more efficient fatty acid metabolic environment (Piquereau, Caffin et al. 2013). In conclusion, the 0.1 mM LA treatment may not be sufficient to activate Srebp1, in order to further enhance PUFA metabolism in cardiomyocytes.

As shown in result section, FADS2 itself was not able to regulate gene expressions of adr in cardiomyocytes (Figure 2.3, 2.4). Instead, silencing Srebp-1c (encoded by Srebf-1), an upstream transcription factor, increased adrα-1a, and completely blocked LA bioconversion, from both gene (deprived FADS2 gene expression) and protein levels (reduced GLA and ARA production) (Figure 2.6). Therefore, blocking FADS2 alone may not be strong enough to alter adr response in cardiomyocytes. On the contrary, fibroblasts do not have distinct preference to energy sources. This indicates that fibroblasts are more sensitive to fatty acid, compared to cardiomyocytes. In this chapter, we showed that same concentration of LA (0.1 mM) was much easier to stress fibroblasts, leading to the amplified adr expression with the inhibition of FADS2 (Figure 2.2). This study indicated that the adr response on fatty acid varied in different cell types. Cardiomyocytes have higher metabolic rate of fatty acids, compared to fibroblasts. Hence, we can infer that the ‘ratio’ of LA level to LA metabolic potential, but not LA itself, may determine the adr expression. Dietary LA is excessive in Western diet. This ultimately may increase this ‘ratio’, so that LA bioconversion is relatively inefficient. This might be the root cause of increased adr responses in mice receiving a diet rich in n-6 PUFA (observation from previous lab work).

We previously observed that supplementing with fish oil attenuated high corn oil diet induced adr responses. Referring to the conclusion of this chapter, this beneficial mechanism
might be a more balanced ‘ratio’ of LA level to LA bioconversion potential. We first discovered that additional EPA and DHA promoted PUFA bioconversion by increasing the expression of PUFA bioconversion genes (FADS1 and Elovl5), at the same time activating antioxidant redox cycle (Figure 2.7). Interestingly, EPA+DHA did not alter FADS2 expression. Elovl5 directs the bioconversion from GLA to DGLA, and FADS1 metabolizes DGLA to ARA. This represented an accelerated metabolism from GLA onwards. As a result, the accumulation of LA is reduced, as well as the ‘ratio’ of LA level to LA bioconversion efficiency.

In conclusion, globally blocking PUFA bioconversion by Srebp1 increased adr expression in cardiomyocytes exposed to LA (Figure 2.8). Additional EPA+DHA accelerated PUFA bioconversion, with a higher level of antioxidant genes like Gpx1. This potentially improved oxidative stress and indicated a vascular protective role, in the context of LA overload. This novel mechanism may explicit another possible protective pathway of well-noted fish oil benefits.
Figure 2.8. The mechanism of accumulating LA on regulating the expression of α-1 adrenergic receptor. LA: linoleic acid; GLA: γ-linolenic acid; ARA: arachidonic acid; EPA eicosapentaenoic acid; DHA: docosahexaenoic acid; FADS2: fatty acid desaturase 2; Elov5: elongase 5; FADS1: fatty acid desaturase 1; SREBP1c: sterol regulatory element binding protein 1c.
Chapter 3: Elovl5 positively regulates blood pressure and cardiac hypertrophy

3.1 Background

Debate continues on the pros and cons of n-6 PUFA consumption. Numerous clinical studies have demonstrated that n-6 PUFA attenuated hypertension (Wolters, Pala et al. 2016), prevented cardiovascular diseases (Hodson, Skeaff et al. 2001), and improved insulin resistance (Kurotani, Sato et al. 2012). However, in chapter 2, we showed that LA, the predominant fatty acid in corn oil, activated the pro-contracting genes (adr) through the central transcription factor Srebp1 in vitro. In fact, Srebp1 not only controls FADS2, but also regulates elongases at gene level (Shikama, Shinozaki et al. 2015). Srebp1 binds to SRE which is located at the enhancer region of elongase 5 (Elovl5) that directly promotes GLA elongation (Shikama, Shinozaki et al. 2015). Elovl5+/− mice showed a highly activated Srebp1 in response to inhibited long-chain PUFA production, which further induced fatty liver (Moon, Hammer et al. 2009). However, conversely over-expression of Elovl5 was not able to increase Srebp1 expression due to the accelerated production of long chain n-6 PUFA, including ARA, EPA and DHA, in vitro (Yoshikawa, Shimano et al. 2002) and in vivo (Tripathy, Torres-Gonzalez et al. 2010). To clarify the effect of Elovl5 on cardiac contractility, Elovl5+/− mice were used here to investigate vascular contractility.

To date, seven elongase isoforms have been discovered (Uchida 2011). Elovl5 is believed to elongate unsaturated fatty acids, particularly from C18 to C20 (Jump 2009). Additionally, Elovl5 promoted fatty acid catabolism, by breaking down hepatic triglycerides in obese C57/BL6 mice (Tripathy, Lytle et al. 2014). Similarly, post obesity surgery
improved fatty acid metabolism of patients who suffered from obesity, by increasing Elovl5 level, but not FADS1 and FADS2, in adipose tissue (Walle, Takkunen et al. 2017). This indicated the substantial role of Elovl5 in accelerating fatty acid catabolism, which potentially altered circulatory fatty acid profile. One recent study in rodents with diabetes came to a similar conclusion, but in an opposite way. In db/db and ob/ob C57/BL6 mice livers, Elovl5 expression was elevated through DNA auto-methylation, in the context of insulin resistance and enhanced inflammatory response (Hwang, Lee et al. 2018). Another study examined the role of Elovl5 in obese mice under a high fat diet. The authors concluded that Elovl5 was beneficial to maintain fasting blood glucose and to improve insulin sensitivity (Tripathy, Torres-Gonzalez et al. 2010). Therefore, Elovl5 might be a novel therapeutic target to correct abnormal blood glucose and to reshape circulatory fatty acid composition in CVD. The challenge is that most studies that examined Elovl5 were completed in liver and adipose tissue. The effects of Elovl5 on the cardiovascular system are not known. We hypothesize that *Elovl5 deficiency may cause elevated vascular contractility in the context of a high n-6 PUFA diet*. In this chapter, we specifically focused on heart and aorta function of Elovl5<sup>+/−</sup> mice.

It is well known that insulin resistance and hypertension have a co-existing relationship (Salvetti, Brogi et al. 1993). Patients with hypertension are at high risk of developing vascular dysfunction and cardiometabolic diseases such as CVD and diabetes. The root cause of these complications is believed to be the result of increased oxidative stress and systemic inflammation (Dinh, Drummond et al. 2014). Our lab and many other studies had demonstrated that LA, the primary fatty acid in corn oil, promoted oxidative stress in the heart (Laher, Beam et al. 2013; DiNicolantonio and O’Keefe 2018), vascular endothelial
cells (Toborek, Blanc et al. 1997) and red blood cells (Yuan, Fan et al. 2015). Opposite to LA, GLA is considered to be an anti-inflammatory n-6 PUFA, which can alleviate oxidative stress (Kapoor and Huang 2006). Since Elovl5 controls the n-6 PUFA bioconversion from GLA to DGLA, regular corn oil diet was supplemented with additional borage oil (2% w/w), in order to investigate the correlation between upstream n-6 PUFA (LA and GLA) and inflammation in Elovl5<sup>+/−</sup> mice.
3.2 Materials and methods

3.2.1 Animal care

Researchers have suggested that knocking out Elovl5 (Elovl5+/−) mice had impaired reproduction system in female, but males showed the normal fertility (Moon, Hammer et al. 2009). It is known that females accumulate more fat in the perspective of reproductive purpose, with higher level of long-chain PUFA, compared to males (O’Neill and Minihane 2017). However, females experience lower risk of cardiovascular diseases (Palmisano, Zhu et al. 2018). Therefore, in this study, six-month old female Elovl5+/− mice (KOMP, University of California, Davis, CA, USA) were used to evaluate the effect of dietary fat on cardiac responses. Genotyping was completed following KOMP’s protocol, to confirm the gene knockout (Appendix H). Six-month old partial knockout Elovl5+/− and Elovl5+/+ female mice were fed with isocaloric, iso-nitrogenous high-fat diets, with additional 20% w/w fat coming from various vegetable oils and fed for six weeks. The basal diets were prepared by Harlan Teklad (Envigo, CA, UK. # TD.88232). Then the basal diet was mixed with either olive oil (OO), corn oil (CO) or corn oil plus borage oil (CO+GLA; GLA supplemented LA diet) (Appendix F). The quantity of borage oil in CO+GLA diet was applied by referencing the commercial guidelines of borage oil supplements (240 mg/day). Therefore, 24% borage oil (w/w) was added to 76% (w/w) corn oil to offset the high LA diet. Composition and nutritional information for the diets are given below in Table 3.1 and Table 3.2. Mice were weighed twice a week and fasting glucose was measured every two weeks. Food and water were provided ad libitum. After six weeks of feeding, mice were sacrificed. The heart, aorta and blood were collected for further analysis. The aorta and heart were flash frozen in liquid
nitrogen, or stored in RNA stabilizer solution. Serum was separated from blood by centrifuging at 1000 rpm for 10 minutes.

Table 3.1 Nutrients composition of high fat diets

<table>
<thead>
<tr>
<th>Formula</th>
<th>OO Composition (g/kg)</th>
<th>CO Composition (g/kg)</th>
<th>CO+GLA composition (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>240.0</td>
<td>240.0</td>
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<tr>
<td>DL-Methionine</td>
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<td>75.0</td>
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<td>3.6</td>
</tr>
<tr>
<td>Vitamin Mix</td>
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<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Oil</td>
<td>190.0 (olive oil)</td>
<td>190.0 (corn oil)</td>
<td>140 (corn oil) 60 (borage oil)</td>
</tr>
</tbody>
</table>

Diet consisted of basal mix plus additional oil as indicated. CO, corn oil; OO, olive oil, GLA, γ-linolenic acid.

Table 3.2 Final diet composition (%)

<table>
<thead>
<tr>
<th></th>
<th>by weight %</th>
<th>by energy %</th>
</tr>
</thead>
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<tr>
<td>Carbohydrate</td>
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<tr>
<td>Protein</td>
<td>21.2</td>
<td>19.0</td>
</tr>
<tr>
<td>Fat</td>
<td>20.2</td>
<td>40.8</td>
</tr>
</tbody>
</table>

Energy Density/g of finished diet: 13.81 kJ/g

3.2.2 Fasting glucose

Fasting glucose was measured in all experimental animals every two weeks throughout six-week feeding study duration. Animals were fasted for five hours and the blood glucose was measured by collecting blood from their tails. Glucose test strips (OneTouch Ultra® test strips) and glucometer (One-Touch UltraMini® meter) were used in this test.
3.2.3 Echocardiography

A HP/Phillips Sonos 5500 ultrasound system (Phillips, NL) was used to examine the heart function in mice by non-invasive transthoracic echocardiography. All experiments were completed by following HP/Phillips Sonos 5500 ultrasound system manual. Mice were anaesthetized with 2% isoflurane on a warming platform to maintain body temperature at 37 °C (referring to CACC regulation). By using probe 15-L, M-M mode and pulsed-wave (PW) Doppler mode were applied to analyze heart and aortic valve function, respectively.

3.2.3.1 M-M mode

The probe was settled in the mitral valve to acquire images. Multiple readings were taken to ensure accuracy. Images were transferred from the machine to the computer for further analysis. Digimizer Image Analysis Software was used to measure parameters including left ventricular internal diameter end diastole (LVIDd) and end systole (LVIDs), posterior wall thickness diastole (PWd) and systole (PWs), interventricular septal end diastole (IVSd) and end systole (IVSs). By using these parameters, ejection fraction (EF), fractional shortening (FS), left ventricular mass (LV mass), end diastolic volume (EDV), end systolic volume (ESV) and stroke volume (SV) (Yang, Bazhin et al.) was calculated using the equations listed below. The cardiac output (CO) and heart rate (HR) were measured by echocardiography. By adding HR function in the pre-set list, HR can be measured under M-M mode or PW mode with a selection of the calliper tool. EF and FS, and CO indicate the efficiency of the contractility of heart muscles, LV in particular. LV mass provides the information of the density of myocardial density.

\[
EF\% = \frac{(\text{LVID}_d - \text{LVID}_s)}{\text{LVID}_d}
\]
FS% = (LVIDd − LVIDs)^3 ÷ LVIDd^3

LV mass = 1.05 * ((LVIDd + PWd + IVSd)^3 − LVIDd^3)

EDV = (7 ÷ (2.4 + LVIDd)) × LVIDd^3

ESV = (7 ÷ (2.4 + LVIDs)) × LVIDs^3

SV = EDV − ESV

CO = SV × HR

EF%: ejection fraction percentage; FS%: fractional shortening percentage; LV mass: left ventricular mass; EDV: end-distole volume; ESV: end systole volume; SV: stroke volume; CO: cardiac output.

3.2.3.2 Pulsed-Wave Doppler

After acquisition of images, calculations were performed using the ‘left ventricular outflow tract (LVOT)’ package analysis under ‘aorta’ option. Within the LVOT package, ‘left ventricular outflow tract velocity time integral (LVOT VTI)’ was selected and the trace tool was used. Through the use of this tool, individual cardiac cycle was traced. The length of the trace-line gave the value of LVOT VTI. Other parameters including blood flow velocity and aortic blood pressure were both measured under the LVOT VTI function. Multiple cardiac cycles were measured separately to minimize human error. To measure the aorta diameter, ‘LVOT DIST (LVOT distance)’ was selected under the LVOT package. By using the calliper tool, aorta diameter was obtained. LVOT, heart rate, systolic blood pressure (Sys. BP) and diastolic blood pressure (Dia. BP) were measured by using trace-line (dot line showed in Figure 3.1). Once the heart rate measurement was completed, mean arterial pressure (MAP) and pulsatility index (PI) were calculated using the following formula (Pinsino, Castagna et al. 2018):
\[
\text{MAP} = \frac{2}{3} \times \text{Dia. BP} + \frac{1}{3} \times \text{Sys. BP}
\]

\[
\text{PI} = \frac{V \text{ sys.} - V \text{ dia.}}{V \text{ sys.}}
\]


Figure 3.1: Sample echocardiography pulsed-wave Doppler mode. White dotted trace-line represent one cycle of aortic valve. A, B, C, D on the left side represented four readings from different aortic valve cycle. VTI was the length of white dot trace-line. First max and min indicated the blood flow velocity. The second max and min showed the systolic blood pressure and diastolic blood pressure, respectively. Abbreviations: HR, Heart Rate.

3.2.4 Quantitative polymerase chain reactions (qPCR)

Quantitative PCR was performed as described in section 2.3.8. Primer sequences were listed below in Table 3.3.
Table 3. 3 qPCR primer list

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward (5'-3')</td>
<td>Reverse (5'-3')</td>
</tr>
<tr>
<td>18s</td>
<td>CGGCTACCACATCCAAGGAA</td>
<td>R: GCTGGAATTACCGCGGCT</td>
</tr>
<tr>
<td>Adra-1a</td>
<td>GCGGTGGACGTCTTATGCT</td>
<td>TCACACCAATGTATCGGTGA</td>
</tr>
<tr>
<td>Adra-1d</td>
<td>TGGTATCTGTTGGGACCGCTA</td>
<td>ACATACACGGCCAGTACAT</td>
</tr>
<tr>
<td>Adra-b1</td>
<td>GAACCGCTGCAACCTGTCGT</td>
<td>CACGTTACCCACCAGTGA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TACTGAACCTTCGGGATTGGCTCC</td>
<td>CAGCCTGTCCCTTGAAGAGAACC</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TGAACGCTACACACTGCATCTTG</td>
<td>CGACTCCTTTTCCGCTTCTGAG</td>
</tr>
</tbody>
</table>

3.2.5 Fatty acid analysis in aorta

Fatty acid analysis was performed as described in Chapter 2.

3.2.6 Cytokine analysis in blood serum

Animal whole blood was collected and centrifuged at 4 °C, 1000 rpm for 10 minutes. The serum was separated from the red blood cells. 10ul of serum were diluted with 30ul of sterile water for ELISA test. The test was completed by Ray Biotech, GA, USA.

3.2.7 Statistical analysis

Statistical analysis was performed as described in 2.2.12. ‘Sectional statistical analysis’ is available before each figure.
3.3 Results

As previously shown in Chapter 2, blocking the n-6 PUFA bioconversion increased expression of adr-α in both cardiomyocytes and fibroblasts. However, the inhibition of LA to GLA bioconversion, or FADS2, did not demonstrate similar results in these two cell lines. Compared to control cells, we noticed that silencing FADS2 did not reduce GLA production (Figure 2.4). However, blocking n-6 PUFA bioconversion by Srebp1c siRNA diminished the production of both GLA and ARA (Figure 2.6). As addressed earlier, Srebp1c not only regulates FADS2, but also attaches to the enhancer of Elovl5, to directly promote Elovl5 expression. Therefore, Elovl5+/− mice were used in this chapter, to discover the effect of Elovl5 on aortic and cardiac function, following the consumption of various high fat diets.

3.3.1 Elovl5 deficiency increased aortic blood pressure

Elovl5+/− mice decreased Elovl5 gene expression in heart (Figure 3.2B), without altering other genes involved in n-6 PUFA bioconversion (Figure 3.2A, 3.2C-E). No difference of body weight was detected between Elovl5+/+ and Elovl5+/− mice over the six-week high fat feeding (20% w/w) period (Figure 3.2F).

Aortic blood pressure and blood velocity were evaluated by PW Doppler echocardiography. Elovl5+/− mice showed an elevated mean arterial pressure, regardless of the diet (Figure 3.3B). Elovl5+/− mice receiving OO and CO diets increased systolic blood pressure in the aorta, but diastolic pressure remained the same (Figure 3.3C). Elovl5+/− mice supplemented with GLA exhibited a tendency to increase blood pressure, however, no statistical significance was found.
Section statistical analysis: Figure 3.2A-E were analyzed by applying t-test, \(^{*}p<0.05\) vs Elovl5\(^{+/+}\). Figure 3.2F was analyzed by applying two-way ANOVA. \(n=7\). Figure 3.3 B and C were analyzed by applying one-way with post-hoc Tukey test. \(^{*}p<0.05\) vs Elovl5\(^{+/+}\). \(n=7\).
*Figure 3.2: Elovl5<sup>+-</sup> mice did not affect other n-6 PUFA bioconversion genes. (A-E) expression of PUFA bioconversion genes in Elovl5<sup>++</sup> mice and Elovl5<sup>+-</sup> mice. F) Body weight of Elovl5<sup>++</sup> and Elovl5<sup>+-</sup> mice following six-week high fat feeding. Elovl2: elongase 2; Elovl5: elongase 5; FADS1: fatty acid desaturase 1; FADS2: fatty acid desaturase 2; Srebp1c: sterol regulatory element-binding protein 1c; OO: olive oil; CO: corn oil; CO+GLA: corn oil + γ-linolenic acid.*
Figure 3.3: Elovl5 deficiency increased aortic blood pressure. A) Pulsed-wave Doppler graphs in Elovl5+/+ and Elovl5+-/ mice. B) MAP in Elovl5+/+ and Elovl5+-/ mice. Elovl5+-/ mice increased MAP, regardless of the diet. C) Pulsed-wave Doppler parameters table by detecting the physiological function at aortic valve. Elovl5: elongase 5; OO: olive oil; CO: corn oil; CO+GLA: corn oil + γ-linolenic acid; LVOT: left ventricular outflow tract; LVOT VTI: left ventricular outflow tract velocity time integral; max vel.: maximum velocity; min vel: minimum velocity; sys. BP: systolic blood pressure; dia. BP: diastolic blood pressure; MAP: mean arterial pressure.

3.3.2 High corn oil feeding altered cardiac physiology in Elovl5+-/ mice

High blood pressure normally increases the risk and development of numerous cardiovascular diseases (Tajalizadekhoob, Sharifi et al.). In order to determine how Elovl5 affects cardiac function, M-M mode echocardiography was used to examine corresponding parameters. Ejection fraction (EF), fractional shortening (FS) and stroke volume didn’t change across all groups (Figure 3.4A, 3.4B, 3.4C). However, cardiac output decreased in CO fed Elovl5+-/ mice (Figure 3.4D). In addition, a smaller left ventricle size was found in
CO fed Elovl5+/− mice (Figure 3.4E), which also showed a decreasing trend of LV mass to body weight ratio (Figure 3.4F).

Section statistical analysis: Figure 3.4 were analyzed by applying one-way ANOVA with post-hoc Tukey test. *p<0.05 vs Elovl5+/− OO. n=7.

Figure 3.4: Heart High corn oil feeding altered cardiac physiology in Elovl5+/- mice
Ejection fraction (EF) (A), fractional shortening (FS) percentage (B), Stoke volume (C), cardiac output (CO) (D) and heart LV mass (E) measurements were completed by selecting M-M mode of echocardiography. (F) LV mass to body weight ratio was calculated by dividing LV mass by individual mouse body weight. Elovl5: elongase 5; CO: corn oil; OO: olive oil; GLA: γ-linolenic acid.

3.3.3 High n-6 PUFA diets increased adr expression and inflammatory markers in aorta

We investigated the genetic responses in the Elovl5+/− aorta, following three different high fat diets. Total fatty acid composition in the aorta matched the type of fats that mice
received, with a high LA in mice with CO diet and a high GLA in mice receiving GLA+CO diet (Figure 3.5F). High n-6 PUFA diets increased gene expressions of adrα-1 and adrβ-1 in Elovl5+/− mice aorta, compared to OO group. Mice fed with CO showed a higher adrα-1a expression (Figure 3.5A), whereas adrα-1d expression was increased in CO+GLA group (Figure 3.5B). However, a higher adrβ-1 expression was also detected in CO+GLA group, which suggested vascular relaxation (Figure 3.5C). Despite the finding of the similar aortic blood pressure across all three diets in Elovl5+/− mice, genetic responses were vastly different between the high n-6 PUFA diets and high MUFA diets.

High blood pressure is also associated with inflammation. Here, we examined the local inflammatory responses of the aorta, by evaluating cytokines’ expression. Mice receiving high n-6 PUFA diets showed a higher IFN-γ and TNF-α expression, compared to OO fed Elovl5+/− mice (Figure 3.5D, 3.5E).

Section statistical analysis: Figure 3.5 was analyzed by applying one-way ANOVA with post-hoc Tukey test. *p<0.05 vs Elovl5+/− olive oil. *p<0.05 vs Elovl5+/− corn oil. n=4-6. Statistical significance of Figure 3.5D was detected by applying nonparametric Kruskal-Wallis test (non-ordinary one-way ANOVA).
Figure 3.5: High n-6 PUFA diets increased adr expression and inflammatory markers in aorta A-C) major adrenergic receptors’ gene expression (adra-1a, adra-1d, and adra-β1). D) IFNγ and E) TNF-α gene expressions. F) major PUFA distribution in aorta. adra-1a: adrenergic receptor α1-a; adra-1d: adrenergic receptor α1-d; adrb1: adrenergic receptor β1; TNF-α: tumor necrosis factor α; IFN-gamma: interferon γ; OO: olive oil; CO: corn oil; CO+GLA: corn oil + γ-linolenic acid.
3.3.4 Dietary n-6 PUFA did not strongly stimulate systemic inflammation in Elovl5+/− mice.

Diets high in n-6 PUFA induced local inflammation in the Elovl5+/− aorta. To evaluate the systemic inflammatory response, blood serum was collected to examine the expression of cytokine and chemokines by applying ELISA. Expression of cytokines and chemokines had a detection limit at 4 ng/ml (dotted line), which was provided by the company. It is known that IL-15 binds to IL-2/15 receptor β, to promote T-cell proliferation (Meghnem, Morisseau et al. 2017). Among all cytokines tested, IL-15 showed an increased expression only in CO fed Elovl5+/− mice (Figure 3.6D). Other cytokines remained the same (Figure 3.6). Besides, keratinocyte chemoattractant (KC), a chemokine that attracts neutrophils, increased in Elovl5+/− mice receiving high n-6 PUFA diet, compared to OO fed mice (Figure 3.5E). Although CO group did not show statistical significance, it displayed an increasing trend of KC. Neutrophils are considered to be the first immune cells to counter against xenobiotic, which induce an oxidative burst and initiate an inflammatory response (Selders, Fetz et al. 2017). However, TNF-α did not change in all three groups (Figure 3.6F), which was opposite of the aortic local TNF-α expression showed in Figure 3.5B. Therefore blocking PUFA bioconversion may not necessarily affect the systemic inflammatory response.

*Section statistical analysis:* Figure 3.6 was analyzed by applying one-way ANOVA with post-hoc Tukey test. n=6 *p<0.05 vs Elovl5+/− OO.*
Figure 3.6: Dietary n-6 PUFA did not strongly stimulate systemic inflammation in Elovl5+/− mice. IL-1a (A), IL-2 (B), IL-13 (C), IL-15 (D), KC (E) and TNF-alpha (F) expression in blood serum were detected by ELISA. IL-1a: interleukin 1a; IL-2: interleukin 2; IL-13: interleukin 13; IL-15: interleukin 15; KC: keratinocyte chemoattractant; TNF-alpha: tumor necrosis factor alpha.

3.3.5 Supplementation with EPA+DHA increased PUFA bioconversion gene expression in the aorta of Elovl5+/− mice

Supplementation of EPA+DHA promoted LA removal in C57/Bl6 mice aorta (Chapter 2). A similar *ex vivo* study was conducted with aortas isolated from Elovl5+/− mice. Isolated aortas that were cultured with LA+EPA+DHA (Krejsa, Franklin et al.) increased the
expression of Elovl5 (Figure 3.7C). Similar to Figure 2.7, gene expressions of three major antioxidants were examined. SOD2, the mitochondrial SOD, was the only one increased in aorta treated with LED, compared to LA group (Figure 3.7E). Although LED treated aorta culture increased Elovl5 expression, the PUFA profile didn’t support this gene response (Figure 3.7G). Opposite to aorta culture, LA was higher in the medium supplemented with EPA and DHA (Figure 3.7G), which may indicate a different energy preference.

Section statistical analysis: Figure 3.7 was analyzed by applying t-test. *p<0.05 vs Elovl5+/− LA. n=5. In Figure 3.7G, *p<0.05 vs LA in culture medium.
Figure 3.7: Supplementation with EPA+DHA increased PUFA bioconversion gene expression in the aorta of Elovl5+/− mice A-C). Expression of PUFA bioconversion genes in isolated aortas that were treated with 0.1 mM LA or LED. D-F) Expression of three major antioxidant genes’ in isolated aortas. G) Major PUFA distribution in ex vivo aorta culture and medium. FADS1: fatty acid desaturase 1; FADS2: fatty acid desaturase 2; Elovl2: elongase 2; Elovl5: elongase 5; SOD1: superoxide dismutase 1; SOD2: superoxide dismutase 2; Gpx1: glutathione peroxidise; LA: linoleic acid; LED: LA+EPA+DHA.
3.4 Discussion

Chapter 2 indicated that blocking LA bioconversion increased the expression of adra-1 which potentially indicated higher cardiomyocyte contraction. However, expression of pro-contraction genes can not reflect the outcome of systemic blood supply, or vascular contractility. In this chapter, Pulsed-Wave Doppler and M-M mode of echocardiogram were applied in Elov15+/+ and Elov15+/- mice, to directly investigate the connection between PUFA bioconversion and vascular performance. The role of Elov15 in PUFA metabolism is to elongate C18 to C20. Other than PUFA, Elov15 also regulates triglycerides (Ishiyama, Taguchi et al.) and MUFA metabolism (Shi, Du et al. 2018). Functional mitochondria, where acetyl-CoA is produced, are critical to maintain all the elongation reaction by adding two additional carbons to existing carbon chain (Nakamura and Nara 2003). Heart uses fatty acid as its’ primary energy source, as it has the highest mitochondrial density in our body system (Berg, Tymoczko et al. 2002). These make heart metabolize fatty acids more efficiently. In cardiac disease state, fatty acid utilization is interrupted, leading to the accumulation of fatty acids in heart. PUFA are the most vulnerable group of fatty acids due to the number of double bonds, which increase the risk of being oxidized and cause lipotoxicity (Borradaile and Schaffer 2005). Hence, maintaining PUFA elongation is important to preventing high dietary LA accumulation induced heart problems, as well as clearing out excess storage of TAGs.

Mice lacking Elov15 separated out the upstream and downstream n-6 PUFA, with accumulated LA and GLA, and inhibited DGLA and ARA yield. ARA traditionally has been considered to be the most detrimental n-6 PUFA, which was blamed for causing negative
consequences of current Western diet (Adam, Lie et al. 2017; Thomas, Paris et al. 2017). However, opposite to this classic opinion, this data suggested a more damaging effect of LA and GLA over the downstream n-6 PUFA (DGLA onwards) (**Figure 3.3B**). Although aortic MAP was the same in the Elovl5^+/−_ mice receiving all three high fat diets (OO, CO, CO+GLA), gene expression of adr was significantly elevated by high n-6 PUFA diets. High n-6 PUFA diets (CO and CO+GLA) increased adr expression in the aorta, compared to OO (**Figure 3.5A**). This indicated that upstream n-6 PUFA accumulation along with impeded n-6 PUFA bioconversion were equally important to regulate vascular contractility. This might further stimulate local inflammatory biomarkers such as TNF-α and IFN-γ (**Figure 3.5D, 3.5E**). However, diet alone, or the duration of the feeding, may not be strong enough to alter physiological parameters.

Despite similar cardiac output across all dietary groups (**Figure 3.4B**), CO fed mice had smaller LV mass (**Figure 3.4C**). In order to obtain the same amount of blood pumping out of the heart per minute, a stronger pressure has to be pushed on aortic valve. This indicated that LA accumulation, through dietary and genetic aspects, pushed harder on the heart, resulting in a stronger cardiac contractility. This may prime for further heart hypertrophy or other cardiovascular associated diseases.

In chapter 2, additional EPA and DHA (primary fatty acids from fish oil) increased Elovl5 gene expression in _ex vivo_ aorta culture. Similarly, LED incubated aortas from Elovl5^+/−_ mice also elevated expression of Elovl5 (**Figure 3.7C**) and SOD2 (mitochondrial SOD) (**Figure 3.7D**). This suggested that EPA+DHA increased n-6 PUFA bioconversion primarily by elevating Elovl5 expression. Interestingly, although Elovl5 increased in aorta
that was exposed to LED, LA significantly accumulated in the medium (Figure 3.7G). EPA and DHA levels were similar between LED and LA groups, in both aorta culture and medium. It is possible that LED treated aorta tended to avoid LA as the energy source in Elovl5+/− aorta culture, to protect against of LA induced toxicity. Hence, EPA+DHA may benefit the system by selecting appropriate fatty acids as the prior energy source. However, further studies are needed to confirm and to better address this result.

In conclusion, Elovl5 deficiency increased mean arterial pressure, independent of the diet. The increased vascular contractility also possibly triggered local and systemic inflammatory responses. Therefore, Elovl5 might be the main target of Srebp1 shown in Chapter 2, resulting in increased adr expression in cardiomyocytes. In addition, fish oil supplement (DHA+EPA) accelerated LA removal by elevating the expression of Elovl5. Taking together, these data indicated that Elovl5 might be a novel therapeutic target to improve heart associated problems as a result of our current Western diet.
Chapter 4: GSH attenuated LA induced heart dysfunction by improving LA bioconversion

4.1 Background

Western pattern diet, or western diet, refers to the diet heavy with sugar and fat (Cordain, Eaton et al. 2005). Looking back to the Second Industrial Revolution, the economic in USA was largely improved, which promoted further agricultural modernization. This substantially altered dietary pattern, with much more processed food, refined carbohydrates and fibre-free fats, available in the market. Historical statistics also indicated that the Industrial Revolution Period marked the increase of body mass index (BMI), and concurrent obesity incidence (Caballero 2007). Researchers have been blaming saturated fat as the leading driver of obesity since 1978, resulting in limited option of saturated fats in the market (Keys 1980). As a result, fatty acid composition in Western Diet largely changed from saturated fat dominated diet to n-6 PUFA rich diet (Hamley 2017). However, the incidence of obesity and its’ complications such as cardiometabolic diseases kept rising (Kelly and Fuster 2010). This observation contradicted A. Keys’ theory. Interestingly, recent emerging evidence showed that ‘healthy n-6 PUFA’, instead of SFA, actually promoted the risk of cardiovascular diseases (Ramsden, Hibbeln et al. 2010; Hamley 2017) and obesity (Massiera, 2010).

As described in chapter 3, LA is the most available n-6 PUFA, which primarily drives the increase of n-6 PUFA in the diet. Meta-analysis studies provided compelling evidence that LA promoted the development of CVD (Hodgson, Wahlqvist et al. 1993; Schwertner and Mosser 1993). LA, instead of saturated fats, was also supported to be the most abundant
fatty acid in artherosclerosis plaque (DiNicolantonio and O’Keefe 2018). More importantly, CVD normally coexists with high oxidative stress and low grade inflammatory responses (Patterson, Wall et al. 2012; DiNicolantonio and O’Keefe 2018). Excess free radicals provoked LA peroxidation (Brooks, Harland et al. 1970), which entered the dead-end circle that magnified the oxidative stress even further (Turpeinen, Basu et al. 1998). Comparing to healthy individuals, patients with artherosclerosis had higher level of oxidized LA in LDL (Brooks, Harland et al. 1970; Jira, Spiteller et al. 1998), indicating a vicious circle between oxidative stress and LA peroxidation.

Heart prefers to use fatty acid over glucose as the energy source. β-oxidation is the last and the most crucial step to generate energy from fatty acid in mitochondria (Tajalizadekhoob, Sharifi et al.). Impaired mitochondria impeded β-oxidation process, predisposing interrupted energy metabolism (Wajner and Amaral 2016). This may exaggerate the development of existing heart diseases (Fillmore, Mori et al. 2014). Additionally, in heart pathological conditions, fatty acid transportation and utilization were inhibited, by representing restricted activity of β-oxidation (Lopaschuk, Ussher et al. 2010). Managing energy metabolism had been considered to be a possible therapy to improve heart function (van der Vusse, van Bilsen et al. 2000; Tuunanen, Ukkonen et al. 2008). However, the mechanism and its’ outcome still need to be further investigated.

As discussed earlier, a functional mitochondrion is needed to ensure fatty acid catabolism. In this chapter, EndoG+/− (endonuclease G) mouse, a mitochondrial mouse model, was selected to study the effect of mitochondrial health on modulating LA-derived heart toxicity. Studies relating to EndoG mostly focused on its’ caspase-independent apoptosis (Li,
Luo et al. 2001; David, Sasaki et al. 2005). The insight of EndoG on affecting heart function is still largely lacking. Apoptosis is usually known as a self-protective pathway to avoid further tissue damage (Jorgensen, Rayamajhi et al. 2017). Opposite to other somatic cells, heart muscle cells are not able to divide, which denies the protective role of apoptosis on supporting heart health. Indeed, high apoptosis activity increased the loss of myocytes, so that heart was unable to sustain contractility (Orogo and Gustafsson 2013). Numerous studies have shown that cardiomyocytes apoptosis increased the progression of heart diseases such as ischemic heart diseases and myocardial infarction (Wencker, Chandra et al. 2003; van Empel, Bertrand et al. 2005). Surprisingly, LA was characterized as an inducer of apoptosis, by blocking PGE₂ production (Choi 2014). Additional antioxidants such as α-tocopherol were able to restore LA derived cell death (Gavino, Miller et al. 1981). Particularly, LA induced the loss of mitochondrial SOD, mitochondrial DNA damage (Ghosh, Kewalramani et al. 2006), and GSH dysregulation (Ghosh, Sulistyoningrum et al. 2011). These potentially characterized a damaged mitochondrial integrity under excess LA (Ballinger, Patterson et al. 2002; Shokolenko, Venediktova et al. 2009), indicating a reduction of fatty acid oxidation (β-oxidation).

In chapter 2 and 3, blocking n-6 PUFA bioconversion decreased expression of antioxidant genes. On the contrary, fish oil promoted LA removal, and at the same time increased expression of mitochondrial antioxidant genes. Therefore, balancing oxidative stress might be an effective approach to reverse LA induced cardiac damage. **Hence, we hypothesize that supplementing with antioxidant, GSH in particular, accelerates LA metabolism, simultaneously improving LA-induced heart dysfunction.**
4.2 Methods and materials

4.2.1 Animal care

Six-month old partial knockout Elovl5+/− (female, n=7), EndoG+/− (mixed gender, n=6) (Appendix I), Gpx1+/− (mixed gender, n=4) (KOMP, University of California Davis, CA, USA) and wildtype (C57/Bl6) mice (The Jackson Laboratory, USA) feeding was performed as previously described in 3.2.1, with either 20% w/w olive oil (OO) or corn oil (CO) (Appendix F). Composition and nutritional information for the diets are given in Table 3.1 and Table 3.2 in Chapter 3. Mice were weighed twice a week and fasting glucose were measured every two weeks. Food and water were provided ad libitum. Blood glucose and heart physiological test were performed as describe in 3.2.2 and 3.2.3. Gpx1+/+ and Gpx1+/− mice genotyping and feeding were completed by other lab members.

4.2.2 in vitro experiment

4.2.2.1 GSH study

NIH/3T3 and differentiated H9c2 cells were cultured as described in 2.3.1. To investigate the effect of GSH on high LA treatment, cells were treated with 25 uM N-acetylcysteine (Guil-Guerrero, Tikhonov et al.) (VWR, Cat. No. 616-91-1) and 2 mM GSH ethyl ester (GSH-ee) (Cayman, CAS no. 92614-59-0) for 6 hours, followed by 18 hours incubation with 0.1 mM BSA or LA. Cell viability, mitochondrial potential, ROS and GSH assays were measured as described in 2.3.6.

4.2.2.2 EndoG study

NIH/3T3 cells were seeded in 48-well plates and transfected with EndoG Orf clone (Cat No.: MR204047, OriGene) or pCMV6 vector (Cat No.: PS100001, OriGene). The
procedure for Orf clone transfection was the same as 2.3.6, but with Attractene (Cat No./ID: 301005, QIAGEN), instead of DharmaFECT 1 transfection reagent. Cell viability, mitochondrial potential, ROS and GSH assays were completed as described in 2.3.6. Due to the EndoG’s property on regulating apoptosis, three caspase assays were performed as shown below. All these caspase assays were designed, tested and modified based on Sigma caspase-3 assay kit (product code: CASP-3-C; Sigma, MI, USA).

Caspase-3 Assay

Caspase-3 is the central caspase that directs both intrinsic and extrinsic apoptosis. This assay is based on the reaction between external caspase-3 substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-Methylcoumarin (Ac-DEVD-AMC) and enzyme caspase-3 in cells. Positive control (1ug/ml staurosporine) was performed before proceeding to this assay. Since staurosporine was dissolved in DMSO (Dimethylsulfoxide), DMSO medium was used as the negative control. When cells were ready, control wells were prepared by replacing growth medium with either 200 ul staurosporine medium (duplicate) or DMSO medium (duplicate). After 4 hours incubation, the mediums was removed and cells were placed on ice. 75 ul 1×LDH lysis buffer was added into each well and the 96-well plate was incubated on ice for 20-30 minutes. A 96-well fluorimeter plate was set up as Table 4.1. Set fluorimeter with 360 nm excitation and 460 nm emission. Plates were read in a kinetic mode every10 minutes for 40–60 minutes at room temperature. The reading reached the maximum signal at 60 minutes. Therefore the data point at 60 minutes was used to do further data analysis.
Table 4. 1 Caspase 3 reaction scheme for 96-well plate reaction

<table>
<thead>
<tr>
<th>1x assay buffer</th>
<th>Ac-DEVD-AMC</th>
<th>Cell lysate</th>
<th>Caspase 3 inhibitor (200 uM)</th>
<th>Reaction Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent blank</td>
<td>5 ul</td>
<td>-</td>
<td>-</td>
<td>100 ul</td>
</tr>
<tr>
<td>positive control</td>
<td>-</td>
<td>5 ul</td>
<td>-</td>
<td>100 ul</td>
</tr>
<tr>
<td>positive control+</td>
<td>-</td>
<td>5 ul</td>
<td>2 ul</td>
<td>100 ul</td>
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<tr>
<td>Caspase3 inhibitor</td>
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<td>-</td>
<td>5 ul</td>
<td>100 ul</td>
</tr>
<tr>
<td>Samples</td>
<td>-</td>
<td>-</td>
<td>5 ul</td>
<td>100 ul</td>
</tr>
<tr>
<td>Sample+inhibitor</td>
<td>-</td>
<td>-</td>
<td>2 ul</td>
<td>100 ul</td>
</tr>
</tbody>
</table>

Caspase 8 and Caspase 9

Caspase 8 assay was performed with the same protocol as caspase 3 assay. Plate was set up as shown in Table 4.2, and read under 400 nm excitation and 505 nm emission every 20 minutes for 2 hours. The signal reached plateau at 100 min. IETD-AFC was used as caspase-8 substrate (see Table 4.2 for details).

Table 4. 2 Caspase 8 reaction scheme for 96-well plate reaction

<table>
<thead>
<tr>
<th>1x assay buffer</th>
<th>IETD-AFC (1mM)</th>
<th>Cell lysate</th>
<th>Reaction Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent blank</td>
<td>5 ul</td>
<td>-</td>
<td>100 ul</td>
</tr>
<tr>
<td>positive control</td>
<td>-</td>
<td>10 ul</td>
<td>100 ul</td>
</tr>
<tr>
<td>Samples</td>
<td>-</td>
<td>5 ul</td>
<td>100 ul</td>
</tr>
</tbody>
</table>

IETD-AFC: N-Acetyl-Ile-Glu-Thr-Asp-7-amino-4-Trifluoromethylcoumarin

Caspase 9 assay was performed with the same protocol as caspase 3 assay. Plate was set up as shown in Table 4.3. The signal reached plateau at 100 minutes. LEHD-AFC was used here as caspase 9 substrate (see Table 4.3 for details).

Table 4. 3 Caspase 9 reaction scheme for 96-well plate reaction

<table>
<thead>
<tr>
<th>1x assay buffer</th>
<th>LEHD-AFC (1mM)</th>
<th>Cell lysate</th>
<th>Reaction Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent blank</td>
<td>5 ul</td>
<td>-</td>
<td>100 ul</td>
</tr>
<tr>
<td>positive control</td>
<td>-</td>
<td>10 ul</td>
<td>100 ul</td>
</tr>
<tr>
<td>Samples</td>
<td>-</td>
<td>5 ul</td>
<td>100 ul</td>
</tr>
</tbody>
</table>

LEHD-AFC: N-Acetyl-Leu-Glu-His-Asp-7-amino-4-Trifluoromethylcoumarin
4.2.4 Quantification of mRNA by quantitative PCR

Quantitative PCR was performed as described in section 2.3.8. Primer sequences were listed below in Table 4.4.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward (5'-3')</strong></td>
<td><strong>Reverse (5'-3')</strong></td>
</tr>
<tr>
<td>18s (H9c2)</td>
<td>TTGATTAAGTCCCTGCCCCTTTGT</td>
</tr>
<tr>
<td>18s (NIH/3T3)</td>
<td>CGGCTACCACATCCAAGGAA</td>
</tr>
<tr>
<td>FADS1</td>
<td>ACGCGCTACTTTTACTTGGGA</td>
</tr>
<tr>
<td>FADS2</td>
<td>CACTATGCCACGTCACAAC</td>
</tr>
<tr>
<td>Elovl5</td>
<td>CGAGGCATCCTGCAG TTG TA</td>
</tr>
<tr>
<td>β-actin</td>
<td>CTGCCTGACGGCCAGG</td>
</tr>
<tr>
<td>ND5</td>
<td>ACAGCTATTTTGTGCCCTACCCCAA</td>
</tr>
<tr>
<td>aldh2</td>
<td>GCTGGGCTGACAAGTACCAT</td>
</tr>
<tr>
<td>Gpx1</td>
<td>GTTCGGACACCAGGAGAA</td>
</tr>
<tr>
<td>Gclc</td>
<td>ACATCTACCACGCAGTCAAG</td>
</tr>
<tr>
<td>Gelm</td>
<td>CCGATTTAGTCAGGGAGTTTC</td>
</tr>
<tr>
<td>Gsr</td>
<td>ACGTGGATTACGGCTTTTC</td>
</tr>
<tr>
<td>Gss</td>
<td>CTGCAGTAAATGCGGTGGTG</td>
</tr>
<tr>
<td>SOD1</td>
<td>GGCTTCTCGTCTTTGCTCTC</td>
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<td>SOD2</td>
<td>GGCCAAGGGAGATGTTAC</td>
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<td>SOD3</td>
<td>AACCTCACGAGGAAAAGAG</td>
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<tr>
<td>catalase</td>
<td>AGAAGCCTAAGAAGCGCAATTCC</td>
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</tbody>
</table>
4.2.5 Gas chromatography

Fatty acids were extracted from cells and hearts. The extraction and detection protocols were described in section 2.3.9.

4.2.6 Echocardiography

Heart function was measured by using ultrasound M-M mode. The detailed protocol was described in section 3.2.3.

4.2.7 Statistical analysis

Statistical Analysis was performed as described in 2.2.12. ‘Sectional statistical analysis’ is available before each figure.
4.3 Results

4.3.1 The expression of cardiac antioxidant genes decreased in Elovl5+/− mice following high n-6 PUFA feeding

In order to investigate the effects of LA accumulation on antioxidant level, CO fed Elovl5+/+ and Elovl5+/− mice hearts were used here to examine the expressions of major antioxidant correlated genes. Catalase is a dismutase enzyme that reduces H₂O₂ to water. It mostly resides in peroxisome but it can travel to mitochondria in the context of excess free radicals (Ighodaro and Akinloye 2018). Knocking out catalase caused mitochondrial damage and induced obesity, whereas over-expressed catalase resolved these metabolic problems (Amos, Robinson et al. 2017). In my study, Elovl5+/− mice decreased catalase expression in the heart (Figure 4.1A). Aldehyde dehydrogenase 2 (aldh2) is a mitochondrial specific antioxidant which is only involved in oxidative pathway of alcohol metabolism (Ohta, Ohsawa et al. 2004). Many studies indicated that aldh2 attenuated mitochondrial oxidative stress (Ohta, Ohsawa et al. 2004), and prevented cardiac diseases (Zhang, Mi et al. 2014; Guo, Yu et al. 2015). In this study, Elovl5+/− mice showed a decreased aldh2 expression in the heart (Figure 4.1B). As mentioned earlier, SOD catalyzes O₂− to either O₂ or H₂O₂ (Ighodaro and Akinloye 2018). Elovl5 deficiency also reduced SOD2 (mitochondrial SOD) expression in Elovl5+/− mice heart (Figure 4.1D).

Other than catalase and SOD, glutathione (GSH) is another major and most abundant antioxidant in mammals. Although GSH is the most well-known molecule of first-line defence antioxidants, glutathione peroxidase 1 (Gpx1) is the enzyme that catalyzes the GSH reaction in the heart (Li, Yan et al. 2000). Glutamate—cysteine ligase catalytic subunit (Gclc)
and glutamate—cysteine modifier subunit (Gclm) are two required enzymes to biosynthesize GSH (Krejsa, Franklin et al. 2010). Interestingly, the expression of these three genes (Gclc, Gclm and Gpx1) all decreased in Elovl5+/− mice heart (Figure 4.1H-J). This indicated the impeded GSH biosynthesis and its utilization in Elovl5+/− mice hearts.

Section statistical analysis: Figure 4.1 were analyzed by applying t-test. *p<0.05 vs Elovl5+/− CO. n=5.
Figure 4.1: Elovl5<sup>+/−</sup> decreased the expression of antioxidants associated genes. Catalase (A), Aldh2 (B), SOD1 (C), SOD2 (D), SOD3 (E), Gsr (F), Gss (G), Gpx1 (H), Gclc (I) and Gclm (J) expressions in Elovl5<sup>+/−</sup> and Elovl5<sup>+/+</sup> mice hearts. All data were normalized to Elovl5<sup>+/+</sup> CO. Abbreviations: Aldh2: aldehyde dehydrogenase; SOD: superoxide dismutase; Gsr: glutathione-disulfide reductase; Gss: glutathione synthetase; Gpx1: glutathione peroxidase 1; Gclc: glutamate cysteine ligase catalytic; Gclm: glutamate-cysteine ligase.
4.3.2 Additional GSH rescued high LA derived oxidative stress *in vitro*

Our lab previously showed that high n-6 PUFA (corn oil) feeding decreased GSH to GSSG ratio, and at the same time reducing the expression of Gpx1 and Gclc (data not published). In 4.3.1, partially knocking out Elovl5 dampened the expression of GSH associated genes, in the context of high n-6 PUFA diet. Therefore, recovering GSH might be an effective approach of reversing LA induced heart toxicity. In order to increase the GSH biosynthesis, 25uM NAC was added to NIH/3T3 and differentiated H9c2 cells. NAC is the acetylated cysteine that has been demonstrated to increase levels of GSH *in vivo*. Although there were no differences of GSH level detected between control and NAC treatment groups (Figure 4.2A), additional NAC increased mitochondrial health in cells exposed to LA (Figure 4.2B).

*Section statistical analysis:* Figure 4.2 were analyzed by applying two-way ANOVA with post-hoc Tukey test. *p*<0.05 vs Control LA; *p*<0.05 vs Control BSA. n=4-6.

**Figure 4.2:** NAC rescued mitochondrial function following LA incubation in fibroblasts. Fibroblasts were treated with or without 25 uM NAC. A) GSH level. All data were normalized to control BSA. B) mitochondrial potential. Abbreviations: BSA: bovine serum albumin; OA: oleic acid; LA: linoleic acid; NAC: n-acetyl cysteine; AU: arbitraty units. GSH: glutathione.
Unlike NAC, GSH-ee provides additional and usable GSH to cells directly. Additional GSH increased lysosomal integrity in OA treated H9c2 cells (Figure 4.3A), and improved mitochondrial potential following both OA and LA incubation (Figure 4.3B). It was shown earlier that blocking Elovl5, PUFA elongation gene, decreased the expression of GSH associated genes (Figure 4.1). So there might be a mutual regulation between LA bioconversion and GSH metabolism. n-6 PUFA profiles of both cardiomyocytes (Figure 4.3C) and medium (Figure 4.3D) showed higher production of GLA with the treatment of GSH-ee. This indicated an increased LA bioconversion rate in cells supplemented with additional usable GSH.

*Section statistical analysis:* Figure 4.3A and B were analyzed by applying two-way ANOVA with post-hoc Tukey test. *p<0.05 vs Control LA; #p<0.05 vs Control OA. n=4-6.

Figure 4.3C and D were analyzed by applying multiple t-test (LA, GLA, DGLA and ARA): Control vs GSH-ee. *p<0.05 vs Control. n=4.
Figure 4.3: GSH-ee promotes n-6 PUFA bioconversion by improving mitochondrial function. Lysosomal integrity (A) and mitochondrial potential (B) in cells with or without 2 mM GSH-ee pre-treatment. Data in (A) was relative to control OA. n-6 PUFA distribution in cells (C) and medium (D) was measured by GC. Abbreviations: GSH-ee: GSH ethyl ester; OA: oleic acid; LA: linoleic acid; GLA: γ-linolenic acid; DGLA: dihomo-γ-linolenic acid; ARA: arachidonic acid.
4.3.3 n-6 PUFA bioconversion decreased in mice with genetically inhibited GSH metabolism

In order to verify the benefit of active GSH metabolism on improving n-6 PUFA bioconversion, Gpx1 \(^{+/+}\) and Gpx1 \(^{+/-}\) mice were used. Gpx1 deficiency decreased the expression of Elovl5 (Figure 4.4C) and FADS1 (Figure 4.4A), but not FADS2 (Figure 4.4B). This indicated that the deletion of Gpx1 impeded the bioconversion from GLA to ARA. n-6 PUFA profile showed an accumulation of LA and decreased GLA production in Gpx1 \(^{+/-}\) mice heart (Figure 4.4D).

Section statistical analysis: Figure 4.4 was analyzed by applying t-test. Figure 4.4D had multiple t-test included: Gpx1 \(^{+/+}\) CO vs Gpx1 \(^{+/-}\) CO, n=3-5.
Figure 4.4: Gpx1<sup>+/−</sup> decreased PUFA bioconversion. A-C). expression of PUFA bioconversion genes (FADS1, FADS2, Elovl5) in Gpx1<sup>+/+</sup> and Gpx1<sup>+/−</sup> mice hearts. D) n-6 PUFA profile in Gpx1<sup>+/+</sup> and Gpx1<sup>+/−</sup> mice hearts. Abbreviations: CO: corn oil; Gpx1: glutathione peroxidise 1; LA: linoleic acid; GLA: γ-linolenic acid; DGLA: dihomo-γ-linolenic acid; ARA: arachidonic acid; FADS1: fatty acid desaturase 1; FADS2: fatty acid desaturase 2; Elovl5: elongase 5.
4.3.4 PUFA bioconversion efficiency was highly improved in mitochondrial genetically modified mice model

As we saw in 4.3.1, all mitochondrial antioxidants were decreased in Elovl5+/− mouse heart, including Aldh2, SOD2 and Gpx1 etc (Figure 4.1). In addition, GSH supplement increased PUFA bioconversion and improved mitochondrial potential (Figure 4.3). Hence, mitochondrial health seems to be crucial to secure PUFA bioconversion. To investigate the connection in between, EndoG+/− mouse model was used here. EndoG is located in the inner membrane of the mitochondrion. It is encoded by mitochondrial DNA (mtDNA) which can induce caspase-independent apoptosis once it’s cleaved in cells under oxidative stress (Li, Luo et al. 2001). EndoG+/+ and EndoG+/− mice were fed with high n-6 PUFA diet for six weeks. Fasting glucose (Figure 4.5A) and heart function (Figure 4.5B) did not change following high n-6 PUFA feeding. Although statistical differences were detected at week 2 and 4, heart function at the starting time point (week 0) and end time point (week 6) remained the same. No difference regarding of the heart function was found between EndoG+/+ and EndoG+/− genotype.

Despite similar heart function, PUFA bioconversion had a spike in EndoG+/− mice, with an increased FADS1 (Figure 4.6A), FADS2 (Figure 4.6B) and Elovl5 (Figure 4.6D) expression in heart. Although GLA and DGLA levels stayed the same in EndoG+/+ and EndoG+/− mice, a lower LA accumulation and an increased C20:2n6 were detected in EndoG+/+ heart (Figure 4.6E). C20:2n6 is the product of an alternative bioconversion pathway from LA through Elovl5. Other than n-6 PUFA bioconversion, EndoG+/− mice heart had a significantly higher DHA production (Figure 4.6F). As mentioned in 4.3.2, our lab
previously found that GSH level was significantly decreased in C57/Bl6 mice receiving CO diet, compared to high olive oil diet. Interestingly, lacking EndoG did not alter the expression of GSH related genes, in the context of high CO diet (Figure 4.6G-1). This indicated healthier mitochondria in EndoG +/- mice heart.

Section statistical analysis: Figure 4.5A and 4.6B were analyzed by applying multiple two-way ANOVA with post-hoc Tukey test (row). *p<0.05 vs 0 week. #p<0.05 vs 6 weeks. n=10-12. P and F values were not presented.

Figure 4.6 was analyzed by applying t-test. *p<0.05 vs EndoG +/- CO. n=4-6.

<table>
<thead>
<tr>
<th></th>
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<tr>
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<td></td>
<td>0 (mM/ml)</td>
<td>2 (mM/ml)</td>
<td>4 (mM/ml)</td>
</tr>
<tr>
<td>EndoG+/- CO</td>
<td>10.31±2.97</td>
<td>8.65±1.44</td>
<td>8.49±0.96</td>
<td>9.72±1.17</td>
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<tr>
<td>EndoG +/- CO</td>
<td>10.25±2.59</td>
<td>9.15±2.19</td>
<td>8.58±1.52</td>
<td>9.68±1.88</td>
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</table>

<table>
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</thead>
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<td>Measurements</td>
<td>Time (weeks)</td>
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<td>4</td>
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<tr>
<td>Ejection Fraction (%)</td>
<td>EndoG+/- CO</td>
<td>62.05±7.56</td>
<td>59.48±10.26</td>
<td>62.97±5.31</td>
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<tr>
<td></td>
<td>EndoG +/- CO</td>
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<td>60.24±6.21</td>
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<tr>
<td>Fractional Shortening (%)</td>
<td>EndoG+/- CO</td>
<td>27.88±4.89</td>
<td>26.47±6.29</td>
<td>28.35±3.63</td>
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<tr>
<td></td>
<td>EndoG +/- CO</td>
<td>22.91±5.27</td>
<td>26.64±3.84</td>
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<tr>
<td>Left-ventricular Mass (mg)</td>
<td>EndoG+/- CO</td>
<td>5.66±2.27</td>
<td>8.62±2.10*</td>
<td>7.90±1.51*</td>
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<tr>
<td></td>
<td>EndoG +/- CO</td>
<td>3.47±0.77</td>
<td>8.15±1.48*</td>
<td>5.98±1.51*</td>
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<tr>
<td>Stroke Volume (ml)</td>
<td>EndoG+/- CO</td>
<td>3.01±1.58</td>
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<td>3.18±0.58</td>
</tr>
<tr>
<td></td>
<td>EndoG +/- CO</td>
<td>2.17±0.36</td>
<td>3.25±0.67</td>
<td>2.72±0.46</td>
</tr>
</tbody>
</table>

Figure 4.5: EndoG +/- did not alter blood glucose and cardiac function. A) Bi-weekly fasting glucose in both EndoG+/- and EndoG +/- mice, following high corn oil diet feeding. B) Bi-weekly EF, FS, LV mass, and stroke volume of EndoG+/- and EndoG +/- mice measured by M-M mode of echocardiography. Abbreviations: EF: ejection fraction; FS: fractional shortening; LV mass; left ventricular mass.
Figure 4.6: **EndoG**+/− increased PUFA bioconversion in heart. A-D) PUFA bioconversion genes’ expression (FADS1, FADS2, Elovl2 and Elovl5) in EndoG+/− and EndoG+/+ mice heart. Data were normalized to EndoG+/+. E) complete n-6 PUFA profile in EndoG+/+ and EndoG+/− heart measured by GC. F) DHA (%) level in EndoG+/+ and EndoG+/− mice heart. G-I). GSH associated genes’ expression (Gpx1, Gclc, Gss). Abbreviations: CO: corn oil; FADS1: fatty acid desaturase 5; FADS2: fatty acid desaturase 6; Elovl2: elongase 2; Elovl5: elongase 5. LA: linoleic acid; GLA: γ-linolenic acid; DGLA: dihomo-γ-linolenic acid; ARA: arachidonic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; Gclc: glutamate—cysteine ligase; Gss: glutathione synthetase; Gpx1: glutathione peroxidase 1.
4.3.5. EndoG induced mitochondrial damage and apoptosis

EndoG+- mice were shown to have healthier mitochondria due to the high DHA accumulation (Figure 4.6F). To study the role of EndoG on mitochondrial function, EndoG overexpressor (EndoG OE) was used in NIH/3T3. Cells were transfected with EndoG Orf clone, followed by OA or LA incubation. EndoG OE decreased mitochondrial reductase activity (Figure 4.7A), at the same time increased reactive oxygen species (Figure 4.7B). Decreased mtDNA copy number in EndoG OE cells also indicated the reduced healthy mitochondrial number (Figure 4.7C). Furthermore, the activity of all three caspases (caspase 3 (Figure 4.7D), caspase 8 (Figure 4.7E) and caspase 9 (Figure 4.7F) was increased in EndoG OE group with LA treatment.

Section statistical analysis: Figure 4.7 was analyzed by applying two-way ANOVA, with post-hoc Tukey test. *p<0.05 vs Control LA; #p<0.05 vs Control OA; +p<0.05 vs EndoG OE BSA. n=4-6.
Figure 4. EndoG induced mitochondrial damage and extrinsic apoptosis.
Mitochondrial reductase activity (A) and ROS (B) levels in NIH/3T3 cells were transfected with EndoG overexpressor or empty vector, following by 0.1 mM OA or LA incubation. A) was relative to Control OA; B) was normalized to Control OA. (C) Mitochondrial copy number was measured by qPCR with genomic DNA template. Caspase 3 (D), caspase 8 (E), and caspase 9 (F) activity were measured based on their own substrate. Data were relative to Control OA. Abbreviations: OA: oleic acid; LA: linoleic acid; EndoG OE: EndoG overexpression.
4.4 Discussion

LA accumulation has been demonstrated to induce oxidative stress, with an imbalanced ratio of free radicals and antioxidants in mitochondria. Our lab previously showed that CO fed mice decreased GSH level, as well as the expression of GSH related genes (data not published). Mice accumulating LA (Elovl5+/−) also reduced the expression of GSH associated genes, which were involved in both GSH biosynthesis and GSH redox cycle (Figure. 4.1). This indicated a complete blockage of GSH production and utilization. Hence, accumulating LA seemed to impair GSH metabolism in the heart. GSH depletion (Ghosh, Sulistyoningrum et al. 2011; Kim, Kim et al. 2013) and excess LA (Beam, Botta et al. 2012; Beam, Botta et al. 2015) has been positively linked with the progression of heart dysfunction. However, the interaction of LA accumulation, GSH depletion and heart function remains elusive. To verify this hypothesis, GSH metabolism was modified in vitro and in vivo. GSH: GSSG ratio has been widely used as the indicator of thiol redox state, which can represent mitochondrial health (Mari, Morales et al. 2009). Additional GSH helped to clear out upstream n-6 PUFA in vitro, therefore improving mitochondrial health in cardiomyocytes and fibroblasts (Figure 4.2, 4.3). Conversely, deletion of Gpx1 blocked GSH oxidation to GSSG, indicating decreased reductive potential. Gpx1+/− mice accumulated LA in the heart, accompanied with decreased FADS1 and Elovl5 gene expressions (Figure 4.5). Therefore, Elovl5 and GSH were mutually regulated.

Previous study has demonstrated that high LA diet induced mitochondrial dysfunction and deprivation of mitochondrial GSH (Ghosh, Kewalramani et al. 2006). Hence, maintaining mitochondrial health can be taken as a potential strategy to attenuate high n-6
PUFA diet caused antioxidant depletion. However, no determinant clinical strategy has been confirmed to secure mitochondrial health, so far. Here, we used a mitochondrial mouse model (EndoG) (Li, Luo et al. 2001), to investigate the role of mitochondrial health on regulating high LA diet induced GSH reduction. Compared to wildtype mice, EndoG deficient mice displayed similar expressions of GSH associated genes in the heart, independent of the diet (Figure 4.6). This observation was opposed to the result observed in wildtype mice (C57/BL6, data not published). In addition, lacking EndoG increased DHA accumulation in the heart (Figure 4.6), indicating more active β-oxidation process and healthier mitochondria. On the contrary, increasing EndoG level elevated mitochondrial oxidative stress, and damaged mitochondrial integrity (Figure 4.7).

In conclusion, GSH was proved to be the central factor that benefitted mitochondrial function and improved LA bioconversion rate. Improving mitochondrial health accelerated LA metabolism, resulting in decreased LA accumulation. This lowered the negative effects of LA accumulation on provoking mitochondrial oxidative stress, and further heart damage. Therefore, managing GSH metabolism was able to terminate the endless loop of LA-mitochondrial damage-heart dysfunction.
Figure 4.8: Schematic pathway of the mutual regulation between GSH metabolism and n-6 PUFA bioconversion.
Chapter 5: Discussion and conclusions

5.1 Discussion

Consumption of LA-rich vegetable oils like sunflower and corn oil, as well as farmed animals fed LA-rich oilseeds has skyrocketed in North America and other westernized societies over the last several decades. Several recent studies and meta-analyses suggest that an increased n-6 PUFA can induce adverse cardiac events in humans, but with unidentified mechanisms hidden behind. In previous study, we demonstrated that additional fish oil attenuated high n-6 PUFA induced vascular α1-AR expression in C57/Bl6 mouse model (data are not published). However, we are unsure about the mechanisms involved. As described earlier, LA is the primary source of high n-6 PUFA diet. Therefore, different approaches were used to evaluate the effect of LA accumulation on adr expression in vitro (Chapter 2). Globally blocking LA bioconversion (Srebp-1 siRNA) increased the expression of adr-α, which supported the previous observation in vivo.

Besides, of the same previous study, we also observed that additional fish oil (EPA+DHA) attenuated high LA diet induced adr-α expression (data are not published). Here, using ex vivo aorta culture (Chapter 2 and 3), EPA and DHA were shown to accelerate PUFA bioconversion by elevating the expression of bioconversion genes (FADS1 and Elovl5). This consistency between in vivo and ex vivo study led us to see if increased PUFA bioconversion was the reason of EPA and DHA that attenuated LA-induced α1-Adr expression. Therefore, we hypothesized that Elovl5 might be the internal target that can help to remove excess LA, as a result of attenuated vascular contractility. Using Elovl5 deficient mice (Elovl5+/−) and wildtype mice (Elovl5+/+), we are able to separate out the upstream n-6
PUFA (LA and GLA), and other downstream PUFA (DGLA onwards). This study showed that LA and GLA elevated α1-AR expression and local inflammatory response in the aorta (Chapter 3). Up to here, Elovl5 can be considered to be an effective target to overcome n-6 PUFA rich Western diet induced hypertension.

Other than PUFA bioconversion genes, we also found that the expression of Gpx1 was increased under additional EPA and DHA treatment in Elovl5+/− mice (Chapter 3). Gpx1+/− mice were used to evaluate the level of Elovl5. Gpx1 is an enzyme that catalyzes H₂O₂ by converting glutathione (GSH) to glutathione disulfide (GSSG). This is a crucial process that prevents PUFA from lipid peroxidation. Hence, Elovl5, GSH metabolism, and EPA/DHA are mutually regulated. To specially investigate the interaction between Elovl5 and GSH metabolism (Gpx involved antioxidant), Following the high n-6 PUFA diet, Gpx1 decreased Elovl5 expression and accumulated LA, whereas additional GSH increased LA bioconversion (Chapter 4). Similarly, Elovl5+/− mice showed to decrease all antioxidant associated genes (Chapter 4), which supported the finding of Gpx1+/− directed Elovl5 reduction. Therefore, in the context of n-6 PUFA, or LA overload, maintaining the GSH availability and its metabolism determines the LA bioconversion efficiency, which may further indicate the elevated vascular contractility.

Due to the nature of cellular respiration, mitochondrion is the major organelle that produces endogenous ROS. Heart muscle cells have the highest amount of mitochondria across the whole body system because of the high ATP demand for maintaining cardiac muscle contraction. As described in Chapter 1, LA is showed to induce mitochondrial dysfunction. We have seen that additional Gpx1 and Elovl5 are mutually regulated (Chapter
3 and 4). As we know, Elovl5 elongates fatty acids by introducing one acetyl-CoA, which is generated in mitochondria. Therefore, mitochondrial health is pre-condition to ensure the PUFA bioconversion, which may further regulate the level of antioxidants. To start from mitochondria, we used in vivo (EndoG+/−) and in vitro (EndoG overexpression) model, to investigate the role of mitochondrial health on regulating GSH metabolism, as well as LA bioconversion. EndoG is the mtDNA coded protein which directs the DNA degradation. Removing EndoG theoretically is protective, whereas additional EndoG increases the stress of mitochondria. Our lab previously showed that high n-6 PUFA feeding decreased GSH level in hearts of C57/BL6 mice (data are not published), compared to iso-caloric olive oil diet. However, in chapter 4, EndoG deficient mice had the same expressions of GSH associated genes. This indicated that removing EndoG keeps the antioxidants at a higher level, which increases the potential of mitochondria to scavenge excess free radicals. In addition, EndoG+/− mice also showed to increase Elovl5, which increased LA removal. Similarly, over-expressing EndoG in vitro showed the consistent results in an opposite way, with increased mitochondrial damage and oxidative stress. In conclusion, maintaining mitochondrial health can be the effective target to ensure the metabolism of GSH, as well as accelerate the LA bioconversion. These two pathways are equally important to overcome the high LA Western diet induced vascular contractility.

5.2 Conclusions and significance

Numerous studies have presented evidence opposing high n-6 PUFA in the diet. Unlike n-6 PUFA, the universal health strategy, from researchers to health authorities, is to increase n-3 PUFA in the diet, preferably through the intake of fish. The whole idea is to
narrow down the imbalanced n-6 to n-3 PUFA ratio, thereby boosting the beneficial effects of n-3 PUFA and limiting the adverse effect of n-6 PUFA on heart. However, PUFA is a group of highly unsaturated fatty acids which is vulnerable to free radicals. It has been demonstrated that oxidized metabolites worsen the cardiac problems even further (Spiteller 1998; Jenkinson, Franklin et al. 1999). As previously described in chapter 1, it is difficult to avoid source of n-6 PUFA in our current diet. Therefore, accelerating n-6 PUFA removal might be a better strategy to diminish its negative outcome. It is known that dietary LA does not increase ARA production in red blood cells and blood serum (Rett and Whelan 2011). Besides, in diseases states, LA to ARA ratio is higher in blood serum, indicating a relatively higher LA accumulation (Seigneur, Freyburger et al. 1994; Simon, Hodgkins et al. 1995; Novgorodtseva, Karaman et al. 2011; Yamaguchi, Kinoshita et al. 2018).

In this study, we demonstrated that accumulating LA elevated aortic blood pressure (Figure 3.3), and increased the gene expression of adrenergic receptors (Figure 2.6). This was also accompanied by diminished expression of antioxidant related genes. Particularly, the entire GSH to GSSG metabolism was inhibited in Elovl5+/− mouse heart (Figure 4.1). Interestingly, supplementation of DHA and EPA also recovered the expression of antioxidant genes and LA bioconversion genes in ex vivo aorta culture exposed to LA (Figure 2.7, 3.7). Among all such genes, Elovl5 had the highest increase. It is well known that n-3 PUFA reduces mitochondrial oxidative stress, therefore protecting against heart diseases. Here, our data indicates a novel mechanism of cardio-protection via accelerated removal of LA in the heart.
In most chronic heart diseases, heart displays impaired fatty acid β-oxidation due to the mitochondrial oxidative damage. We hypothesized that additional GSH can attenuate oxidative damage and improve fatty acid β-oxidation. Both in vivo and in vitro approaches consistently showed that improved GSH metabolism increased LA bioconversion, in the context of high LA (Figure 4.3, 4.4). A mice model lacking a mitochondrial endonuclease (EndoG+/−) fed with CO, which prevented further mitochondrial damage, explicated a beneficial role of accelerating LA removal, without affecting GSH metabolism. This may indicate that the primary role of GSH, following high LA feeding, is to attenuate mitochondrial damage. In support of this idea, previous research in our lab demonstrated a loss of GSH in wildtype mice receiving high CO diet (data not published). In summary, supplementing GSH or protecting the mitochondria might be a novel and effective therapeutic target to increase LA removal through Elovl5, therefore attenuating vascular contractility (Figure 5.1).

As discussed earlier, PUFA is vulnerable to free radicals due to its double bonds. Patients with metabolic diseases such as CVD, diabetes and obesity have high levels of oxidative damage. Therefore, taking fish oil or fish daily may cause adverse effect on their existing diseases due to higher oxidizability of multiple double bonds present in this class of fatty acids (Albert, Derraik et al. 2015). Indeed, in recent years all trials with fish oil have failed in clinical studies on prevention of heart diseases (Kromhout, Yasuda et al. 2011). On the other hand, excess dietary LA increases its accumulation in adipose tissue (Guyenet and Carlson 2015), which further promotes the development of obesity (Kiefer, Clement et al. 2004). More importantly, LA is one of the most prevalent fatty acids found in adipose tissue of obese patients which can be metabolized into its harmful oxidative metabolites (Alvheim
Similarly, patients with heart diseases showed to have a decreased ratio of LA to ARA, which indicated a decelerated n-6 PUFA bioconversion (Wang, Folsom et al. 2003). Therefore, increasing n-3 PUFA by supplementing with fish oil may not be a solution to remove this excessive LA accumulation. In this study, we claim that strategies that enhance LA bioconversion, thus facilitating its removal or GSH augmentation strategies can be the therapeutic targets to reduce the detrimental cardiovascular and metabolic effects of n-6 PUFA, particularly LA.

Figure 5.1: The schematic mechanism of GSH on regulating LA bioconversion. α1-Adr: adrenergic receptor α1; FADS2: fatty acid desaturase 2; Elolv5: elongase 5; EndoG: endonuclease G; GSH: glutathione.

5.2 Limitations of the work

The work presented in this thesis was to provide a potential strategy to improve high n-6 PUFA diet (LA in particular) caused vascular contractility. We concluded that GSH was the crucial factor to attenuate high n-6 PUFA induced cardiac toxicity, by accelerating LA removal. However, mitochondrial oxidative damage or GSH level was not measured in any model. In addition, parameters of blood pressure and heart physiological function were not
fully measured in mitochondrial protective mice model (EndoG$^{+/}$). Similarly, heart physiological output result was lacking in Gpx1$^{+/}$ mice. Finally, lack of clinical data is the biggest shortcoming of this study, as humans and rodent animals have distinct metabolic mechanisms.
References


Botta, A. (2017). Glutathione Dysregulation, Cardiac Oxidative Stress, and Inflammation during n-6 Polyunsaturated Fatty Acid Overload, University of British Columbia.


Canada, H. (2012). "Do Canadian adults meet their nutrient requirements through food intake alone?".


foundation, C. h. a. s. (N/A). "The DASH Diet to lower high blood pressure." from https://www.heartandstroke.ca/get-healthy/healthy-eating/dash-diet.


Kurotani, K., M. Sato, et al. (2012). "High levels of stearic acid, palmitoleic acid, and dihomo-γ-linolenic acid and low levels of linoleic acid in serum cholesterol ester are associated with high insulin resistance." Nutrition research 32(9): 669-675. e663.


randomized, placebo-controlled study." European archives of psychiatry and clinical neuroscience 261(8): 539-549.


Appendices

Appendix A: Fatty acid analysis of H9c2 treated with or without FADS2 Inhibitor (sc-29196).

Full fatty acid profile in differentiated H9c2 cells treated with or without FADS2 inhibitor, following LA incubation. Data were analyzed using t-test, *p<0.05 vs Control LA. n=3-4. Abbreviations: LA: linoleic acid; FADS2: fatty acid desaturase 2.

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<td>C20:4n6</td>
<td>0.20 ± 0.01</td>
<td>0.03 ± 0.01</td>
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Appendix B: Fatty acid analysis of H9c2 transfected with or without FADS2 siRNA.

Full fatty acid profile of differentiated H9c2 cells transfected with or without FADS2-siRNA, following LA incubation. Data were analyzed using t-test, *p<0.05 vs Control LA. n=3-4.

Abbreviations: LA: linoleic acid; FADS2: fatty acid desaturase 2.

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Appendix C: Fatty acid analysis of H9c2 transfected with or without Srebp1c siRNA.

Full fatty acid profile of differentiated H9c2 cells transfected with or without Srebp1c-siRNA, following LA incubation. Data were analyzed using t test. *p<0.05 vs Control. n=3-4.

Abbreviations: LA: linoleic acid; Srebp1c: sterol regulatory element-binding protein 1c.

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Appendix D: FADS2 gene expression in NIH/3T3 (left) and H9c2 (right) transfected with or without FADS2 siRNA.

Data were normalized to Control cells, and were analyzed by applying t-test. *p<0.05. n=4. FADS2 gene expression was decreased by 20% (left) and 23% (right).

Appendix E: Srebp1c gene expression in cells transfected with or without Srebp1c siRNA.

Data were normalized to Control cells, and were analyzed by applying t-test. *p<0.05 vs Control. n=3-4. Srebp1c gene expression was decreased by 23%.
Appendix F: Fatty acid profile in high fat diets.
All diets contained extra 20% w/w fat. n=3. Data were analyzed using one-way ANOVA. *p<0.05 vs OO; **p<0.05 vs CO.

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</table>
Appendix G: Fatty acid analysis of ex vivo aorta culture of C57/Bl6.

Full fatty acid profile of ex vivo aorta treated with or without FADS2 inhibitor, in the context of 0.1 mM LA. Data were analyzed using t test. *p<0.05 vs Control. n=3-4.

ex vivo aorta culture fatty acid profile.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FADS2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c10:0</td>
<td>5.28±0.37</td>
<td>13.44±3.84*</td>
</tr>
<tr>
<td>c11:0</td>
<td>1.11±0.13</td>
<td>2.92±1.09</td>
</tr>
<tr>
<td>c12:0</td>
<td>0.95±0.48</td>
<td>2.02±0.62</td>
</tr>
<tr>
<td>c15:0</td>
<td>1.54±0.06</td>
<td>7.02±3.17</td>
</tr>
<tr>
<td>c16:0</td>
<td>15.66±0.30</td>
<td>16.93±1.26</td>
</tr>
<tr>
<td>c17:0</td>
<td>0.08±0.01</td>
<td>0.13±0.03</td>
</tr>
<tr>
<td>c18:0</td>
<td>6.81±0.64</td>
<td>7.46±0.90</td>
</tr>
<tr>
<td>c20:0</td>
<td>0.30±0.04</td>
<td>0.30±0.03</td>
</tr>
<tr>
<td>c22:0</td>
<td>0.13±0.04</td>
<td>0.02±0.03</td>
</tr>
<tr>
<td>c24:0</td>
<td>0.14±0.02</td>
<td>0.19±0.17</td>
</tr>
<tr>
<td><strong>Monounsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c14:1</td>
<td>0.03±0.04</td>
<td>0.02±0.02</td>
</tr>
<tr>
<td>c15:1</td>
<td>0.08±0.00</td>
<td>0.44±0.22</td>
</tr>
<tr>
<td>c16:1</td>
<td>1.12±0.04</td>
<td>2.39±0.48*</td>
</tr>
<tr>
<td>c17:1</td>
<td>0.05±0.01</td>
<td>0.05±0.09</td>
</tr>
<tr>
<td>c18:1</td>
<td>20.85±0.11</td>
<td>27.64±6.51</td>
</tr>
<tr>
<td>c22:1</td>
<td>1.06±0.01</td>
<td>0.08±0.13</td>
</tr>
<tr>
<td>c24:1</td>
<td>0.16±0.01</td>
<td>0.34±0.07</td>
</tr>
<tr>
<td><strong>n-3 Polyunsaturated Fatty</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c18:3n3</td>
<td>0.59±0.44</td>
<td>0.30±0.03</td>
</tr>
<tr>
<td>c20:3n3</td>
<td>0.75±0.06</td>
<td>1.44±0.24</td>
</tr>
<tr>
<td>c22:6n3</td>
<td>0.24±0.08</td>
<td>0.21±0.07</td>
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<tr>
<td><strong>n-6 Polyunsaturated Fatty</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c18:2n6</td>
<td>29.03±0.37</td>
<td>14.81±2.39*</td>
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<tr>
<td>c18:3n6</td>
<td>11.66±0.52</td>
<td>0.36±0.06*</td>
</tr>
<tr>
<td>c20:3n6</td>
<td>0.12±0.06</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>c20:1</td>
<td>2.08±0.18</td>
<td>0.64±0.20</td>
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<tr>
<td>c20:2</td>
<td>0.12±0.00</td>
<td>0.08±0.14</td>
</tr>
<tr>
<td>c22:2</td>
<td>0.00±0.00</td>
<td>0.33±0.43</td>
</tr>
<tr>
<td>c20:4n6</td>
<td>0.13±0.04</td>
<td>0.21±0.09</td>
</tr>
</tbody>
</table>
Appendix H: EndoG gene expression in cells transfected with or without EndoG Orf Clone.

Data were normalized to Control cells, and were analyzed by applying t-test. n=3. Srebp1c gene expression was increased by 24%.

![Graph showing EndoG gene expression](image)

$$p=0.06$$

Appendix I: Elovl5 knockout mice genotype verification.

Following manufacture’s protocol (KOMP, University of California, Davis), they offered two sets of primers (Elovl5 wildtype and insertion). If bands presented with Elovl5 wildtype primer, the target mouse was wildtype. If bands presented with insertion primer, the target mouse was heterozygous or homozygous. If bands presented with both insertion and wildtype primers, the target mouse was heterozygous. These bands were shown in the figure below. Data interpretation was shown in the table below.

![Genotype verification gel](image)

Line 1: 100bp marker; line 2, negative control, line 3, Elovl5\(^{+/+}\); line 4; Elovl5\(^{+-}\).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Elovl5 wildtype (200bp)</th>
<th>Insertion (581bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elovl5(^{+/+})</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Elovl5(^{+-})</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Elovl5(^{-/-})</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Appendix J: EndoG knockout genotype verification.

Following manufacture’s protocol (KOMP, University of California, Davis), they offered two sets of primers (EndoG wildtype and insertion). If bands presented with EndoG wildtype primer, the target mouse was wildtype. If bands presented with insertion primer, the target mouse was heterozygous or homozygous. If bands presented with both insertion and wildtype primers, the target mouse was heterozygous. These bands were shown in the figure below. Data interpretation was shown in the table below.

![Image of gel electrophoresis](image)

Line1: 100bp marker; line 2 Insertion band; line 3: negative control; line 4: EndoG wildtype band. If line 2 was appeared, the mouse was EndoG<sup>−/−</sup>, if line 2 and line 4 were both appeared, the mouse was EndoG<sup>+/−</sup>; if line 4 was appeared, the mouse was EndoG<sup>+/+</sup>.

<table>
<thead>
<tr>
<th>Primer</th>
<th>EndoG wildtype (112bp)</th>
<th>Insertion (917bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EndoG&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>EndoG&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EndoG&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>