THE ROLE OF GLUTATHIONE PEROXIDASE 1 IN THE HEART FOLLOWING HIGH FAT FEEDING

by

Lisa Renaud

B.Sc., University of British Columbia Okanagan, 2016

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

MASTER OF SCIENCE

in

The College of Graduate Studies

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Okanagan)

October 2019

The following individuals certify that they have read, and recommend to the College of Graduate Studies for acceptance, a thesis/dissertation entitled:
INVESTIGATING THE ROLE OF GLUTATHIONE PEROXIDASE 1 IN THE HEART FOLLOWING HIGH FAT FEEDING

submitted by Lisa Renaud in partial fulfillment of the requirements of

the degree of Master of Science.

Dr. Sanjoy Ghosh, Irving K. Barber School of Arts and Sciences, University of British Columbia Okanagan

Supervisor

Dr. Mark Rheault, Irving K. Barber School of Arts and Sciences, University of British Columbia Okanagan

Supervisory Committee Member

Dr. Michael Deyholos, Irving K. Barber School of Arts and Sciences, University of British Columbia Okanagan

Supervisory Committee Member

Dr. Ismail Laher, Anaestheology, Pharmacology and Therapeautics, University of British Columbia Vancouver

University Examiner

External Examiner

Additional Committee Members include:

Supervisory Committee Member

Supervisory Committee Member

Abstract

Over the last century, the composition of dietary fats has changed in Western countries leading to an increased ratio of n-6:n-3 polyunsaturated fatty acid (PUFA) consumption. It has been suspected that this altered ratio may contribute to the increase in incidence of cardiovascular diseases in Western countries. One of the classical ways how diet can impact diseases is through increasing oxidative stress. Previous studies have shown that linoleic acid (LA), a common dietary n-6 PUFA, is able to induce oxidative stress. Oxidative stress in the heart can be mitigated by antioxidants. In this regard, glutathione peroxidase 1 (GPx1) is an important antioxidant involved in protecting the heart. My project was to investigate the role of GPx1, in protecting the heart especially following a high n-6 fatty acid Western diet. The first objective was to investigate the effects of high fat diets *in vivo* in GPx1 deficient mouse hearts (Chapter 1). At the onset, we determined that $GPx1^{+/-}$ mouse hearts had lower mitochondrial copy number indicating metabolic abnormality. Upon feeding of various diets, we established that a GPx1 heterozygous KO does not affect linoleic acid induced cardiotoxicity in mice Further, $GPx1^{+/-}$ mouse hearts did not have reduced cardiac function, thereby indicating that even in reduced amounts, GPx1 is able to provide protection. Regarding antioxidants the $GPx1^{+/-}$ mouse hearts had upregulated gene expression of other antioxidants such as GPx4, superoxide dismutase (SOD) 1, SOD2, and catalase when compared to the wildtype control mice, indicating that alternate antioxidants did maintain antioxidant status in these mice. The second objective was to investigate the interactions between GPx1 and fatty acids *in vitro* on antioxidant regulation in H9c2 cardiomyocytes, one of the main heart cell types, in causing cell death (Chapter 2). GPx1 overexpression increased cellular uptake of neutral red indicating higher cell number. However, with incubation with fatty acids, we found that GPx1 overexpression increased caspase 3/7 activity with lower LDH release, thereby indicating a more controlled mode of cell death. Furthermore, we determined that GPx1 overexpressed cells incubated with linoleic acid, and not oleic acid, had increased glutathione, possibly an adjustment to cope with the increased oxidative stress that occurs after n-6 PUFA exposure. Overall, these results contribute to understanding the role of GPx1 in the heart under various dietary fatty acids.

Lay Summary

It is believed that dietary imbalances may contribute to increases in heart disease, that which is observed in Westernized countries. In recent years, Western diets have increased the consumption of omega-6 polyunsaturated fatty acids (PUFA), which mainly found in corn and sunflower oils. Studies have shown that excess omega-6 PUFAs may damage heart muscle and lead to cell death, however; the underlying mechanisms are unknown. Furthermore, the heart utilizes antioxidants, like glutathione peroxidase 1 (GPx1), to prevent heart damage. I propose that when GPx1 is reduced, the heart is at risk for incurring damage from a Western diet. *In vivo*, we observed that various antioxidant gene expressions were altered but there was no change in heart function. *In vitro*, we observed that GPx1 overexpression led to changes in the type of cell death occurring. These results contribute to understanding the role of GPx1 in the heart following a Western diet.

Preface

Jiayu (Daisy) Ye assisted with obtaining echocardiogram data and conducting appropriate analyses. Euthanasia was assisted by Jiayu Ye, John (Jake) Winkler, and Svetlana Simtchuok. I am largely responsible for generation of all experimental data; however, Mitchell Figura kindly completed qPCR on the wildtype mouse heart samples. All animal experiments completed in this thesis were reviewed and approved by the UBC Animal Care Committee (certificate # A13-0305).

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AKT	Protein kinase B
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
ARA	Arachidonic acid
BSA	Bovine serum albumin
BMK1	Big mitogen-activated protein kinase 1
CAT	Catalase
cDNA	Complimentary deoxyribonucleic acid
CHD	Coronary heart disease
CO	Corn oil
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DHA	Docosahexaenoic acid
DE-TBARS	Diethyl thiobarbituric acid
EF	Ejection fraction
EPA	Eicosapentaenoic acid
FA	Fatty acid
FFA	Free fatty acid
FS	Fractional shortening
GSH	Glutathione (reduced)
GPx	Glutathione peroxidase
GPx1	Glutathione peroxidase 1
GPx4	Glutathione peroxidase 4
GR	Glutathione (oxidized)
GSSG	Glutathione reductase
H_2O	Water
H_2O_2	Hydrogen peroxide
HFD	High fat diet
IVS	Interventricular septum thickness
KO	Knock out

LA	Linoleic acid
LDH	Lactate dehydrogenase
LVH	Left ventricular hypertrophy
LVID	Left ventricle internal dimension
MAPK	Mitogen-activated protein kinase
MUFA	Monounsaturated fatty acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NC	Normal control
ND5	NADH-ubiquinone oxidoreductase chain 5
OA	Oleic acid
O_2	Oxygen
O_2^-	Superoxide radical
^-OH	Hydroxide radical
OO	Olive oil
OPA	o-Phthaladehyde
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acid
PWT	Posterior wall thickness
qPCR	Quantitative polymerase chain reaction
RIPA	Radio-immunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SFA	Saturated fatty acid
SOD	Superoxide dismutase
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
WT	Wild type

Acknowledgements

First, and foremost, I would like to express my appreciation to my supervisor, Dr. Sanjoy Ghosh. In your lab, I developed several skills that will eventually lead to a successful career in science. Furthermore, in your lab I was given the opportunity to be independent and was given ample opportunity to grow. My graduate degree experience has greatly influenced my life and has led to several opportunities, and for these and other reasons I would like to say thank you. In addition, I would also like to extend a heartfelt thank you to my supervisory committee members, that which were able to provide valuable perspectives and advice. First, Dr. Michael Deyholos; for helping to greatly improve my public speaking and presentation skills. Your perspective and guidance have contributed invaluable insight throughout this process. Next, Dr. Mark Rheault; your assistance with helping to fix the Promega GloMax when my plates once became stuck, as well as your continual support throughout the duration of my program has been greatly appreciated. Furthermore, I appreciate that an effort was always made to address my questions. For all above mentioned reasons and more, I am extremely grateful to my supervisory committee.

Second, I would also like to acknowledge my fellow undergraduate and graduate students in the Department of Biology and Department of Chemistry at UBCO. I would also like to extend a sincere thank you to all of the graduate and undergraduate in Dr. Ghosh's and Dr. Gibson's laboratories for readily helping when needed. I would especially like to thank Jiayu (Daisy) Ye for the great amount of assistance and support during my degree.

Third, I would like to thank Dr. Trudy Kavanagh for her support and guidance regarding improvements in writing clarity. Direction provided by Dr. Kavanagh helped to greatly improve my writing style.

Lastly, I would like to thank my family for their continual support and love. A special large thank you to Anthony Haines for the enormous amount of support and patience. Thank you to Henry and Stanley for keeping me grounded.

Dedication

For my family.

Chapter 1: Introduction

1.1 *Overall Problem*

Cardiovascular disease (CVD) was the top cause of global mortality in 2013 [1]. Currently in Canada, CVD mortality is ranked second in occurrence, only to be surmounted by cancerous tumors [2]. Unsurprisingly, CVD places a heavy burden on Canadians due to the prevalence and financial cost of this disease [2]. Heart disease and atherosclerosis, the latter of which is a dangerous accumulation of plaque within arterial walls, are categorized as circulatory system diseases, that which totaled a cost of $13.1 billion in Canada in 2010 [3]. With the Canadian predictions on population growth and aging, it is expected that these costs and rates of CVD incidence will continue to increase, however; the etiology of these diseases are still not fully understood [4].

1.2 *Cardiovascular Disease*

CVD encompasses several diseases of the heart or circulatory system including coronary artery disease, stroke, coronary heart disease, cardiomyopathy, heart failure and several others. [3]. While CVD has been researched heavily over the years, there is still debate upon absolute causes of CVD [5]. There are several known risk factors for developing CVD including obesity and diet [6,7]. For chronic diseases, it is recommended to increase intake of fruits and vegetables; however, in westernized countries there is a movement towards processed, quickly prepared foods, that which are rich in seed oils [6,8]. While these recommendations to eat the appropriate amount of vegetables and fruits are known, there are added complexities related to certain chronic diseases [9,10].

Being a multifactorial disorder, there are various contributing factors like diet, exercise, or an underlying genetic component [11] that lead to the development of CVD [12]. To add to the rich complexity of understanding CVD; illnesses typically exist as co-morbidities, where an individual suffers from multiple clinical manifestations of disease [13]. While some diseases have similar origins, cardiomyopathy is typically independent of coronary vascular disease [14].

1.2.1 *Cardiomyopathy*

Cardiomyopathies are diseases of the heart and can be further distinguished between various types including dilated cardiomyopathy and hypertrophic cardiomyopathy [14]. Unsurprisingly, the

negative effects include ventricular dysfunction, such as by dilation or hypertrophy [14]. Both of which have enlargement of the ventricles and the causes are considered to be predominantly genetic [14].

Hypertrophic cardiomyopathy, one of the most common forms of cardiomyopathies, is typically an inherited genetic mutation [15,16]. It is common for cardiomyopathy patients have left ventricular hypertrophy [15]. Left ventricular hypertrophy (LVH) is an increase in wall thickness and mass of the ventricle as a cellular adaptation that occurs to cope with wall stress [17–19]. Therefore, hypertension is a main cause of LVH[17,20]. In aged populations, there are similar changes in the heart that occur by pressure induced hypertrophy [21]. Both have an increase in the volume fraction of myocytes and a decrease in interstitium [21]. The link between age-related cardiomyopathy though is controversial [22]. Unique to cardiomyopathy among several CVDs however; is the potential occurrence of disease during any life stage [15].

Mitochondrial disease has the capability to manifest as a cardiomyopathy by mutations to mitochondrial DNA [16]. This is because the contractile cells within the heart contain plentiful amounts of mitochondria and therefore abnormalities can lead to cardiac cell impairment [16]. While the prevalence is not as common as hypertrophic cardiomyopathy, the severity of mitochondrial cardiomyopathy varies and can lead to heart failure and death [16]. More recently, mitochondrial changes have been attributed to obesity due to a faulty diet [23].

1.2.2 *Obesity, Oxidative Stress and Cardiomyopathy*

Obesity, a chronic disease prevalent in western countries, is characterized as an accumulation of adipose tissue and a minimum body mass index above 30kg/m^2 [11,24]. Adipose tissue, which is composed of adipocytes, secretes hormones and cytokines in part of homeostatic regulation. In an obese individual, with increased adipose tissue, cytokines can produce ROS and thereby result in an inflammatory response[24]. Eventually the amount of ROS cannot be equivocated by antioxidants and oxidative stress results [25]. Furthermore, obese individuals typically have increased plasma levels of free FAs (FFA) due to inhibited clearing of FFA in addition to adipose tissue releasing more FFA [26]. Heightened FFA then is able to negatively affect the insulin-signaling pathway and contribute to insulin resistance [26]. Obese rats had impaired cardiac

function with elevated FFA levels [27]. Subsequently, obesity is a risk factor for developing additional oxidative-induced disease such as diabetes mellitus, CVD, and cancer [24].

In addition, obesity is capable of straining the cardiovascular system by increasing blood volume and cardiac output due to the addition of body mass [28,29]. Cardiac output is increased from stroke volume and therefore the heart rate is typically elevated as well [30]. The occurrence of these factors contributes to dysfunction in several other cardiovascular areas leading to heart failure [28,29]. In obese individuals, impaired antioxidant function leads to onset of other chronic oxidative diseases [25]. It has been shown that individuals with metabolic syndrome, not just obesity, have lowered amounts of antioxidants [25]. Obese individuals however, are more at risk for death by progressive heart failure [31].

1.3 *High Fat Diets*

Western diet is categorized as one with high amounts of fat, sugar and salt[8]. The fat is typically around 40% of total caloric intake [32] and has been steadily on the rise over the past 100 years due to agricultural advancements [8]. Dietary fat is thought to be one of the main contributors to obesity, which is a foundation for developing several other diseases[33]. It is now understood that the types of fat are important for disease propagation and not necessarily the amount of fat[34]. Types of fat and therefore types of fatty acids (FAs), vary in their chemical composition[8].

1.3.1 *Saturated Fatty Acids*

Saturated fatty acids (SFAs) contain no double bonds and therefore are saturated with hydrogens[35]. Some examples of foods rich in SFA are fatty meats, butter, milk, and cheese[8]. In the 20th century, the recommended intake of SFA has decreased due to the possible links to CVD[36]. In 1986, Menotti et al., published a paper indicating a positive association between SFA intake and death rates from CHD[37]. Later, it was shown that SFA increases low density lipoprotein which are associated with atherosclerotic plaques[38,39]. As previously stated, hypertension contributes to left ventricle hypertrophy, therefore additional supporting evidence was published in 1997 stating that saturated fat intake increased blood pressure[40].

After further review, the role of SFAs and the onset of CVD has been heavily debate [36,41]. Unfortunately, there are few randomized control trials that support these claims pertaining to

SFA intake and CVD risk[9]. Clinical trials have often reduced SFA but increased other types of fat in the diet, thereby complicating interpretation of the results[38]. Some studies reduced fat content but it has been shown that carbohydrates can cause abnormal lipid profiles as well[38,42]. In a meta-analysis from 2010, it was concluded that there is no association in increased CHD or CVD risk with SFA intake [36]. In addition to this, several other studies have been published concluding similar results that the link is weak between CVD and SFA intake[36,42,43].

1.3.2 *Polyunsaturated FAs*

PUFAs have multiple double bonds as opposed to MUFAs, which contain one double bond [44]. Depending on where the first double bound is in relation to the terminal methyl group designates the n value of the PUFA. As an example, linoleic acid (LA) has a total of 18 carbons, has its first double bond at the 6th carbon from the methyl group [44] and a total of 2 double bonds. Hence LA is termed as 18:2n-6 and is the predominant FA present in vegetable oils like corn, safflower and sunflower oils[44].

Interestingly, although proteins and carbohydrate intakes generally remained the same, the amount of total fats and oils and the consumption of specific FAs has changed due to changes in consumption of certain newer kinds of fats and oils. Of the three FA types shown, there was a large increase in consumption of polyunsaturated FAs (PUFAs) (Table 1). A small increase was seen in monounsaturated fatty acid (MUFA) consumption, whereas SFAs has decreased since 1976 (Table 1).

Table 1. *Canadian food disappearance data from 1986-2009. Combined from Wong et al., 2015 and Data Statistics Catalogue number 21-020-X*

	1986	1996	2006	2009	% Increase
Total fats (g)	88.31	97.98	94.17	91.02	**3%**
Total PUFA (g)	14.08	17.78	17.69	17.06	**21%**
Total SFA (g)	27.37	26.60	26.54	25.7	**0%**
Total MUFA (g)	40.70	47.31	43.62	42.10	**3%**
Carbohydrates (g)	288.74	302.60	299.62	295.65	**2%**
Proteins (g)	68.36	68.59	71.03	69.85	**2%**

Investigating the effects of certain types of FAs has been a large focus in nutrition research recently. n-3 PUFAs are thought to suppress inflammation compared to n-6 PUFAs which drives an anti-inflammatory response[8]. However, in Western countries, large amounts of n-6 PUFA compared to n-3 PUFA are consumed[8]. LA is converted to arachidonic acid (ARA) through chain elongation and desaturation [45]. ARA is a precursor for several eicosanoid metabolic products such as thromboxanes and prostaglandins[45], which in cardiovascular system can promote atheromas and thrombus, that which can lead to autoimmune and inflammatory responses[45]. While ARA is well known as a pro-inflammatory mediator, it can also lead to the production of anti-inflammatory mediators such as lipoxins [46,47]. Lipoxins act synergistically to resolvins to reduce inflammation [48].

n-3 PUFAs like the marine fish derived docosahexaenoic acid (DHA; C22:6n3) and eicosapentaenoic acid (EPA; C20:5n3) encourage anti-inflammatory processes by promoting synthesis of resolvins[46,49]. Resolvins can reduce the severity and duration of inflammation directly by decreasing neutrophil infiltration and increasing macrophage phagocytosis of apoptotic cells [46,50]. For EPA specifically, production of differing series prostaglandins, thromboxanes, and leukotrienes are produced[49]. A study in mice hearts found that n-3 PUFA diet, including DHA and EPA, was able to increase antioxidant enzyme expression and decreased

mitochondrial ROS [51,52]. In humans it was shown that within 2-3 weeks of EPA and DHA supplementation there were changes within the human heart such as enhancement of antioxidant capacity [51]

The production of anti and pro inflammatory mediators is important for homeostasis and therefore represents a complex relationship shaped by consumption of n-3 and n-6 PUFAs[46]. Finally, as n-3 and n-6 PUFAs share the same enzymatic pathway to produce longer chain PUFAs, it is thought that a western diet rich in n-6 PUFAs would have increased pro-inflammatory mediators compared to anti-inflammatory mediators[45,53].

Regarding structural roles, LA and ARA and other PUFAs are necessary components for cell membranes[45]. Dietary FAs are able to replace existing FAs within membranes[45], which can increase the risk of potential oxidation of double bounds in membranes. PUFAs, thereby containing the most double bonds, are most at risk for lipid peroxidation and continual ROS damage[54]. This can lead to a damaged cell membrane from accumulating oxidative damage. Mitochondrial membranes have high amounts of PUFAs in their membranes and are the main producers of reactive oxygen species (ROS). This makes mitochondria at risk for lipid peroxidation in addition to the already present inflammatory processes from ARA production[54]. The increases in chronic disease, such as CVD and diabetes, is well understood to be from possible increased dietary ratio of n-6 to n-3 PUFAs[45].

It should be mentioned that several studies support that n-6 PUFAs, such as LA, are good for cardiovascular health [44,55]. However, increasingly it is shown that consumption of certain plant oils, such as those containing n-6 PUFAs can lead to higher mortality when compared to SFAs [38,41]. While recommendations are made to consume plants for reducing incidence of disease, it becomes complex when considering oils deriving from various plants [9,56–58]. Furthermore, not all plant based diets are nutritionally equal and while it is recognized that plant based diets can reduce incidences of certain diseases, the “less-healthy” (for example including fruit juices or refined grains) plant based diets do put individuals more at risk for developing CVD when compared to a healthier plant based diet [10]. While corn is considered a healthy vegetable, corn oil contains large amounts of LA [10,41]. It is well established that LA is a precursor for inflammatory molecules by conversion into ARA, necessary for production of eicosanoids, therefore a

reduction of dietary n-6 PUFAs such as LA can decrease inflammation[44,48]. This is shown in a reanalysis of the Sydney heart study where LA had a 17.8% chance of death compared to the 11% chance in the control group[41,59]. Additionally, the negative effects associated with LA were shown recently when mice were provided varying amounts of LA directly to the left ventricle before ischemia[50]. The infarct sizes were recorded and found that increasing LA increased myocardial infarct size[50]. A paradigm shift is far from occurring in the field because the effects of LA on heart health has not been well established[60].

1.4 *Reactive Oxygen Species and Oxidative Stress*

ROS are reactive oxygen containing molecules, which can interact with proteins, lipids, and DNA and cause oxidative changes [61,62]. The cellular oxidative damage eventually can lead to tissue damage and death[61]. Production of ROS can occur endogenously (respiration, lipid metabolism) or exogenously (UV light, toxins) [61]. Due to the high-energy demand and a high concentration of mitochondria, the mitochondria are responsible for approximately 90% of ROS production in the heart muscle[63,64]. Additionally, ROS is also produced in response to cytokines or bacterial invasion to transduce signal to initiate a defense mechanism [65].

ROS production and scavenging systems are highly regulated. An alteration of these systems can change various signaling pathways including host defense and the aging process[61,66]. Under normal conditions is it estimated that ~2% of total oxygen consumed produces ROS and thereby maintains these vital signaling pathways[61]. Alternatively, excess ROS causes the negative effects such as cell damage and death. One of the primary ROS is the superoxide anion (O_2^-), as shown below in Figure 1[67]. The superoxide anion is produced by single electron reduction of molecular oxygen [66]. The two main points where ROS are produced within the mitochondria are complex I and complex III during the electron transport chain (ETC)[61]. The superoxide anion produced from these processes will harm numerous cell constituents in addition to the mitochondria's own DNA[62]. A self-perpetuating cycle can be induced where a defective mitochondrion is producing more ROS and cannot combat ROS due to a lack of antioxidants (Figure 1).

Hydrogen peroxide (H_2O_2) is considered the strongest molecule for cellular signaling [68]. Unique to H_2O_2, is the enzymatic production and degradation, that which is not present in other ROS (superoxide, hydroxyl radical, etc.). [68]. H_2O_2 has a considerably long half-life and able to oxidize

cysteine residues easily [69]. Next, the cystine is able to continue the cascade of events by interacting with another cystine, glutathione (GSH), an amide in a protein backbone, or another hydroperoxide [68]. The hydroxyl radical is a product of hydrogen peroxide decomposition and is thought to cause the most oxidative damage [69].

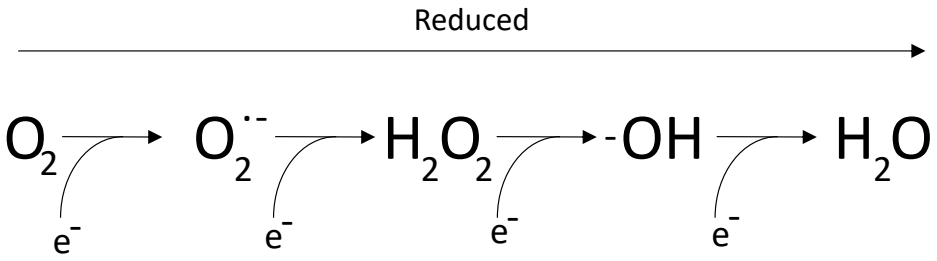


Figure 1. *Simplification of types of ROS. Adapted from Kiley & Storz, 2004.*
ROS originate as a normal by-product of cellular respiration from molecular oxygen [70]. The most reduced product is water, while working towards the products on the left are more oxidized. Abbreviations: O_2, molecular oxygen; O_2^-, superoxide anion; H_2O_2, hydrogen peroxide; $^{\cdot}OH$; hydroxyl radical, and H_2O, water.

There are several antioxidants acting to alleviate oxidative stress. There are however, three main intracellular antioxidants: superoxide dismutase, catalase, and glutathione peroxidase [71]. Superoxide dismutase converts O_2^- into hydrogen peroxide (H_2O_2). Next, catalase converts hydrogen peroxide into water and oxygen. Glutathione peroxidase also converts H_2O_2 into water by using two molecules of reduced glutathione, which becomes oxidized glutathione (GSSG) [71]. Further discussion on antioxidants will be continued in a later section, however; the reactions for these three particular antioxidants are shown below [71].

Superoxide Dismutase:	$O_2^- + O_2^- + 2\ H^+$	→	$H_2O_2 + O_2$
Catalase:	$H_2O_2 + H_2O_2$	→	$2\ H_2O + O_2$
Glutathione Peroxidase:	$H_2O_2 + 2$ GSH	→	$2\ H_2O$ + GSSG

Oxidative stress is a result of an imbalance between ROS and antioxidants (Finkel & Holbrook, 2000). Without sufficient antioxidant levels, ROS clearing is impaired the accumulation of ROS

produces oxidative damage. Increased oxidative stress can damage tissues and lead to various diseases such obesity or CVD[70].

1.4.1 *Oxidative Stress and CVD*

An imbalance between antioxidant systems and ROS production is seen in various chronic pathologies including diabetes, insulin resistance, and cardiovascular disease [62,63]. Cardiovascular disease (CVD) is present worldwide; however, it is more prevalent among westernized countries and is the top cause of death in diabetic individuals [62,72]. CVD, similarly, to other chronic diseases, is a result of numerous complex variables including diet, exercise, genetic predisposition, sex, and other factors [62]. Atherosclerosis, a type of CVD, once was thought to simply be the accumulation of fat in vessels; however, now it is understood to be complex multi-stage chronic inflammatory process triggered by endothelial damage [62]. ROS in this case damages the epithelial tissue and primes for atherosclerotic lesions by exposing the underlying cells to inflammatory processes[62]. Atherosclerosis is harmful to an individual and unfortunately can worsen and induce myocardial infarction[73,74]. The fatty inflamed plaques decrease the amount of oxygen rich blood reaching the heart (ischemia) these ischemic tissues become damaged and necrotic and can lead to heart complications[73].

While there are several contributors to developing CVD, a large proportion of the risk factors for CVD are preventable and include factors such as smoking, diabetes, and lipid intake[74]. Lipid intake, as we now understand is more complex than previous assumed, and therefore this supports the notion that in order to help patients with CVD that the n-6:n-3 ratio could be reduced in an attempt to allow cardiovascular protection provided by cellular pathways [50]'.

1.4.2 *Importance of ROS*

ROS production and scavenging systems are highly regulated [75]. An alteration of these systems can change various signaling pathways including host defense and the process of aging [61,66]. Under normal conditions is it estimated that ~2% of total oxygen consumed produces ROS and thereby maintains these vital signaling pathways [61]. Alternatively, unregulated ROS has negative outcomes such as cell damage and death [76].

Most of the discussion in this thesis revolves around ROS levels remaining exceedingly high and

causing oxidative damage. While ROS have the capacity to be harmful, their strength as signaling molecules is unequivocal. The roles of ROS in several homeostatic pathways are well established, such as in the mitogen-activated protein kinase (MAPK) pathway [65]. MAPK broadly encompasses processes that result in cell growth, development and differentiation [65]. Specifically, ROS has been shown association in the big MAP kinase 1 pathway (BMK1/ERK5), which delegates action for cell survival, motility, differentiation and proliferation [65]. ROS can activate precursor molecules that activate this pathway [65]. In regard to the human immune system, macrophages play a key role in eliminating bacterial cells by oxidative burst [77]. The mechanism is still not fully understood, however; involves production of ROS and reactive nitrogen species by the phagolysosomes [77].

ROS play valuable roles in several aspects of homeostasis ranging from vascular considerations, apoptosis, and the immune system, to name a few [68,75,78]. One example of vascular physiology reliance on ROS is for maintenance of a differentiated contractile phenotype, that which is a result of H_2O_2 interaction with embryonic stem cells by activating certain transcription factors [69]. Apoptosis, a programmed cell death as a result of an interaction with a death stimulus [78]. H_2O_2 activates tumor suppressor protein p53 which then activates transcription of apoptotic genes involved in intrinsic apoptosis [79]. Additionally, ROS are able to damage mitochondrial DNA and disrupt mitochondrial function, thereby activating mitochondrial apoptosis [79]. ROS are key secondary messengers for activation of both the innate and adaptive immune systems [76]. To mention briefly, there are several antimicrobial modes of action utilizing ROS [80]. As previously stated, ROS are able to damage protein, DNA and lipids; therefore, it is unsurprising that it is used as chemical warfare against pathogens by the host's immune system [80].

Exercise is an excellent way to combat the preventable risk factors for several chronic diseases. Exercise does increase ROS and can still diminish diabetes, cancer, and other ROS-related diseases [76]. From an inflammatory perspective, the decrease in visceral fat thereby reduces pro-inflammatory molecules associated with visceral fat and therefore can reduce inflammation [81]. ROS are regular by-products of cellular metabolism, that could be upregulated during exercise [82]. In a healthy individual, adaptations to the exercise stressor can occur such as increases in antioxidant activity [82]. Furthermore, it is suggested that the exercise adaptation process can lower baseline of ROS and therefore reduce ROS-related disease such as Alzheimer's and diabetes [83]. It

should be mentioned that although exercise is able to improve some patient health outcomes, excessive exercise counteracts these positive effects[83].

1.5 *Antioxidants*

Antioxidants are classified as enzymatic and non-enzymatic [84]. Enzymatic antioxidants are reducing agents that delay or prevent oxidative damage from ROS accumulation and examples include superoxide dismutase and glutathione peroxidase[84,85]. Non-enzymatic antioxidants include glutathione and uric acid [84]. Endogenous antioxidants, such as glutathione, thioredoxin, and coenzyme Q, are considered to be principal intracellular reducing agents[84,85]. Antioxidant enzymes constitute the remainder of the endogenous antioxidant category[84]. Examples are glutathione peroxidase and superoxide dismutase[84]. These enzymes have several activities in relation to detoxifying harmful ROS but also in regeneration of glutathione or NADPH [84]. The antioxidant system though relies heavily on exogenous uptake of antioxidants due to the synergistic nature of both the endogenous and exogenous sources of antioxidants in maintenance of redox homeostasis[84].

1.5.1 *Exogenous Antioxidants*

Exogenous antioxidants typically from diet and include vitamins (A, C and E), plant polyphenolic compounds (flavonoid sand tannins), and carotenoids (beta-carotene), and trace elements (zinc and selenium), to mention a few [84]. These compounds can be consumed; however, large amounts can lead to toxicity or impairment of cellular ROS signaling [84]. Exogenous reducing compounds supplement the endogenous primary defense systems, for example selenium is present in several antioxidant compounds [84,86]. Flavonoids are naturally occurring compounds derived from plants [87,88]. Of the many roles within plants, these compounds influence color and are used in signaling for pollinators in addition to their role as ROS scavengers [87]. Red wine and tea contain high levels of phenolic compounds [87,88]. While endogenous and exogenous work together, endogenous antioxidants play a more powerful role in redox homeostasis in humans [84]. Several studies have investigated the role of plant phenols and decreasing the incidence of coronary heart disease and atherosclerosis [88–90]. For atherosclerosis, polyphenols inhibit low-density lipoprotein (LDL) oxidation to prevent development of lesions [88,90]. For other CVDs, it is that the protective effects are provided by improving endothelial function and anti-inflammatory effects [88,91].

1.5.2 *Glutathione*

Glutathione (GSH) acquires its redox capabilities from its thiol group located on the cysteine residue within the tripeptide [92,93]. In addition to its role in redox homeostasis, GSH is involved in signal transduction and several other cellular pathways [94]. GSH is a ubiquitously expressed antioxidant that scavenges free radicals and reduces H_2O_2 [92]. GSH is a highly prevalent antioxidant as there are 100-1000-fold more glutathione molecules than thioredoxin, another thiol rich protein, in mammalian systems[85].

Various tissue types contain differing amounts of glutathione ranging from 0.1-15mM, with the highest concentration at the synthesis site within the liver [70,92,93]. Around 10-15% of total GSH moves from the cytosol to the mitochondria for first line of defense against ROS [92]. Glutathione exists in bound or unbound forms [92]. The unbound form has two redox states: oxidized glutathione disulfide (GSSG) and the active, reduced glutathione (GSH), both of which are visualized below in Figure 2. The ratio of GSH/GSSG is an indicator of redox status within the cell or specific organelles because as the ratio decreases the cells are more at risk for oxidative damage[92]. GSSG is returned to its reduced state by glutathione reductase utilizing NADPH as an electron donor [70,95].

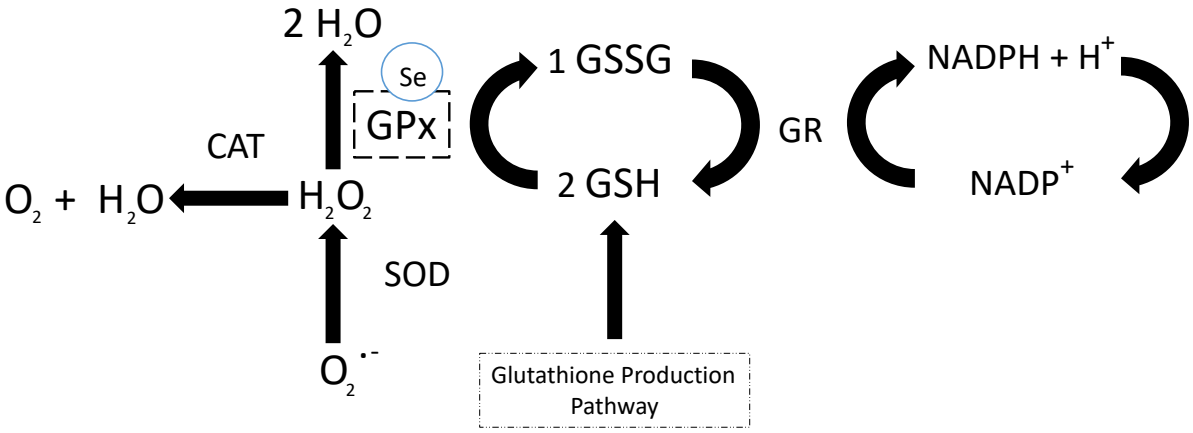


Figure 2. *Glutathione metabolism adapted from Weydert & Cullen, 2010.*
Following glutathione production, GSSG (oxidized glutathione) is reduced by the antioxidant enzyme GPx to form 2 molecules of GSH (reduced glutathione). GPx, in addition to SOD and CAT are the main intracellular antioxidant enzymes. The superoxide anion (O_2^-), a byproduct of normal cellular metabolism, is reduced by superoxide dismutase (SOD). GPx, a selenium (Se) containing enzyme, reduces H_2O_2 into water. Similarly, CAT similarly reduces H_2O_2 into water

and oxygen (O_2). Glutathione reductase (GR) replenishes the glutathione pool by oxidizing 2 molecules of GSH to produce GSSG, using NADPH as the electron donor.

Cancer, neurodegenerative diseases, and CVD are all associated with GSH deficiencies, which therefore helps to establish the importance of this antioxidant[94]. Importantly, individuals with a deficiency in systemic glutathione have been shown to develop worsening heart failure and structural abnormalities[96] as well as cardiomyopathy[97]. GSH utilizes enzymes such as glutathione peroxidase (GPx) and glutathione reductase to maintain redox homeostasis[85]. GPx is of particular interest as they utilize GSH to reduce H_2O_2 and lower oxidative stress[67,85]. While GSH is able to reduce ROS, antioxidant enzymes target harmful products as a result of ROS interactions[92].

1.5.3 *Glutathione Peroxidases*

GPxs are a family of antioxidant enzymes that reduce hydroperoxides into corresponding alcohols utilizing the antioxidant glutathione typically [86]. Interestingly, not all GPxs utilize GSH and have been known to have specificity for thioredoxin or other thiol oxidoreductases [98]. The 8 isoforms are listed below in Table 2 as well as their corresponding primary tissue location by species. The table was generated using NCBI to determine the primary locations of each GPx isoform. The search was conducted using the gene function specific to each species of interest. Lists are not conclusive but display the predominant locations.

The family can be differentiated based on the presence of selenium in the catalytic site [86]. The four selenium proteins were determined following analysis of the selenoproteome [86,99]. Later, GPxs containing only a cysteine in the active site were discovered [86]. GPx1, GPx2, GPx3, GPx4, and GPx6 make up the selenoenzyme group whereas GPx5, GPx7 and GPx8 contain cysteine [98,100]. The locations of the various GPx isoforms is detailed in Table 2 below.

Table 2. *Glutathione peroxidase isoforms and their highest expressed organ or tissue location in Homo sapiens and Mus musculus.*

Isoform	*Homo sapiens* Accession	*Homo sapiens* location	*Mus musculus* Accession	*Mus musculus* location
GPx1	AAH07865	All cell types[1]	P11352	All cell types[7], predominantly in erythrocytes[7], kidney[7], liver[7]
GPx2	NP_002074	Gastrointestinal tract[2]	Q9JHC0	Gastrointestinal tract[8]
GPx3	P22352	Plasma[2], lung[2], kidney[2]	P46412	Kidney[6], plasma[10]
GPx4	P36969	Most cell types, predominantly in testis[2]	O70325	Testis[11], sperm[11], brain[13], liver[12], heart[12], kidney[12]
GPx5	O75715	Epididymis[3]	P21765	Epididymis[9]
GPx6	P59796	Olfactory epithelium, embryos[4]	Q91WR8	Olfactory epithelium[6]
GPx7	Q96SL4	Esophageal epithelial cells[5]	Q99LJ6	Vault of skull bone[6]
GPx8	Q8TED1	(Putative), kidney[6]	Q9D7B7	Humerus cartilage[6]

1 - [101]
2 - [102]
3 - [103]
4 - [104]
5 - [105]
6 – Uniprot.org
7 - [106]
8 - [107]
9 - [108]
10 - ncbi.nlm.nih.gov
11- [109]
12 - [110]
13- [111]

1.5.3.2 *GPx1*

Of all GPxs, GPx1 was the first to be discovered in 1953 and in 1973 and was categorized as a selenoenzyme [102,112,113]. It is a tetrameric protein containing 22-23 kDa identical subunits with 4 identical subunits [102,113]. GPx1 is an intracellular antioxidant enzyme that reduces FA hydroperoxides in phospholipids under the presence of phospholipase A2 [113]. It is limited to hydrogen peroxide predominantly due to the presence of a loop structure restricting access for bulk phospholipid hydroperoxides [98,102]. Catalase and GPx proteins both remove hydrogen peroxide; however, GPxs have a lowered K_m (high affinity for H_2O_2) and are considered to be the

main removers of hydrogen peroxide as a result [106].

The mechanism of reduction by GPx1 is established. In GPx1, the selenocysteine reactive site becomes a selenic acid intermediate before becoming reduced by 1 molecule of GSH[98]. GSH becomes a glutathionylated senol intermediate which undergoes a subsequent reaction with a second GSH molecule[98]. Finally, the selenocysteine activity site is restored and GSSG is a result[98].

When discussing antioxidants, it is beneficial to understand what benefits or detriments could occur during alterations of activity. GPx1 overexpression is protective against apoptosis in scenarios involving a disruption in normal redox balance[102]. Some tumor cells undergoing chemical induced apoptosis were not able to circumvent apoptosis with the addition of increased GPx1 expression[102]. This result is not unexpected as increasing an antioxidant in the presence of a stressor is typically beneficial and furthermore, specifically GPx1 is a cytosolic enzyme present within the mitochondria and therefore could play a role in decreasing intrinsic apoptosis[113].

1.5.3.3 *GPx1 and Heart Disease*

As previously mentioned, when attempting to understand oxidative stress in the heart, that former of which that underlies several diseases, it could be beneficial to look to GPx1 as it has been shown to play a unique role in the cardiovascular system[113,114]. The catalyst for some of the GPx1 research interest was a paper that measured antioxidant activity in coronary artery disease patient's red blood cells [115]. The authors found that erythrocyte GPx1 activity was associated with lower risk of cardiovascular events in coronary artery disease patients[115]. This result was not shown in other antioxidants tested which included catalase and superoxide dismutase [115]. As seen in table 2, GPx1 is in high levels predominantly in the fat and spleen; however, its role in the heart and cardiovascular system should not be neglected[113].

Additionally, it has been shown that deficiencies of GPx1 in mouse models has accelerated atherosclerosis following a western diet[116]. Furthermore, H_2O_2, the primary target of GPx1, has been shown to accelerate contractile dysfunction of the heart, therefore in a reduced GPx1 individual there may be heart function abnormalities[114]. Therefore, GPx1 seems to play an important role in maintenance of cardiovascular health. However, the effect has not been

investigated fully following consumption of a n-6 PUFA diet. We have shown previously the negative effects of excess n6 PUFA consumption on cardiovascular health outcomes[117–119]. Consequently, this study aims to determine the specific role that GPx1, an important class of cardiac antioxidants, plays in preventing CVD following high n-6 fat feeding.

1.5.3.4 *GPx1 and Cell Death*

Apoptosis describes a type of cellular death that involves morphological changes (decrease in cell volume, DNA cleavage, etc.)[120,121]. Apoptosis is important for normal development; however, if apoptosis is unregulated then it can lead to negative effects such as the development of disease[22,122]. Caspase-independent apoptosis undergoes the same cell and nuclear morphology as seen in apoptosis and therefore can be difficult to prove that all caspase activity is inhibited in addition to our potentially limited current understanding of the number of caspases[123,124]. In spite of this, measurement of caspase activity is still considered appropriate to estimate apoptosis activity.

In addition to measurement of apoptosis, necrosis is measured when discussing cellular death. Necrosis is characterized as cell death in which swelling and rupture of the intracellular organelles occurs followed by plasma membrane disintegration[125]. Necrosis is considered to be an inflammatory cellular death in comparison to apoptosis[126]. Necrotic cells do not have mitochondrial membrane permeability and caspase activation while apoptotic cells do not have a reduction in mitochondrial membrane permeability before caspase activation[127].

A common way to evaluate necrosis *in vitro* is by measurement of lactate dehydrogenase (LDH), the latter of which is a soluble cytoplasmic enzyme that is released into extracellular space when the plasma membrane is damaged[125,128]. There are other methods to measure necrosis or viability, such as by measurement of the DNA binding propidium iodide; however, when propidium iodide (PI) staining is used on its own it cannot distinguish between necrotic or apoptotic cells as apoptosis eventually can lead to necrosis[123,129]. Therefore, we selected LDH measurement to estimate necrosis. Complexities can arise when interpreting LDH data though, post-apoptotic cells can release cellular components into the extracellular space[127]. While a macrophage typically engulfs the cell, there is a possibility for apoptotic cells to release LDH and therefore lead to an incorrect interpretation[127]. However, there were no macrophages in our assay.

Caspase is an ATP dependent cellular death and typically when the cell is faced with a large stressor, necrosis exists[123,130]. Increased GPx1 could be seen as an advantage in regard to facing the FA stressors. Therefore, when presented with the highest stressor LA, it is somewhat predicted to have decrease caspase 3/7 activity as perhaps that cell is facing increased oxidative stress and would pursue necrosis instead[57]. This will require further investigation though.

CD95, a receptor in the tumor necrosis family, is established as a death receptor expressed in most tissues and plays a role in the extrinsic apoptotic pathway[131,132]. CD95 activates caspase-8 and thereby triggers the apoptotic cascade [120]. It is also known that fluctuations in glutathione levels can trigger apoptosis through CD95 and therefore early on it has been theorized that ROS plays a role in CD95 apoptotic signalling[131]. GPx1 overexpression in human breast carcinoma T47D cells, a cell type expressing high levels of CD95, was able to reduce ROS production induced by CD95[131].

GPx1 plays a role in the intrinsic apoptotic pathway by decreasing pro-apoptotic proteins. In the human-like endothelial line ECV304, GPx1 overexpression, in the absence of additional ROS, was able to alter the ratios for mRNA and protein levels between Bax to Bcl-2 [133]. By decreasing Bax, that which is a mitochondrial pore forming protein, and not Bcl-2, that which is a pro-survival protein, the cell may be protected from programmed death [134]. Next p53 mRNA and protein levels were measured and the result was no change between GPx1 overexpressed cells and the control [133]. H_2O_2 primarily targets p53 to cause cell cycle arrest or lead to apoptosis [133]. As shown in Figure 2, GPx1 reduces H_2O_2 to water, therefore GPx1 does not confer protection in the absence of a stressor. In addition, GPx4 overexpressed cells (RBL2H3 and M15) were unable to induce the same changes observed in GPx1 overexpressed ECV304 cells, thereby indicting the unique role of GPx1 in mitigating anti-apoptotic signaling [133,135].

Of interest to this project is the effect of GPx1 in the heart. One study found that GPx1 overexpression in diabetic mouse hearts inhibited cardiac remodeling and diastolic dysfunction as a result of cardiac stiffening [136]. Diabetic hearts developed specific cardiomyopathy in the absence of hypertension or other factors [136,137]. Oxidative stress may lead to myocardial

remodeling such as myocyte hypertrophy, apoptosis and fibrosis, all of which impair cardiac function [136]. Furthermore, it has been shown that GPx1$^{-/-}$ mouse hearts experienced more apoptosis, measured by DNA strand breaks, compared to WT hearts following ischemia/reperfusion injury [138]. Similarly, another study measuring apoptosis in GPx1$^{-/-}$ mouse brain samples following ischemia had larger infarct sizes compared to the control mice [139]. There was increased apoptosis in the GPx1$^{-/-}$ mouse brains as well [139]. Additionally, GPx1 overexpressed transgenic mice had reduced tissue damage following ischemia/reperfusion [140,141]. Another study showed that following myocardial infarction, induced via ligation of the left coronary artery, GPx1 overexpressed transgenic mice had reduced cardiac dysfunction, myocyte hypertrophy, and apoptosis [142]. As GPx1 likely conferred extra protection against ROS, we wanted to assess the effects of dietary FA exposure and cell death in cardiomyocytes.

1.6 Hypothesis and Objectives

In concert with the one of the main theories regarding excess dietary n-6 FA consumption, I hypothesize that:

Glutathione peroxidase 1 (GPx1), an important mitochondrial antioxidant enzyme protects the heart following a high fat diet by reducing the accumulation of ROS.

To test this hypothesis, I propose two objectives:

First Objective	Investigate the effects of high fat diets in GPx1$^{+/-}$ mouse hearts.
Second Objective	Investigate the interactions between GPx1 and FAs on cardiomyocytes, one of the main heart cell types, in causing cell death.

Within Chapter 2 of this thesis, the *in vivo* work is described and focuses on the first objective. Chapter 3 of the thesis focuses on the second objective, utilizing *in vitro* techniques.

Chapter 2: GPx1 heterozygous knock out does not further aggravate LA induced cardiotoxicity in mice

2.1 Overview

Over the last century, the composition of diet has changed in Western countries leading to an increased ratio of n6:n3 PUFA consumption[8,143]. Additionally, there has been an increase in chronic diseases in Western countries [57]. It is now suspected that these two events may be linked[8,143]. Furthermore, while GPx1 plays an important role in protecting the heart, it has not been investigated under the high n-6 Western diet [113,114]. In previous research it was shown that CHD patients that had decreased GPx1 activity level in their erythrocytes were more likely to incur a negative cardiovascular event [115]. This observation was not associated with other antioxidant enzymes such as catalase and superoxide dismutase[115]. Therefore, this paper established the important of GPx1 in the cardiovascular system[115].

We obtained GPx1 KO mice, which contains only one functional allele [102] and is considered to be a heterozygous KO and is depicted by $GPx1^{+/-}$. A heterozygous mouse is able to express the gene; however, in reduced amounts compared to a WT [102]. Alternatively, both alleles of the gene can be knocked out and therefore considered a full KO (or called homozygous KO, depicted by $GPx1^{-/-}$) [102], which we did not use. Therefore, the objective of this chapter is to assess cardiovascular changes following the stress of a HFD diet in a heterozygous GPx1 murine model.

In this chapter, we show that there were no changes in lipid peroxidation across the diets in $GPx1^{+/-}$ hearts or alterations in heart functional parameters in $GPx1^{+/-}$ or WT hearts. To determine if other antioxidant systems were present in $GPx1^{+/-}$ hearts, qPCR revealed an increased SOD1 and SOD2 gene expression. Furthermore, in the presence of LA, there was decreased GPx1 expression in the $GPx1^{+/-}$ hearts. Lastly, there were alterations of antioxidant gene expression (catalase, SOD1, SOD2, and GPx4). It was postulated that perhaps the number of mitochondria could have been decreased from the reduction of GPx1. Indeed, there was decreased ND5 gene expression in the $GPx1^{+/-}$ hearts compared to the WT hearts, indicating a decrease in mitochondrial copy number. ND5 gene expression is a commonly used method to determine

mitochondrial copy number as it encodes a subunit of the NADH dehydrogenase complex [144]. This data indicates that although GPx1 KO mice had cardiac morphological abnormalities, n-6 PUFA rich diets and other factors such as duration and age of mice, were not able to cause detectable alterations detected *in vivo*.

2.2 Background

As previously mentioned, sizable changes in diet composition have occurred over the past century, most notably in regards to increased n-6 PUFA consumption, thereby shifting the critical n-6:n-3 ratio[8,57,143]. One of the mechanisms proposed to explain the impact of a high n-6 PUFA diet has been the involvement of oxidative stress. Oxidative stress represents an imbalance between ROS production and its amelioration by antioxidants like GPx1. A paper published in 2003 by Torzewski et al. found that coronary artery disease patients exhibiting higher activity levels of GPx1 in their red blood cells were less likely to incur a cardiovascular event [115]. However, the mechanisms remain unclear as to how GPx1 could be protective. We proposed that it is able to combat the oxidative stress induced by Western style high n-6 PUFA diet.

2.2.1 *GPx1 Transgenic Mouse Models*

Murine models are frequently used for nutrition research due to its similarity to the human and other mammalian systems. Diabetes, obesity, and other chronic diseases are thought to arise from the diet. Mouse models fed high fat are commonly used in diet studies, that which has led to several breakthroughs in our understanding of obesity, diabetes and insulin resistance [145,146]. Thus, CVDs arising from such metabolic diseases are can be investigated using mice models [6].

GPx1 KO mice develop normal phenotypically and are able to reproduce [106]. Several other antioxidant enzymes such as SOD and catalase play similar roles in removing ROS therefore removal of GPx1 does not cause overtly negative effects [106]. Following GPx1 knock out (KO), cells are rendered more at risk for oxidative damage [102,106]. GPx1 is found in all cells and therefore plays an important role in maintaining redox balance [102].

Complete GPx isoform knockouts do not cause lethality with the exception of GPx4 [110]. GPx4 directly reduces lipid hydroperoxides and maintains membrane integrity [147]. Additionally, GPx4 overexpression experiments have shown its importance in several pathways and may retain mitochondrial function by reducing intracellular hydroperoxides [95].

While a complete KO of a particular GPx is relevant for understanding the role of the enzyme, it is not necessarily biologically relevant. Instead, single nucleotide polymorphisms are typically utilized because it is more likely that an individual has decreased expression and not typically a complete ablation [102]. Of the 38 single nucleotide polymorphisms (SNPs), two are typically studied that impact protein coding [102]. One polymorphism inserts 5-7 alanines near the N-terminus while the second SNP has leucine replacing proline at position 198 [102]. One study looked at a polymorphism of GPx1 designated rs1050450 and found that it increased cardiovascular disease risk in an East Asian population [116]. The GPx1 polymorphisms are Pro198Leu and Pro197Leu [116]. The Leu variant has been predicted to reduce GPx1 expression by 40% [116]. I propose that a transgenic mouse model, in addition to *in vitro* techniques, could be a sufficient to model the effects of high fat feeding.

2.2 Materials and Methods

2.2.1 *Rodent Model*

Transgenic mice were purchased from Knockout Mouse Project (KOMP) at the University of California Davis, USA and bred in the In Vivo animal care facility at the University of British Columbia Okanagan, Kelowna, Canada. Male and female mice were weaned at 3 weeks and placed on their experimental diets by 2-3 months of age. A powdered rodent diet (Bio Serv, AIN-76A) was mixed with various dietary oils. The ingredients in the powder diet are as follows: sucrose, casein, corn starch, corn oil, cellulose, mineral mix, vitamin mix, DL-methionine, and choline bitartrate. The food was prepared weekly and stored at 4°C. New food portions were given daily to reduce oxidation of food and each mouse received 5g of food per day. The final diet (basal plus oil) contained 13.89 kJ/g of energy. The % energy by weight and by energy are shown below in Table 3.

Table 3. *Percent composition of major macronutrients in mouse diets.*

	% by weight	% by energy
Carbohydrate	44.7	40.1
Protein	21.2	19.0
Fat	25	50
ENERGY DENSITY /g of finished diet: 13.89 kJ/g		

Mouse mass was recorded every 2-3 days. After 6 weeks of feeding, the cardiac parameters were recorded for each mouse by using echocardiogram (detailed in section 2.2.6). After 6 weeks of feeding, the mice were euthanized, and tissues of interest were collected and flash frozen in liquid nitrogen before storage in -80°C. Half of the frozen heart was used for RNA extraction and the remaining half was utilized for DE-TBARS (section 2.2.5) and gas chromatography (section 2.2.4).

2.2.2 *Total RNA Extraction and qPCR*

Total RNA was extracted from frozen left-ventricle heart sections using the Fibrous Tissue RNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad Laboratories Inc, Hercules, CA, USA). Integrated DNA Technologies (IDT; Coralville, Iowa) synthesized all desired mRNA primers (Table 4). Quantitative polymerase chain reactions (qPCR) were performed using a Bio-Rad CFX Manager 2.0 machine. The reagent for gene amplification used was Sso Fast Eva Green Supermix (Bio-Rad Laboratories Inc, Hercules, CA, USA). Duplicates of each sample were done, and the final volume of each reaction was 10ul. Polymerase chain reactions (PCR) were performed under cycling conditions of 95°C for 30s then 50 cycles of 95°C for 5s, 58°C for 5s and 95°C for 10s. Then a melt curve analysis from 65°C-95°C in 0.5°C increments for 5s was performed. CFX Manager Software version 3.0.1224.1015 was used to quantify gene expression using 18s rRNA as a reference gene for all reactions except for ND5, that which used beta-actin. Primer efficiencies were obtained using LinREG software and are shown below in Table 4. Relative normalized gene expression was quantified via the ΔΔCt method [148].

Table 4. *Primer efficiencies and sequences for primers used.*

Gene	Primer Sequence (5'→3')	Primer Efficiency
GPx1	F: GTTCGGACACCAGGAGAA R: GCCTTCTCACCATTCACTTC	1.93
GPx4	F: GGCAGGAGCCAGGAAGTAAT R: GTGGGCATCGTCCCC	1.92
SOD1	F: GGCTTCTCGTCTTGCTCTCTC R: TTCACCGCTTGCCTTCTG	1.97
SOD2	F: GGCCAAGGGAGATGTTAC R: TGATAGCCTCCAGCAACTC	1.90
Catalase	F: AGAAGCCTAAGAACGCAATTC R: ATCCATCCAGCGTTGATTAC	1.91
ND5	F: ACAGCTATTTGTGCCCTCACCCAA R: GCATGGCTTTGAAGAATGCGTGGG	1.96
18s-rRNA	F: CGGCTACCACATCCAAGGAA R: GCTGGAATTACCGCGGCT	1.97
Beta-actin	F: GCCAACACAGTGCTGTCTG R: TACTCCTGCTTGCTGATCCA	1.91

2.2.3 *Protein Extraction*

500ul of radio-immunoprecipitation assay buffer (RIPA buffer) (containing 1:100 protease inhibitor, see formula below in Table 5) was added to a small section of tissue around 20-30mg in mass. Ingredients found in RIPA buffer are listed below in Table 5. A homogenizer bead was added to the tube and the tissue subsequently underwent homogenization at 30hz for 2 min, followed by a 1 min rest and then a repeat of 30hz for 2 min using a Retsch Mixer Mill M400 homogenizer (Retsch, Germany). Next, the homogenate was centrifuged at 4,000rpm for 5 min at 4 °C. The supernatant was removed and added into a new tube. The isolated supernatant was stored in -20 °C.

Table 5. *RIPA Buffer Recipe used for protein extractions, pH 8.5.*

Component	Amount
Sodium chloride	17.5g
Triton X-100	2mL
SDS (0.1%)	0.2g
Sodium deoxycholate (0.5%)	1g
Tris-Base (50mM)	1.2g

2.2.4 *Gas Chromatography*

To determine the relative proportions of various FA in the cardiac tissue, thereby establishing that the FA specific diets had accumulated in the tissue of interest, was done using a combined FA extraction and methylation protocol [149]. Methylation produces FA methyl esters from glycerolipids and sterol esters [150]. Boron trifluoride was used as an acid catalysis for esterification [150]. Next, 30mg of tissue was added to 1.2ml hexane and 1.2ml boron trifluoride-methanol solution. Samples were then heated on a hot plate to between 90- 100°C for 1 hour. Next, 1ml of water was added to the samples, and the sample was centrifuged for 1 min. The top aqueous layer, containing the extracted and methylated FAs, was removed and placed into a glass vial for gas chromatography analysis. FA methyl esters were analyzed on a Trace 1300 Gas Chromatograph with a flame ionization detector (FID) using a Supelco Famewax column (30m x 0.32 mmID x 0.5 microns). Peaks were identified using a 37-peak standard (Sigma Aldrich, St. Louis, MO, USA). The area under the curve for each identified peak was calculated and then the % area (% FA) was determined for each FA in that sample. Software used for analysis was Chromeoleon7™ Chromatography Data System Software (ThermoFisher Scientific, Waltham, MA, USA).

2.2.5 *DE-TBARS*

Lipid oxidative damage can be obtained using diethyl thiobarbituric acid (DE-TBARS) reactive products, a common lipid peroxidation assay for detecting the presence of TBARS products [151]. Following protein extraction with RIPA buffer (as above mentioned), 80ul of RIPA homogenate was added to 50ul of 50% glacial acetic acid in a 1.5mL tube. The control contained RIPA and

glacial acetic acid only, without the tissue homogenate. Next, the solutions were placed on a shaker for 30 minutes to precipitate the proteins. Once precipitated, the tubes were centrifuged at 3,000rpm for 2 mins to pellet the precipitate. The supernatant was next removed and added to an equal volume of 4.0mM DE-TBA solution. Following a short vortex, the tubes were heated to 95°C for 45 minutes. Once cooled to room temperature, the solutions were pipetted in duplicates into a sterile, clear 96-well plate. Fluorescence was recorded at 490em/510-570 by GloMax plate reader.

2.2.6 *Echocardiogram*

A HP/Philips Sonos5500 ultrasound system (Hewlett Packard Co., NL) was used to measure various heart parameters in mice by utilization of non-invasive transthoracic echocardiography [152]. Mice were initially anesthetized with 2% isoflurane and maintained with 1% isoflurane on a warming platform to maintain a constant body temperature at 37°C. A 15-L transducer (7-15MHz, Hewlett Packard Co.) was used for M-mode and Pulsed-Wave Doppler to investigate heart and aortic valve function, correspondingly.

2.2.6.1 *M-Mode*

The probe was placed in a position to observe the mitral valve to acquire images with multiple readings taken to ensure accuracy. Long-axis scans and 2-chamber view were utilized. Images were then transformed from the machine to the computer for further analysis. Digimizer Image Analysis Software was used to take multiple readings from the stored images 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). Fractional shortening (Equation 1) and ejection fraction (Equation 2) were calculated by using measurements obtained in other published work [153], and together are able to provide left ventricle systolic function. Equation 3 provided left ventricle mass, that which was also obtained from another published article[153]. Stroke volume (Equation 4) was calculated by using M-MODE and utilized the equation from Qi et al. (2017). The following Equations 5-6 were calculated for use in Equation 4[154]

$$Fractional\ Shortening\ (FS) = \left(\frac{\text{LVIDd} - \text{LVIDs}}{\text{LVIDd}}\right) \times\ 100 \quad (1)$$

LVIDd = diastolic left ventricle internal dimension

LVIDs = systolic left ventricle internal dimension

$$Ejection\ Fraction\ (EF) = \left(\frac{(\text{LVIDd})^3 - (\text{LVIDs})^3}{(\text{LVIDd})^3}\right) \times\ 100 \quad (2)$$

$$Left\ Ventricle\ Mass = 1.055[(IVSTd + LVDd + PWT)^3 - (LVDd)^3] \quad (3)$$

1.055 = specific gravity of the myocardium (muscular heart tissue)

IVSTd = diastolic interventricular septum thickness

LVIDd = diastolic left ventricle dimension

PWT = diastolic posterior wall thickness

$$Stroke\ Volume\ by\ M-MODE\ (SV) = EDV - ESV \quad (4)$$

$$End - diastolic\ volume\ (EDV) = \left(\frac{7}{(2.4 + \text{LVIDd})}\right) \times (\text{LVIDd}^3) \quad (5)$$

$$\text{End} - \text{systolic volume}\ \ (ESV) = \left(\frac{7}{(2.4 + \text{LVIDs})}\right) \times (\text{LVIDs}^3) \quad (6)$$

2.2.6.2 *Pulsed-Wave Doppler Mode*

After acquisition of the images, calculations were performed using the left ventricular outflow tract (LVOT) package analysis. Within the LVOT package, left ventricular outflow trace velocity time integral (LVOT VTI) was selected and the trace tool was used to manually trace the areas of interest within the cardiac cycle (Figure 3). The length of the trace-line provides 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

measure the diameter of the aorta, LVOT DIST (LVOT distance) was selected under the LVOT package. By using the caliper tool, aorta diameter was obtained. LVOT, heart rate, systolic blood pressure and diastolic blood pressure were measured using the trace function (dotted line shown in Figure 3). Once the heart rate measurement was completed, cardiac output (Equation 7) and mean arterial pressure (MAP, Equation 8) were calculated. The heart rate (HR) were measured by the echocardiography machine utilizing a caliper tool in M-Mode. Cardiac output was calculated using Equation 7, that which was obtained from J Zhang, Critchley, & Huang (2015). Cardiac output is the amount of blood pumped in one minute [30]. Finally, Equation 8 was obtained from Razminia et al., (2004) and was used to calculate mean arterial pressure (MAP), that which provides the pressure of the vessel in one cardiac cycle.

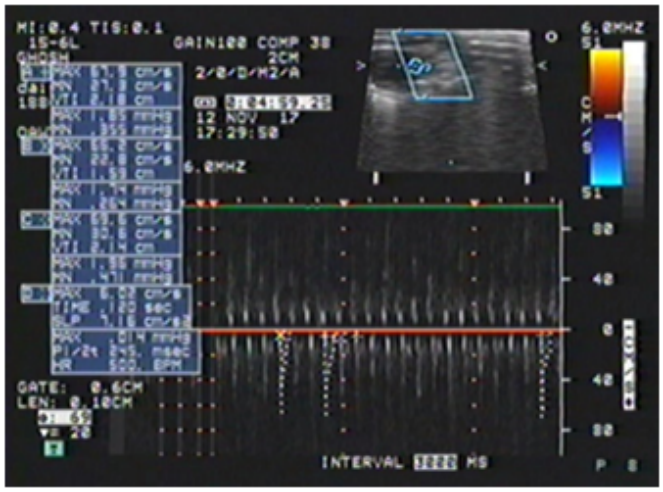


Figure 3. *Echocardiogram reading used for visualizing several recorded measurements, that which were later used in several calculations.*

$$Cardiac\ Output = Heart\ Rate\ \times\ SV \tag{7}$$

$$Mean\ Arterial\ Pressure\ (MAP) = \left(\left(\frac{2}{3}\right) \times diastolic\right) + \left(\left(\frac{1}{3}\right) \times systolic\right) \tag{8}$$

2.2.7 *Statistical Analyses*

Graphical results are visualized in box plot formats, that which easily identify the interquartile range, median, and outliers, if any. For Chapter 2, either a one-way analysis of variance (ANOVA), a two-way ANOVA, or an un-paired T-test was utilized. In the case of non-normal data, a non-parametric test was utilized, in this case a Kruskal Wallis one-way analysis of variance.

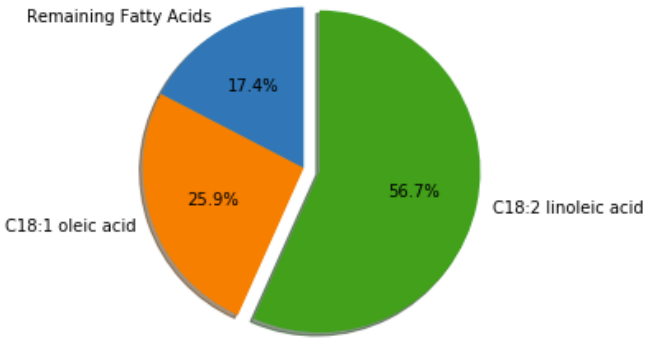
If significance was calculated following a two-way ANOVA, a Tukey's multiple comparisons post hoc test was used. Statistical significance was set at $P < 0.05$. GraphPad Prism 7.0 was utilized for all statistical analyses and graphical representation (GraphPad Software Inc., San Diego, CA, USA), All groups have their respective n values displayed, minimum n=3, except for LDH assay in chapter 2. In Table 6, displaying GC results, standard deviation was displayed, which was calculated using Microsoft Excel.

2.3 Results

2.3.1 *Mice Fed Specific Diets Successfully Demonstrated Differential Fat Accumulation*

To determine the proportions of FAs present in the diet, gas chromatographic analysis was conducted on the rodent diets. The rodent diet was a mixture of a powdered basal mix and a dietary vegetable oil of interest. As expected, the corn oil diet, that which is rich in linoleic acid, contained more LA than the olive oil diet (Figure 4). In contrast, the olive oil diet contained more oleic acid, that which is expected as it is known that this oil contains more oleic acid, as shown in Figure 4 [35]. The gas chromatograph results for linoleic acid and oleic acid are displayed respectively in Figure 4 below.

A



B

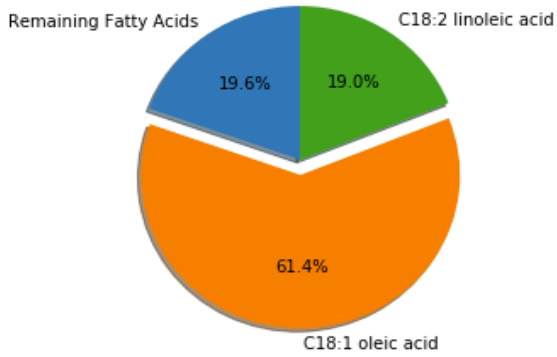


Figure 4. *Relative proportions of FAs present in the corn oil and olive oil diets*.
Gas chromatography result depicting the relative percentages of the FA types of interest within the experimental diet. Figure 4.A depicting the corn oil diet and Figure 4.B depicts the olive oil diet.

After the diets were verified to contain proportions of interest of specific FAs, the feeding began. To ensure that the mice were consuming their experimental diets, their individual body weight were recorded every 3-4 days for the duration of the experiment. At the end of the study, the final body weight was subtracted from initial body weight to determine the changes in weight occurring in the study, that which is represented in Figure 5 across the three diet types. We were able to show that the GPx1$^{+/-}$ mice consuming a basal diet supplemented vegetable oil gained more mass compared to the basal diet alone and there was no significant difference in body weight between OO and CO fed mice.

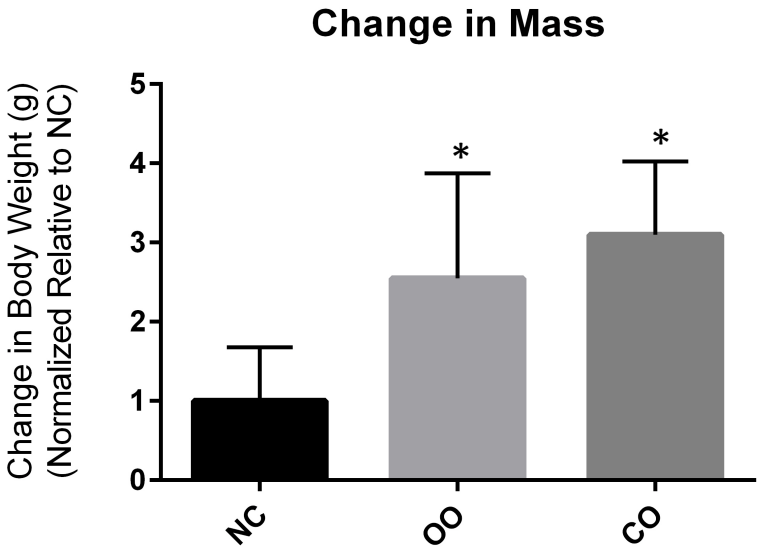


Figure 5. *Change in body mass across the normal control (NC), olive oil (OO), and corn oil (CO) diets.*
Change in body mass was determined by taking the final mass recorded and subtracting from the initial mass (n=6). Data was analyzed using one-way ANOVA (NC-OO $P<0.05$, NC-CO $P<0.005$) and visualized as mean +- standard error of the mean (SEM). Abbreviations: NC, normal control; OO, olive oil; and CO, corn oil.

Gas chromatographic analysis confirmed that the olive oil mice hearts fed specific diets contained more of the specific FAs of interest. The results from the heart tissue FA analysis is displayed in Table 5. A one-way ANOVA was used to determine if there were any significant differences in OA or LA accumulation within the $GPx1^{+/-}$ mouse hearts and it was found only that the olive oil diet contained a statistically significant amount of OA. In the corn oil diet, the amount of LA was not statistically significant.

Table 6. *Relative proportions of certain FAs of interest within heart tissue, obtained by gas chromatography (n=5). One-way ANOVA was used to calculate significance for the corn oil diet containing LA (P=0.0512) and for the olive oil diet containing OA (*P=0.0018). Abbreviations: AVG, average; SD, standard deviation.*

	Normal Control	Olive Oil	Corn Oil
Fatty Acid		**AVG (%) ± SD**	
Palmitic (C16:0)	20.72 ± 1.76	17.52 ± 0.76	20.39 ± 2.37
Stearic (C18:0)	21.17 ± 2.80	20.30 ± 3.14	23.87 ± 1.70
Oleic (C18:1)	15.54 ± 2.27	25.76* ± 2.59	15.41 ± 4.23
Linoleic (C18:2)	25.17 ± 3.41	19.12 ± 4.18	26.11 ± 5.69
Gamma-Linolenic (C18:3n6)	0.22 ± 0.20	1.52 ± 2.88	0.23 ± 0.23
Alpha Linolenic (C18:3n3)	0.14 ± 0.10	0.10 ± 0.04	0.17 ± 0.15
Arachidonic (C20:4n6)	0.02 ± 0.03	0.03 ± 0.02	0.03 ± 0.02
EPA (C20:5)	0.07 ± 0.04	0.05 ± 0.02	0.13 ± 0.11
DHA & Nervonic Acid (C22:6+C24:1)	0.09 ± 0.09	0.01 ± 0.01	0.02 ± 0.01

Next, qPCR was conducted on the heart tissue to determine if GPx1 gene expression was reduced (Figure 6). First, the $GPx1^{+/-}$ mice were compared to $GPx1^{+/+}$ mice to see if a heterozygous KO of GPx1 is able to cause a decrease in GPx1 antioxidant expression (Figure 6). As expected, the transgenic mice had decreased heart GPx1 expression compared to the WT mice (Figure 6). Additionally, the presence of LA in a GPx1 reduced heart resulted in an even further decrease of GPx1 expression when compared to the wildtype (WT) mouse.

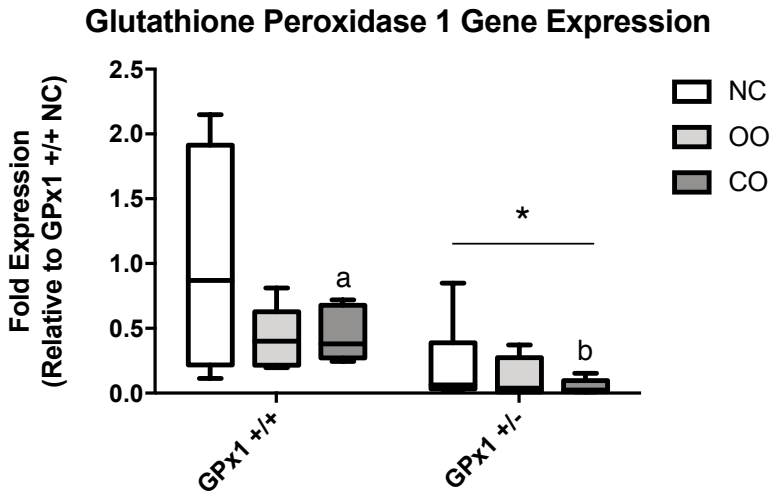


Figure 6. *GPx1$^{+/-}$ mice express significantly less GPx1 in heart tissue.*
qPCR on the GPx1 gene showing GPx1$^{+/-}$ mouse hearts had decreased gene expression. Additionally, in the GPx1$^{+/-}$ mice fed a CO diet, there was a reduction in GPx1 gene expression levels when compared to WT mouse hearts (GPx1 gene factor P<0.0001, GPx1$^{+/-}$ CO-GPx1$^{+/+}$ CO P=0.0088). In comparison to the WT CO group, the GPx1$^{+/-}$ CO group had reduced GPx1 gene expression (P=0.0088). Two-way ANOVA used to determine significance (GPx1$^{+/+}$ n=5, GPx1$^{+/-}$ n=6). Abbreviations: NC, normal control; OO, olive oil; CO, corn oil.

2.3.2 *Dietary differences did not induce lipid peroxidation changes in* GPx1$^{+/-}$ *mouse hearts*

A DE-TBARS assay was used to determine if lipid peroxidation was occurring across the different diet types. This commonly used assay is able to detect lipid peroxidation products and is considered as a biomarker of lipid peroxidation [157]. Only using GPx1$^{+/-}$ heart tissue, we investigated if the diet was able to induce detectable changes in lipid peroxidation due to the presence of more unsaturated FAs while looking at the x-axis moving outwards in Figure 7. There were no statistically significant differences between any of the groups compared.

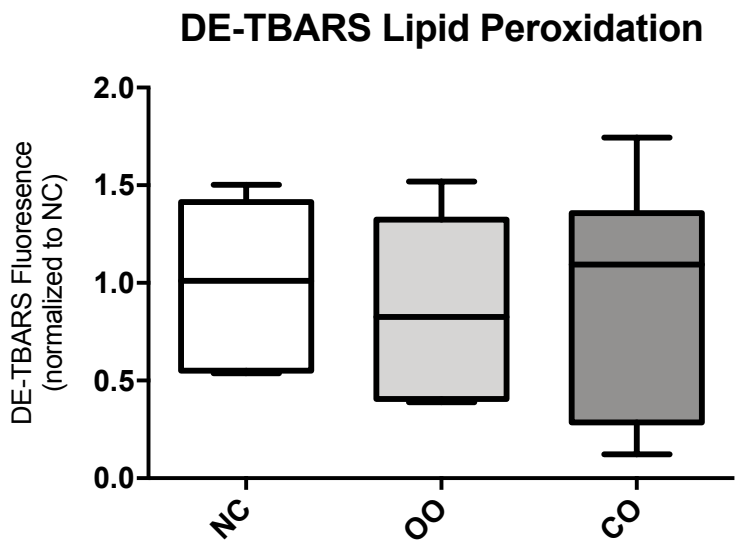


Figure 7. *Lipid peroxidation differences across diet types in GPx1$^{+/-}$ cardiac tissue.* DE-TBARS was utilizes to asses lipid peroxidation in GPx1$^{+/-}$ cardiac tissue (n=6). One-way ANOVA was used to determine significance (P = 0.9033). Abbreviations: NC, normal control; OO, olive oil; and CO, corn oil.

2.3.3 *Antioxidant Gene Expression Alterations and Mitochondrial Copy Number*

The heart tissue of both the WT and GPx1$^{+/-}$ mice was investigated to determine if other antioxidant enzymes would be compensating for the lack of GPx1 in GPx1$^{+/-}$ hearts across diet groups. We demonstrated that GPx4, catalase, SOD1 and SOD2 gene expression varied between the GPx1$^{+/-}$ mouse hearts compared to the WT, that which is shown in Figure 8. There were no statistically significant differences among diet groups with the exception of in the GPx1$^{+/-}$ mouse hearts treated with olive oil, there was increased SOD1 gene expression.

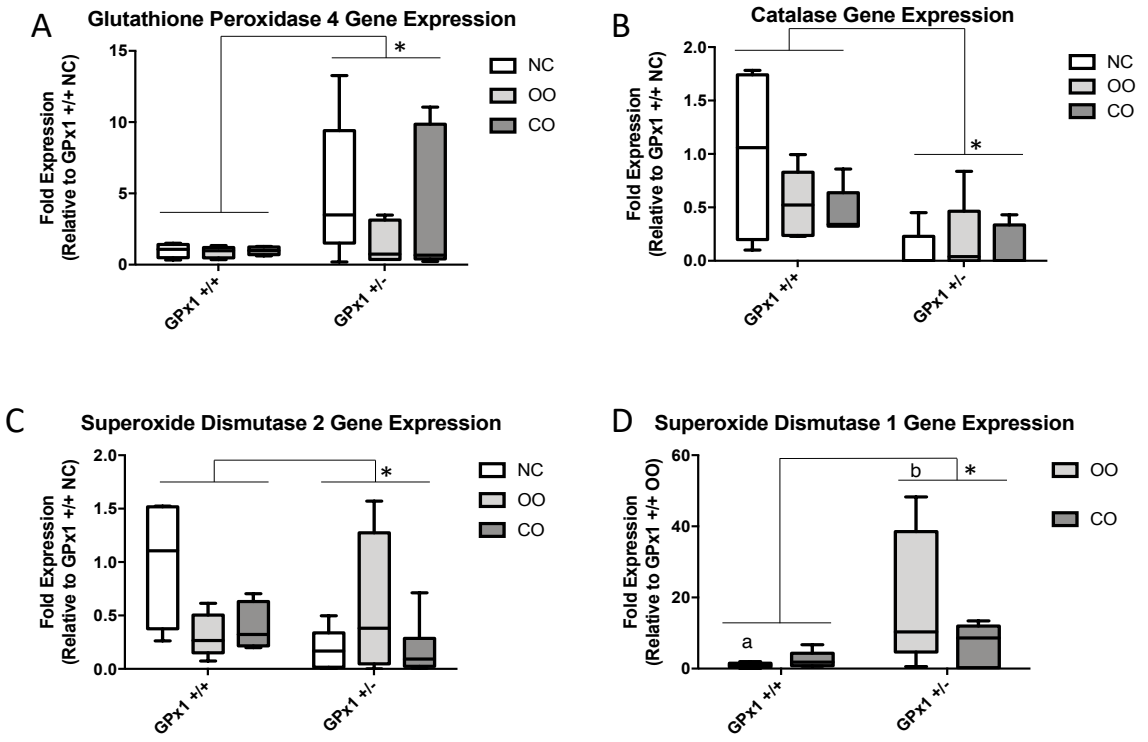


Figure 8. GPx1$^{+/-}$ *mouse hearts have differing expression of antioxidant genes*. qPCR gene expression on WT and GPx1$^{+/-}$ mouse hearts for A) glutathione peroxidase 4 (GPx1 factor P= 0.0404; diet factor P=0.4508; interaction P=0.5037); B) catalase (GPx1 factor P= 0.0092; diet factor P=0.4096; interaction P=0.2557); C) superoxide dismutase 2 (GPx1 factor P= 0.0326; diet factor P=0.5582; interaction P=0.6726); or D) superoxide dismutase 1 (GPx1 factor P= 0.0339; diet factor P=0.2829; interaction P=0.1761, GPx1$^{+/+}$ OO-GPx1$^{+/-}$ OO P =0.0279) (GPx1$^{+/+}$ n=5, GPx1$^{+/-}$ n=6). Two-way ANOVA used for figures A, C-D. Kruskal-Wallis analysis of variance test was used for B. Abbreviations: NC, normal control; OO, olive oil; CO, corn oil.

qPCR determined that there was reduced expression of ND5 in the GPx1$^{+/-}$ mice compared to the control WT mice, as shown in Figure 9. In comparison to the WT OO group, the GPx1$^{+/-}$ OO group had reduced ND5 gene expression. ND5 encodes an essential subunit for the NADH dehydrogenase complex and it used as an indicator of mitochondrial DNA abundance [144,158].

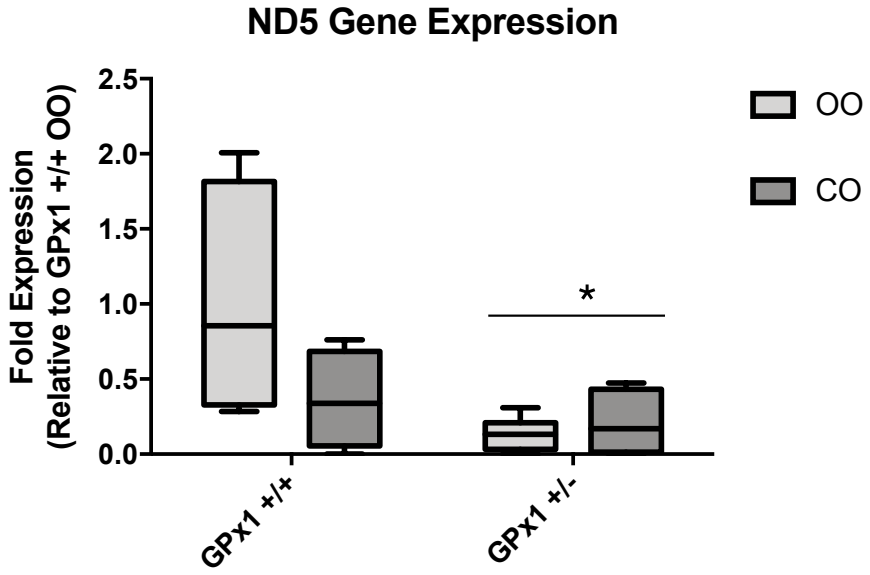


Figure 9. GPx1$^{+/-}$ *mouse heart displays lower ND5 gene expression, possibly due to decreased mitochondrial health.*
ND5 gene expression encoding an important subunit in the NADH dehydrogenase complex. Two-way ANOVA was used to determine significance (GPx1 overexpression factor P= 00115; diet factor P=0.1343; interaction P=0.0623) (GPx1$^{+/+}$ n=5, GPx1$^{+/-}$ n=6). Abbreviations: OO, olive oil; and CO, corn oil.

2.3.4 *Heart Function Measurements as Determined by Echocardiogram*

Expanding beyond molecular techniques, we demonstrated that the heterozygous GPx1 KO was unable to cause detectable heart function changes. By using echocardiogram, various heart parameters were measured and compared. Fractional shortening (Figure 10.A), ejection fraction (Figure 10.B) and stroke volume (Figure 10.D) did not have any statistically significant differences. Left ventricle mass, as shown in Figure 10.C, had increased LV mass in the NC fed GPx1$^{+/-}$ mice compared to the GPx1$^{+/-+}$ mice. Additionally, the GPx1$^{+/-}$ LV mass was decreased following CO diet. Two-way ANOVA determined that the GPx1 overexpression factor and diet factor interaction was of significance as well.

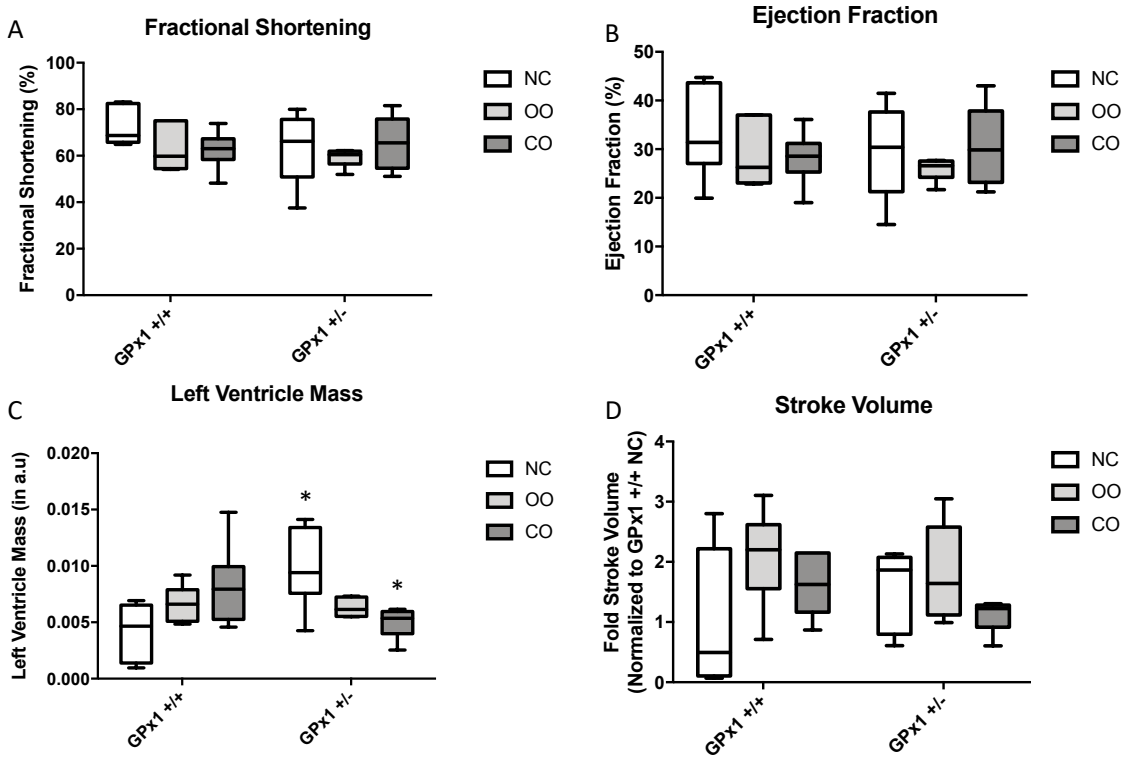


Figure 10. GPx1$^{+/-}$ *mouse left ventricle mass was increased, however; decreased in the presence of corn oil.*
Echocardiogram measurements utilizing M-Mode, from section 2.2.6 and 2.2.6.1. Calculations used were from Equations 1-6 in section 2.2.6.1. Figures as follows: A) Fractional shortening; Figure 10.B, Ejection Fraction; C) Left Ventricle Mass (Interaction between GPx1 overexpression factor and diet factor P=0.005, GPx1$^{+/-}$ NC = P=0.0054, GPx1$^{+/-}$ CO P=0.0215); D) Stroke Volume. Two-way ANOVA was used to determine significance (GPx1$^{+/+}$ n=5, GPx1$^{+/-}$ n=6). Abbreviations: NC, normal control; OO, olive oil; and CO, corn oil.

After measuring various heart parameters, cardiac output and mean arterial pressure were calculated. First, cardiac output, shown in Figure 11.A., did not have any differences. MAP, shown in Figure 11.B also did not have any differences.

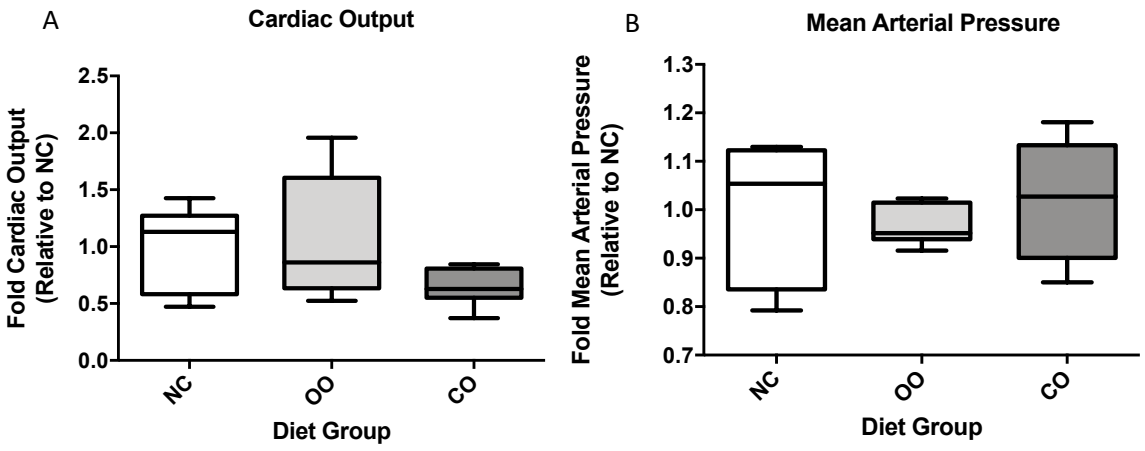


Figure 11. *No change in cardiovascular function across diets in* GPx1$^{+/-}$ *mice hearts*. Doppler mode was utilized to obtain measurements, that which were utilized in calculations using Equations 7 and 8 from section 2.2.6.2. One-way ANOVA was used to determine significance (n=6): cardiac output, P=0.184; mean arterial pressure, P=0.715. Abbreviations: NC, normal control; OO, olive oil; and CO, corn oil.

2.4 Discussion

2.4.1 *Mice Fed Specific Diets Successfully Demonstrated Differential FA Accumulation*

An experiment based upon the effects of diet relies heavily on consumption of the variables of interest and the appropriate delivery of the specific variable, that which have both led to improvements in animal diets in nutrition studies[159]. However, even with such advancements, diet studies are typically not consistent in reporting specific details among studies and typically contain varying amounts of micro- and macronutrient values[160]. A characteristic of unsaturated FAs, that which are found in certain dietary oils, is that they are able to oxidize over time or under improper storage conditions [161]. Oxidation of oils is well established to alter the chemical composition of FAs present and thereby negatively affect one's results[161]. Furthermore, commonly consumed dietary oils can differ in amounts of FAs present[35]. To address these issues, diets were replaced daily to prevent oxidation. Additionally, gas chromatography verified that the diets contained the specific FAs of interest. Weighing the mice ensured proper feeding of mice and was further verified by gas chromatographic analysis on heart tissues, which showed accumulation of the specific FAs present in the diet (Table 6). It was found that when comparing the amounts of LA or OA in the three diet treatments, only the olive oil diet contained a higher

amount of OA compared to the other groups. This was that the corn oil diet did not have a significantly different percent amount of LA as the basal diet does contain corn oil, albeit at lower amounts.

In our own experience 6 weeks of dietary intervention has been sufficient to induce lipid changes in the heart in mice earlier as well. changes while being [117,119]. After considering the length of the dietary intervention, it is important to note the changes in mass shown in Figure 5. The high fat diet fed mice gained more mass compared to the normal control fed mice. The high fat diets contained 25% W/W fat from energy coming from fat and therefore are more calorically dense in comparison to the normal control diet. The normal control diet contained only 5% W/W fat. Energy from fat is 9 calories/g of fat compared to that of protein or carbohydrate, that which are 4 calories/g.

2.4.2 *Dietary Differences Did Not Induce Lipid Peroxidation Changes in* **GPx1$^{+/-}$** *Mouse Hearts*

To assess lipid peroxidation differences driven by diet, a DE-TBARS assay was conducted to indirectly detect thiobarbituric acid reactive substances by spectroscopy. There was no statistically significant change in lipid peroxidation detected by DE-TBARS, as depicted in Figure 7. It is possible that the dietary intervention was too short in duration or the antioxidant system was sufficient to prevent lipid oxidation. Additionally, there is evidence that suggests that aged systems may be more at risk for lipid peroxidation due to decreased antioxidant effects, therefore the younger mice used in this study could have increased antioxidant protection[162].

2.4.3 *Antioxidant Gene Expression Alterations and Mitochondrial Copy Number*

qPCR is a commonly used method across various fields of research due to its relatively quick speed and sensitivity allowing quantification of gene levels [148]. The results in this thesis showed that there were alterations occurring in gene expression following the heterozygous KO of GPx1. This was shown in Figure 8, that which had significant variances due to genotype across all antioxidants tested (SOD1, SOD2, GPx4, and catalase). There are several other antioxidants to

observe in addition to these selected. Furthermore, of those that selected, only catalase has the same function to reduce H_2O_2 to water, however; catalase does not oxidize glutathione as part of the reaction[71]. Catalase has a high K_m for H_2O_2 and therefore serves a smaller role when H_2O_2 is prevalent; however, in high concentrations of H_2O_2, catalase takes over[106].

In regard to GPx1$^{-/-}$ mice, GPx4 was not upregulated at a transcriptional level in the aortic tissue following a high fat diet[163]. This study also saw a change in GPx2 but that is a predominantly kidney associated isoform[163]. The high fat diet used however contained 15% cocoa butter and 50% sucrose, and therefore isn't an accurate representation of the types of fats being commonly consumed[163]. In the absence of GPx1, it is well established to render murine models injected with the oxidative agent paraquat[164,165].

Due to heterozygous GPx1 KO not causing lethality, it has been suspected that other antioxidants are able to act in its place[106,110]. This result is not seen in the homozygous GPx4 KO as it causes lethality and demonstrates the importance of this particular antioxidant isoform[110]. It is predicted that perhaps in the absence of GPx1, that GPx4 is able to provide the same function[166]. Antioxidants, similar to several other biological systems, have biological redundancy where two or more genes are performing the same function and therefore there is no phenotype effect when there is an absence of one[167]. Biological systems are complex and therefore biological redundancy contributes to the difficulty in investigating a single antioxidant enzyme[167].

When it came to differences from dietary intervention, the alterations were less detectable, as shown in Figure 8. This could be due to a multitude of factors including dietary intervention length, the use of male and female mice, or age of the mice used. Some animal studies have feedings for several months, which thereby could lead to larger detectable changes[168]. Furthermore, some diets are able to become obesogenic within shorter ranges of times while others require a longer duration[168]. Next, diet studies typically use male mice, which has led to a gap in our knowledge of the female systems[168,169]. It is suspected that the female estrous cycle has been the factor leading to the decreased use of female mice models when studying obesity[168]. Interestingly though, female murine models are considered better when using a HFD model due to their close similarity to humans, in comparison to male murine models[168]. In this study, both

females and males were used and therefore could lead to mixed results. Lastly, the age of mice is an important consideration in studies. When trying to accurately depict an adult's life stage, it should be considered when planning an animal study as mice that are only a couple months old like in this study, are more so representative of a 20-30 year old human[170]. While there are large cost considerations, it is important to use appropriately aged mice to answer our research questions[170].

Of particular interest in this chapter was the change in expression levels of antioxidants (Figure 8). Several of the antioxidants incurred changes between the WT and GPx1$^{+/-}$ mouse hearts (Figure 9). As GPx1 has an important role within the mitochondria, it was suspected that the mitochondrial health could be decreased and therefore causing alterations in antioxidant gene expression[171]. A commonly used method to determine mitochondrial numbers or function is to measure Mitochondrial DNA (mtDNA)[172]. mtDNA copy number lacks histones and DNA repaid mechanisms present in nuclear DNA, and therefore is more at risk for incurring oxidative damage thereby altering the mtDNA[172]. Of additional interest to investigating mitochondrial copy number is the potential for mtDNA copy number to be used as a biomarker for CVD [172]. This is due to the high number of mitochondria and subsequently mitochondrial DNA present in cardiac tissue, that which is at risk of becoming oxidized and can contribute to negative pathologies such as CVD[172]. A recently published meta-analysis determined that there was a negative correlation between mtDNA copy number and CVD risk[172]. Therefore, to investigate the amount of mtDNA, qPCR was completed on mtDNA.

To detect mtDNA, primers specific to ND5 were used. ND5 is an important subunit of the NADH complex within complex I[144]. Therefore, detection of ND5 can be used to determine mtDNA copy number by qPCR[144,158]. The GPx1$^{+/-}$ hearts had reduced ND5 gene expression (Figure 9). Therefore, this result perhaps indicates there is an important role GPx1 is playing and in the reduced presence of GPx1 there is a consequential effect to mitochondrial copy number, that which is shown in Figure 9. There were no dietary differences observed between the groups.

2.4.4 *Heart Function Measurements as Determined by Echocardiogram*

Due to the importance of GPx1 in the protection of mitochondria, and the heart containing high amounts of mitochondria, it could be predicted that changes in the heart could occur and specific changes in function could be detected by echocardiogram in the $GPx1^{+/-}$ model [171]. By using equations 1-8, we were able to determine various cardiovascular parameters to assess heart function.

In Figure 10, various parameters were measured using M-Mode. Fractional shortening (Figure 10.A) is used to determine left ventricular systolic function [153]. Ejection Fraction (Figure 10.B) is described as the volume pumped from the left ventricle per heart beat [173]. Stroke volume (Figure 10.D) is the volume difference between the diastolic and systolic cycles and therefore provides the volume pumped in one cycle [174]. In all three of the previous heart parameters, there were no statistically significant differences. Figure 10.C calculated left ventricle mass and was found that the interaction between diet and genotype caused variance. The NC fed $GPx1^{+/-}$ mouse had increased LV mass when compared to the WT. Additionally, within the $GPx1^{+/-}$ group, the CO fed mouse had a decreased LV mass when compared to the NC fed mouse. Interestingly, the CO fed $GPx1^{+/-}$ heart had a smaller mass and yet had the same stroke volume as the other mice, perhaps indicating that it has increased strength, however this would require further investigation.

In order to investigate if there was a difference detectable in two commonly used cardiovascular measurements, cardiac output (Figure 11.A) and mean arterial pressure (Figure 11.B) were calculated using Doppler mode in the $GPx1^{+/-}$ mice only. There were no differences across the dietary groups. While $GPx1^{+/-}$ CO fed hearts are perhaps more efficient in that they are smaller but retain the same stroke volume, there is no improvement in cardiac output when compared to the other $GPx1^{+/-}$ hearts.

Using M-Mode to determine stroke volume has been questioned previously and is considered by some to be a poor estimator due to some assumptions when using M-Mode, such as the left ventricle contracting symmetrically[175,176]. Any misalignment with the assumptions leads to error [175]. However; M-Mode is still utilized by some [154]. It is recommended to use two-dimensional or

Doppler to obtain this measurement [175,176]. The equipment utilized in this thesis did not allow choice of method for echocardiogram measurements.

When all taken together, we know that the lipid composition varies amongst the hearts and there are some changes in antioxidant expression; however, it did not elicit a detectable functional response. This could be due to a low sample number (n= 5-6) and sex differences in the cardiovascular system. Male mice are typically used in cardiovascular studies therefore some variance could be introduced when using both sexes in an experiment such as this one[169].

2.5 Chapter Conclusion

In summary, the specific objective for this chapter was to investigate the effects of high fat diets in GPx1$^{+/-}$ mouse hearts. The overall hypothesis was that **glutathione peroxidase 1 (GPx1), an important mitochondrial antioxidant enzyme, which is able to protect the heart following a high fat diet**. To address this hypothesis, it was shown that n-6 PUFA feeding in a GPx1$^{+/-}$ model did not induce large detectable changes by qPCR, or echocardiography. We demonstrated that in GPx1$^{+/-}$ mouse hearts, there were alterations in other antioxidant (SOD1, SOD2, Catalase, GPx4) gene expression, perhaps this result could be due to low mitochondrial health, that which was investigated by ND5 gene expression. In summary, this chapter showed that the heterozygous KO of GPx1 was able to induce minor changes on a cellular level but not detectable *in vivo* even following a HFD.

Chapter 3: Investigating the Effects of Dietary FAs in GPx1 Overexpressed H9c2 Cardiomyocytes

3.1 *Overview*

Over the last century, the composition of diet has changed in Western countries leading to an increased ratio of n6:n3 PUFA consumption, that which has been suggested to contribute to the prevalence of chronic disease [8,57,143]^{8,57,143}. Furthermore, while GPx1 has been established previously to play an important role in protecting the heart, it has not been investigated under the high n-6 Western diet[113,114]. As cardiomyocytes make up the majority of cells within the heart and provide contractility, it was shown in various studies that dietary FAs could lead to changes at the cellular levels[177,178]. An accumulation of oxidative damage from the type and amount of FAs in these cells could eventually lead to death and loss of contractility, which would eventually translate to cardiomyopathy[121]. Therefore, the objective of this chapter is to assess cellular death following the dietary FA exposure in GPx1 overexpressed cardiomyocyte cells (H9c2). The idea behind these experiments was to assess the role of GPx1 in cardiomyocyte health under various fat diets.

In this chapter we show that n-6 PUFA feeding in a GPx1 overexpressed H9c2 cells had increased neutral red uptake, possibly indicating a positive outcome on cellular health. The neutral red uptake assay estimates the number of viable cells by measuring the amount of dye trapped within the lysosomes[179]. The dye becomes charged once entering the lysosome, due to the lowered pH gradient within the organelle, and therefore becomes trapped[179]. The dye is released upon cell death or pH gradient disruption[179]. To determine if there were any differences in cellular death following dietary FA exposure, we measured caspase 3/7 activity and LDH to provide estimations of apoptosis and necrosis respectively. We demonstrated that GPx1 overexpression increased caspase 3/7 activity while LA in both the control and GPx1 overexpression transfected cells had decreased caspase 3/7 activity. Therefore, this result may indicate that H9c2 cardiomyocytes that have GPx1 overexpressed may be undergoing the more controlled apoptotic cellular death pathways more commonly than the control cells. However; when the GPx1 overexpressed cells or empty vector overexpressed cells were provided the stressor LA, there was a decrease in caspase 3/7 activity, thereby creating a FA specific trend.

Interestingly, the control vector overexpressed H9c2 cells though did not have a different LDH measurement compared to GPx1 overexpressed cells, but FA exposure was able to cause a shift. To determine if GSH was contributing to the cell death trends, we measured GSH in its reduced state. We also show that in GPx1 over expressed cells that were exposed to LA, there was an increase in the amount of GSH. Antioxidant systems become upregulated following a stressor[180]. Therefore, following LA treatment, it is not surprising to observe and increase in GSH. This data indicates that n-6 PUFA rich diets and GPx1 overexpression caused differences in our apoptotic measurement, and GSH upregulation could have shifted cell death to a more controlled apoptosis as shown by our lab earlier[181].

3.2 *Background*

In vitro models are key for understanding biological processes due to its ability to investigate the microscopic cellular mechanisms occurring. *In vitro* is most suitable when modeling phenomena that which are typically simpler in nature. While there are limitations of *in vitro* experiments due to the complex nature of the biological systems being modeled, it remains a useful tool for investigations of small size[182].

3.2.1 *In Vitro Cell Lines*

There are three main cell types within the heart: cardiomyocytes, fibroblasts, and endothelial cells[183]. In regard to studies of the heart, typically the cardiomyocyte is of interest due to its role as the muscular contractor and high prevalence within the cardiac tissue[183]. In vitro studies therefore typically utilize H9c2 cells, that which are of ventricular origin from an E13 BDIX rat (*Rattus norvegicus*) heart[184]. While the cell line is undifferentiated, treatment with retinoic acid is able to produce terminally differentiated cells[184]. The usability of H9c2 has been seen recently when the cell line was compared to primary neonatal cardiomyocyte cells and there was found to be similar effects following treatment between the two lines. This therefore helps support the use of H9C2 for molecular studies investigating heart disease[185]. While H9c2 is a rat model, there is homology between humans, rats and mice[186] . Therefore, as long as awareness exists that differences may occur, it remains a useful model in addition to *in vivo Mus musculus* experimentation.

3.2.2 *GPx1 Overexpression*

Overexpression of GPx1 is typically shown to have enhanced protection against oxidative stress [101]. Mice with overexpression of GPx1 in their hearts were found to be more resistant to doxorubicin, that which causes adverse effects in mitochondria by uncoupling of electron transfer and oxidative phosphorylation [121,187]. Overexpression of GPx1 is typically protective of apoptotic stimuli that has a disruption in normal redox balance; however, it should be noted that GPx1 in tumor cells failed to protect against apoptosis when treated with chemicals that did not disrupt redox pathways [102].

3.3 *Materials and Methods*

3.3.1 *Cell Line*

H9c2 (CRL-1446) rat myoblasts were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium in Low Glucose (DMEM; Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. The H9c2 cell line was differentiated into mature cardiomyocytes over 7 days with a daily addition of 0.1 μM all- trans retinoic acid. Differentiation of this cell type is able to produce a more accurate model of the terminally differentiated cardiomyocyte cell present in mammals [184]. The in vitro experiments were conducted in a class 2, type IIA biological safety cabinet. All experiments utilized sterile clear 48- or white 96- well plates, obtained from CellStar.

3.3.1.1 *Overexpression*

Overexpression of the antioxidant enzyme GPx1 (mouse GPx1; Transomic Technologies, Huntsville, AL, USA) H9c2 was achieved using Attractene transfection reagent (Qiagen, Toronto, ON, Canada). Sequences were checked to ensure that the mouse GPx1 would also work in a rat model. Approximately 5000 cells were plated per well of a 48-well plate. After overnight incubation, the media was removed, and cells were washed with PBS to remove traces of FBS. To each well 100 μl transfection solution was added. The transfection solution was prepared using a two-step process. First 0.5 μl of Attractene (Qiagen, Toronto, ON, Canada) was added to

50 µl serum free/antibiotic free media per well to be transfected. A separate solution containing 250 ng plasmid (overexpression or PTCN empty vector) was added to 50 µl serum free/antibiotic free media per well to be transfected. The two solutions were then mixed and incubated at 37°C for 20 minutes. After addition of the transfection solution cells were incubated for a minimum of 6 hours at 37°C under 5% CO_2. Then 100 µl antibiotic free media containing 20% FBS was added to each well. Cells were then incubated for 48 hours before receiving additional treatment.

To verify successful overexpression of GPx1, mRNA was extracted from the cells using RiboZol RNA extraction reagent following manufacturer's instructions (Amresco, Solon, OH, USA). After removal of media, 150 µl of RiboZol was added to each well and then stored at -70°C until needed. For the extraction, the RiboZol cell mixture was transferred to a clean microfuge tube, following a brief thaw on ice. The sample was incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. Next, 60 µl of chloroform was added to the sample tubes, which were then shaken by hand for 30 seconds and incubated for 3 minutes at room temperature. Then tubes were then centrifuged at 12,000 rpm at 4°C for 15 minutes. To precipitate the RNA, the aqueous layer was transferred to a fresh tube and 75 µl isopropanol was added. RNA was precipitated at room temperature for 30 minutes. The samples were then centrifuged at 12,000rpm at 4°C for 15 minutes and the supernatant was discarded. Next, the pellet was washed with 1 ml 70% ethanol. Samples were centrifuged for 5 minutes at 7,500 rpm at 4°C. The supernatant was discarded, and the pellets were air dried then resuspended in nuclease free H_2O. The samples were then incubated at 65°C for 10 minutes. Synthesis of cDNA and qPCR were performed as described above in section 2.2.2.

3.3.2 *FA Treatments*

For all cell culture FA treatments, 100mM stock FAs (bovine serum albumin (BSA), OA, LA) are made for storage in -80°C. The FAs were dissolved in 70% ethanol (made from 100% anhydrous ethyl alcohol and sterile cell culture water). First, 3% FA-free, low-endotoxin BSA solution was prepared in PBS. Then the 3% BSA solution and the 100mM stock FAs solutions were heated to 37 °C (OA, LA). Afterwards, 100mM FAs stock were mixed with 3% BSA solution in a 20-fold dilution to produce 5mM FA stock solutions, that which were subsequently placed in 37°C for 3-4 hours with stirring. Finally, the 5mM FAs were sterile filtered and stored

in -80 °C. Based on previous laboratory experiments, FA concentrations above 0.1mM over 24 hours causes excess toxicity and are not suitable for cell signaling assays. Therefore, the working concentration for FAs in all cell experiments was be 0.1mM for 24 hours. The vehicle control used was 5mM BSA only.

3.3.3 *Total mRNA Extraction*

The mRNA extractions from *in vitro* experiments were conducted using RiboZol RNA extraction reagent following manufactures instructions (Amresco, Solon, OH, USA). Briefly 150 µl of RiboZol was added to each well and then placed at -70°C until needed. For the extraction the RiboZol cell mixture was transferred to a clean microfuge tube and the sample was incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. Then 60 µl of chloroform was added to the sample tubes which were then shaken by hand for 30 seconds and incubated for three minutes at room temperature. Tubes were then centrifuged at 12000 rpm at 4°C for 15 minutes. To precipitate the RNA, the aqueous layer was transferred to a fresh tube and 75 µl isopropanol was added. RNA was precipitated at room temperature for 30 minutes. The samples were then centrifuged at 12000rpm at 4°C for 15 minutes, the supernatant was discarded, and the pellet was washed with 1 ml 70% ethanol. Samples were centrifuged for 5 minutes at 7500 rpm at 4°C. The supernatant was discarded, and the pellets were air dried then resuspended in nuclease free H_2O. The samples were then incubated at 65°C for 10 minutes. Synthesis of cDNA and qPCR were performed as described in previous section 2.2.2.

Table 7. *Primer efficiencies and sequences for primers used.*

Gene	Primer Sequence (5'→3')	Primer Efficiency
GPx1	F: GTTCGGACACCAGGAGAA R: GCCTTCTCACCATTCACTTC	1.937

3.3.4 *Neutral Red Assay*

This commonly used assay is used to quantitively estimate the number of viable cells [179]. The dye is incorporated by live cells and concentrates within the lysosomes [179]. After application of an acidified alcohol solution, the absorbance is read using a spectrophotometer at 540nm. The neutral red media (regular media with 40micrograms/mL neutral red dye) was prepared a day in advance and was incubated at 37°C. Media was centrifuged for 5 min at 1,400 rpm to remove precipitated dye crystals. Next, the normal media was removed from the plate and replaced with 100uL of neutral red media per well. The plate was incubated at 37°C for 2 hours before removal. Cells were then washed with PBS before the addition of 150µL neutral red destain solution (containing 50% ethanol (96% purity), 49% deionized water and 1% glacial acetic acid). Next, the plate was agitated rapidly on a plate shaker for 10 min. The OD was read at 540nm.

Table 8. *Ingredients for solid and liquid Luria-Bertain- Miller (LB) media.*

Ingredients	Solid LB Media (pH 7.2)	Liquid LB Media (pH 7.2)
NaCl	5g	5g
Tryptone	5g	5g
Yeast Extract	2.5g	2.5g
Agar	7.5g	
Ampicillin	100ug/1mL	100ug/1mL

3.3.5 *Electroporation*

Using the electroporator at 2.0 kV, 200 ohms, and 25uF. Recovery media SOC was placed in 37°C. LB antibiotic plates were pre warmed in 37°C while cells were thawed on ice for 10 mins. Next, 1mm electroporation cuvette and microcentrifuge tubes were placed on ice. The tubes containing cells were lightly flicked to mix before removal of 50uL into a microcentrifuge tube occurred. 1-3uL of DNA solution was added to cells in microcentrifuge tube. Next, the mixture was transferred to the cool cuvette, tapped twice on the counter, and the external surface was wiped.

Immediately following electroporation, 950uL of 37°C SOC media was mixed and transferred to a 15mL falcon tube. The solution was shaken at 200rpm at 37°C for 1 hour. Next, the solution was diluted and plated before 18-hour incubation in 37°C. After 18 h, colonies collected were placed in liquid LB media for 24 hours. Once adequate growth occurred (about 24 hours) the plasmid extraction was completed using the Qiagen Plasmid Mini Easy Kit.

3.3.6 *Caspase 3/7 Activity*

This luminescent assay is used to detect apoptosis dependent on caspase 3 and 7 activity, both of which are executioner caspases, in cardiomyocytes (Caspase-Glo 3/7 Assay Systems, Promega). The assay substrate contains Z-DEVD-aminoluciferin, that which undergoes cleavage to remove Z-DEVD- in the presence of caspase 3/7. The remaining substrate is acted on by luciferase. The assay was completed following the manufacturer's instructions allowing cell lyses, caspase cleavage of substrate and luminescence by luciferase. Luminescence is proportional to the amount of caspase activity detected. Luminescence was recorded using the GloMax Multi+ detection system (Promega, Madison, WI, USA).

3.3.7 *GSH Assay*

o-Phthaladehyde (OPA) is a fluorescent probe that can be used for the detection of both oxidized (GSSG) and reduced (GSH) glutathione (Senft, Dalton, & Shertzer, 2000). Cells are first washed with PBS to remove traces of thiols present in the media. As OPA fluoresces in the presence of all thiols, two separate reactions are performed to increase specificity. 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 for detection of background thiols only. The second reaction determines GSH plus background thiols. The first reaction is subtracted from the second reaction to obtain a reading of GSH. Fluorescence was recorded at 365 nm excitation and 410/460 nm emission using the GloMax Multi+ detection system (Promega, Madison, WI, USA).

3.3.8 *Lactate Dehydrogenase Assay*

Cell death causes loss of cell membrane integrity that which releases lactate dehydrogenase (LDH) out of the cell (Ghosh et al., 2004). Media LDH was estimated using a kit (G LDH-Cytox Assay Kit, BioLegend). Manufacturer's procedural instructions were followed to complete this assay. The kit utilizes enzymatic reactions beginning with NAD+ is reduced to NADH when lactate is dehydrogenated to pyruvate by LDH. Furthermore, NADH reduces the water-soluble tetrazolium salt. This reaction results in the conversion of the tetrazolium salt into an orange color formazan. The LDH activity is determined as NADH oxidation. Formazan was measured at 490nm by GloMax Multi+ detection system (Promega, Madison, WI, USA).

3.3.9 *Resazurin Assay*

Resazurin assays are frequently used as an indication of cell viability and relies on the determination of mitochondrial redox status. During the assay, resazurin dye is reduced in the mitochondria to become the fluorescent resorufin (O'Brien, Wilson, Orton, & Pognan, 2000). Stock resazurin dye (Biotium, Fremont, CA, USA) was diluted 10x into cell media and subsequently was used to incubated H9c2 cells for 2 hours at 37°C and 5% CO_2. Fluorescence was measured at 525nm excitation and580/640 nm emission using a GloMax Multi+ detection system (Promega, Madison, WI, USA).

3.3.10 *Statistical Analyses*

For Chapter 3, either a one-way analysis of variance (ANOVA), a two-way ANOVA, or an un-paired T-test was utilized. Analyses was conducted similarly as was described in section 2.2.7. All groups have their respective n values displayed, minimum n=3, except for LDH assay in chapter 2.

3.4 Results

3.4.1 *GPx1 Overexpressed H9c2 Cells Express Significantly More GPx1 Compared to the Empty Vector Overexpressed H9c2 Cells*

To test whether the overexpression was successful, qPCR was conducted from frozen cells. The method utilized is detailed above in 3.2.3 and shown in Figure 13 below. We were able to verify that the overexpression of GPx1 in H9c2 cells was successful by comparing the two treatments groups: empty vector overexpression and GPx1 vector overexpression. By comparing to the control, that which was containing overexpression of an empty vector, it is evident that there was higher GPx1 gene expression in the GPx1 overexpressed cells, shown in Figure 12 below.

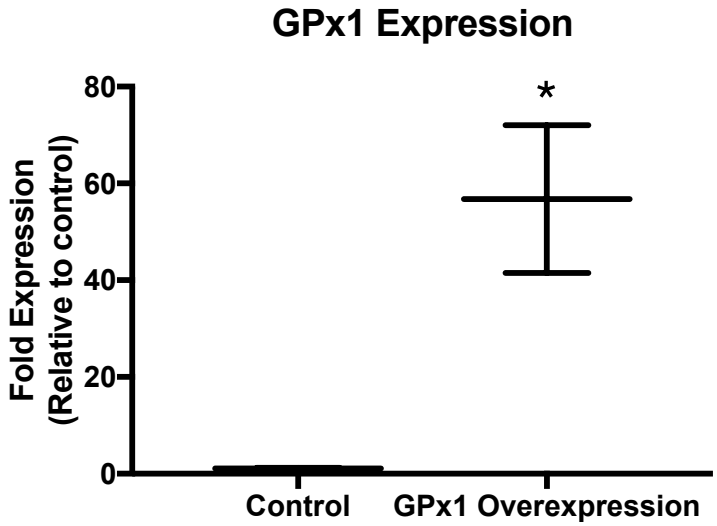


Figure 12. *GPx1 overexpressed H9c2 cells express significantly more GPx1.*
qPCR on GPx1 overexpressed cells ensure that the overexpression technique was successful (n=3). Unpaired test used to determine significance (P=0.0012).

3.4.2 *No Change in Mitochondrial Resazurin Reduction; However, GPx1 Overexpressed H9c2 Cells had More Uptake of Neutral Red*

Next, after establishing that the H9c2 cells were able to successfully undergo GPx1 overexpression, two assays were conducted to asses cellular health. First, neutral red fluorescence was measured, and the result determined that there was a statistically significant difference in the GPx1 overexpressed cells, shown below in Figure 13.A. Next, a resazurin assay was completed, that which measures mitochondrial resazurin reduction. There was no statistically significant different between the two vector types, that which is shown below in Figure 13.B.

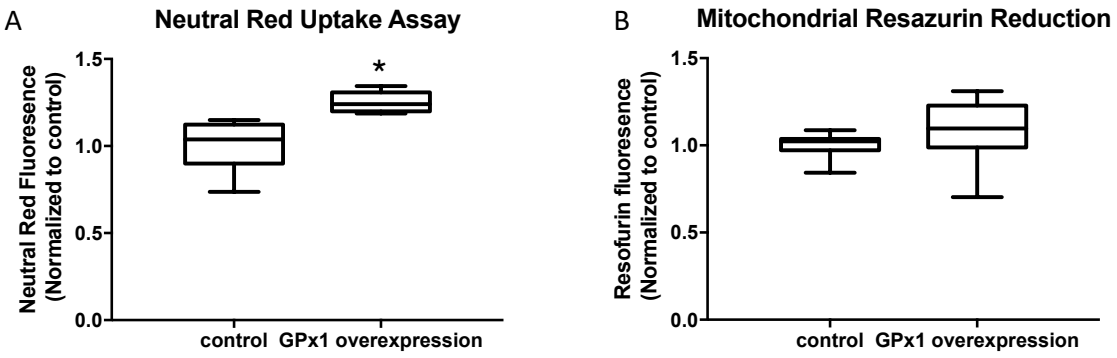


Figure 13. *GPx1 overexpressed H9c2 cells had increased neutral red uptake but no change in Mitochondrial Resazurin Reduction.*
Cell viability assays conducted on transfected H9c2 cells a) Neutral Red Uptake Assay (P= 0.0404); b) Mitochondrial Resazurin Reduction (P=0.261) at the end of a 24-incubation period. Unpaired t-test used to determine significance (control n=3, GPx1 overexpression n=3).

3.4.3 *GPx1 Overexpression Led to Variance in Caspase 3/7 Activity and Lactate Dehydrogenase Detection, While FA Treatment Led to Variation in Caspase 3/7 Activity Only*

To determine if GPx1 is altering cellular death pathways, caspase 3/7 activity was measured, and is visualized in Figure 14.A. The Two-way ANOVA showed that the GPx1 overexpression was source of variation. Additionally, a source of variation occurred from diet but there was no significant variation from the interaction between the two treatments. Tukey's multiple

comparison post-hoc determined that in both the LA treated groups, there was a decrease in caspase 3/7 activity when compared to BSA.

Lactate dehydrogenase (LDH) was measured and a source of variation from FA treatment occurred but no significance of interaction between FA and GPx1 overexpression was determined. There were no statistically significant difference between the treatment groups. The result is displayed in Figure 14.B.

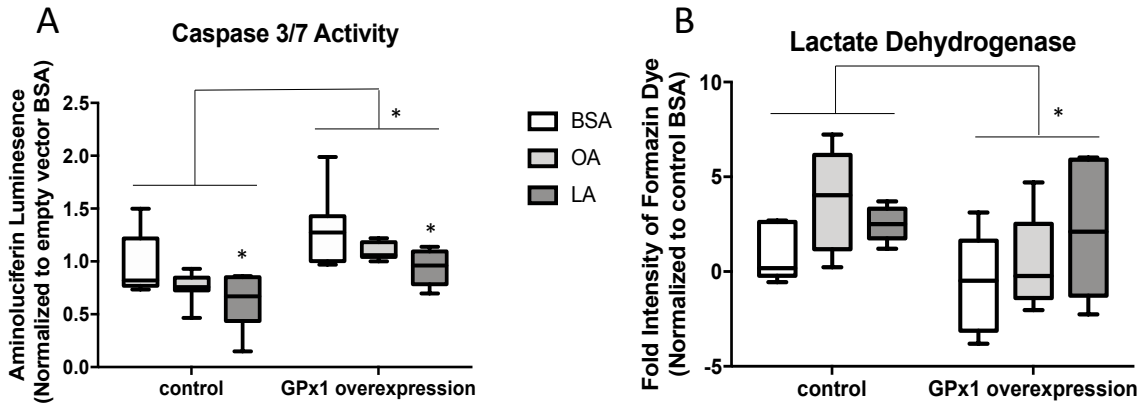


Figure 14. *GPx1 overexpressed cells had increased caspase 3/7 activity, but in the presence of LA in both overexpressed cells there was a decrease in caspase 3/7 activity.*
GPx1 and empty vector overexpressed cells were treated with either a FA or vehicle control (BSA). At the end of a 24-incubation period, two assays were conducted. Two-way ANOVA was used to determine significance (control n=3, GPx1 overexpression n=3). Figures displayed as follows: Figure 14.A, Caspase 3/7 Activity (GPx1 overexpression $P<0.0001$; FA exposure $P=0.0003$; interaction $P=0.9496$; control LA treated $P<0.05$; GPx1 overexpression LA treated $P<0.05$); Figure 14.B, Lactate Dehydrogenase Assay (FA exposure $P=0.0136$; GPx1 overexpression $P=0.0064$; interaction $P=0.3717$). Abbreviations: BSA, bovine serum albumin; OA, oleic acid; and LA, linoleic acid.

3.4.4 *GPx1 Overexpressed H9c2 Cells had Higher Levels of GSH Following FA Exposure*

To investigate what may be underlying the differences between the empty and GPx1 vector overexpressed cells, a GSH assay was completed. As GPx1 (shown in Figure 2) utilizes GSH to reduce its oxidative targets, it was suspected that GSH levels may be depleted and leading to adverse results. It was predicted that GPx1 in such high amounts could be detrimental to the cell by depleting cellular levels of GSH. The GSH assay showed that in the presence of OA and LA

in the GPx1 overexpressed group, there were increased levels of GSH when compared to the BSA treated group. The two-way ANOVA determined that there was significant variance from GPx1 overexpression and FA treatment; however, no significant variance occurring from the interaction of the two treatment types.

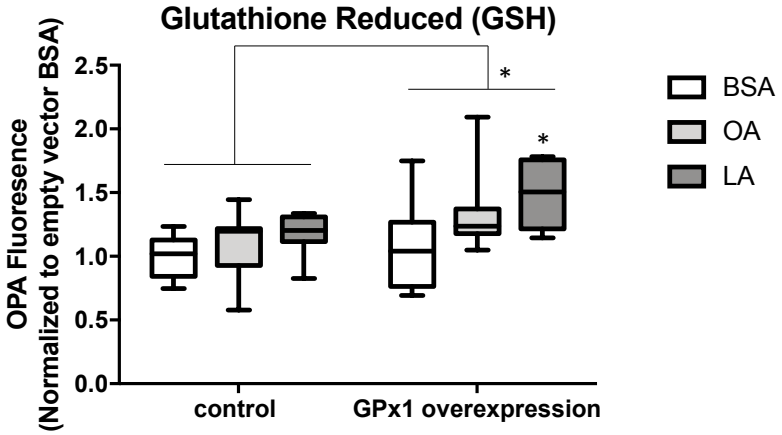


Figure 15. *GPx1 overexpressed cells treated with linoleic acid had increased glutathione in its reduced form.*
Amount of GSH present in its reduced form. The two-way ANOVA determined that there was significant variance from GPx1 overexpression (P=0.0018) and FA treatment (P=0.0019); however, no significant variance occurring from the interaction of the two treatment types (P=0.2725). Additionally, as indicated by the start (*), the GSH assay showed that in the presence of LA (P<0.005) in the GPx1 overexpressed group, there was increased GSH (control n=3, GPx1 overexpression n=3). Abbreviations: BSA, bovine serum albumin; OA, oleic acid; and LA, linoleic acid.

3.5 Discussion

3.5.1 *GPx1 Overexpressed H9c2 Cells Express Significantly More GPx1 Compared to the Empty Vector Overexpressed H9c2 Cells*

As expected GPx1 cells had increased GPx1 expression when compared to the empty vector. Therefore, qPCR validated that the overexpression was successful, as shown in Figure 12.

3.5.2 *No Change in Mitochondrial Resazurin Reduction; However, GPx1 Overexpressed H9c2 Cells had More Uptake of Neutral Red*

The neutral red assay is commonly used due to its independence of enzymatic dye conversion[188]. While the assay is considered to be an indicator of viability, it is dependent on multiple factors[179,188]. If the cell dies or pH gradient is reduced then the dye is released, hence the basis of the assay[179]. Additionally, if there are any cell surface alterations of lysosomal surface modifications it can affect uptake of the dye[179].

GPx1 overexpressed H9c2 cells had higher neutral red absorbance; and more dye was incorporated into the cell (Figure 13.A). At physiological pH, the neutral red dye becomes incorporated into lysosomes[179]. Therefore, a cell that is producing ATP and has a regular pH would incorporate more dye[179]. This result could indicate an improvement over the empty vector overexpressed cells. H9c2 cells, that which provide contractility within the heart, contain high numbers of mitochondria therefore it is predicted that this cell type would benefit from increased GPx1 expression since it has been shown to protect the mitochondria[102,121]. Hence, the GPx1 overexpressing mitochondria may be protected from damage due to GPx1 overexpression in comparison to the empty vector overexpressed cells, and therefore the GPx1 overexpressed cells had increased neutral red uptake absorbance[179].

In contrast to the neutral red uptake assay, the resazurin reduction assay relies on an enzymatic conversion, that which occurs in the mitochondria[188]. Typically, the assay is used to indicate metabolic activity[188]. As shown in Figure 13.B, there were no differences between the two transfected groups. As this assay detects cell proliferation and cytoxicity, it is not expected for there to be a difference between terminally differentiated cells[189]. It is predicted that cell activity drives the conversion of the dye from resazurin to resorufin, that which is a product of cellular metabolism utilizing oxygen[189]. In sperm, the addition of NADH + H^+ increased the reduction of resazurin, thereby demonstrating the importance of the diaphorase enzyme transfer from NADH + H^+ to resazurin[190]. Therefore, it is possible that a similar mechanism may occur in H9c2 cells.

These results taken together may indicate perhaps that the decrease in sensitivity in resazurin assays[188]. Neutral red measures dye uptake in lysosomes while resazurin measures dye in the mitochondria, therefore, mitochondrial redox did not change but lysosomes demonstrated enhanced stabilization with GPx1 overexpression.

3.5.3 *GPx1 Overexpression Led to Variance in Caspase 3/7 Activity and Lactate Dehydrogenase Detection, While FA Treatment Led to Variation in Caspase 3/7 Activity Only*

As GPx1 is likely conferring extra protection against ROS, therefore we wanted to assess the effects of dietary FA exposure and cell death as cardiomyocytes receive exposure to dietary FA within the heart, that which perhaps may play a role in mitochondrial induced cardiomyopathy.

As shown in Figure 14.A, caspase 3/7 activity was measured. Caspases are intracellular cysteine endopeptidases that at by cleaving target proteins at a cysteine amino acid[122,191]. The amino acid is only cleaved if it is followed behind an aspartic acid residue[122]. Caspase-3 and caspase-7 are an executioner caspases that directly leads to cell lysis[122,191,192]. Two-way ANOVA determined that GPx1 overexpression significantly caused variance and FA exposure caused variance as well, but no significance due to their interaction. Tukey's post hoc analyses determined that LA exposure within the control and GPx1 overexpressed cells, decreased caspase 3/7 activity compared to BSA treatment.

As shown in Figure 14.B, LDH was measured to estimate necrosis. Two-way ANOVA determined FA exposure caused variance, but no significance due to their interaction or GPx1 overexpression. Tukey's post hoc analyses determined that LA exposure within the control and GPx1 overexpressed cells, decreased caspase 3/7 activity compared to BSA treatment. Tukey's post doc test did not reveal any significance in its findings.

Previously, we have shown that when GSH was increased, apoptosis was promoted while inhibiting necrosis within fibroblast cells[181]. Taken together, while this work is not sufficient enough to state that a shift is occurring, there is an interesting trend observed.

3.5.4 *GPx1 Overexpressed H9c2 Cells had Higher Levels of GSH Following LA Exposure*

As previously mentioned, we have shown that when GSH was increased, apoptosis was promoted while inhibiting necrosis within fibroblast cells[181]. Therefore, to investigate if there were any differences in the amount of reduced glutathione present, we conducted a GSH assay. The mechanism of action for glutathione peroxidases is well established and uses GSH to produce its reducing power (Figure 2). As shown in Figure 16, the two-way ANOVA determined that there was significant variance from GPx1 overexpression and FA treatment; however, no significant variance occurring from the interaction of the two treatment types. Furthermore, GPx1 overexpressed cells had increased GSH in the presence of LA.

Based on the GPx1 mechanism of action, it is expected that GPx1 overexpressed cells would have decreased GSH levels (Figure 2). This however was not the result obtained in Figure 16. Perhaps this was not observed due to an increase in GSH production, as a result of signaling occurring by increased GPx1 expression. Expectedly, GSH would decrease when there is an oxidative threat. LA was predicted to be a strong enough oxidative opponent but perhaps it was no comparison to the high amount of GPx1 gene expression exhibited by the H9c2 cells. The next step is to determine the amount of oxidized glutathione present to have a better estimation of the glutathione pool.

Likely, ordinary redox signaling has been altered in GPx1 overexpressed cells due to the increased capacity to reduce ROS. As GPx1 utilizes GSH for its action, it is a likely prediction that GSH levels had been altered. This was observed, as variance occurred between GPx1 overexpressed H9c2 cells and empty vector overexpressed H9c2 cells. As we did not measure GSH levels at the early hours following GPx1 overexpression we are unable to say for certain what occurred. Perhaps GSH was reduced to provide GPx1 with reducing power but this requires further investigation. However; as part of many homeostatic pathways, a deficiency leads to an increase to try and compensate for the reduction and to combat further stresses[180]. Therefore, likely GSH was decreased following GPx1 overexpression and furthermore following LA exposure during the early incubation hours, but following complex signaling, the amount of GSH increased. Increases in GSH can occur in one of two ways: regeneration by NADPH production and by generation of new GSH [193]. In addition to a deficiency of GSH, oxidative stress is known

to increase expression of the glutathione synthetic enzymes[180]. As GPx1 utilizes GSH, it is predicted in the absence of a stressor that initially it would be reduced. Normally there are higher levels of GSH compared to GSSG (depending on cellular redox within the cell), therefore if there was an increase in the total glutathione pool, it would exist in the GSH form[180,194].

3.6 Chapter Conclusion

In summary, the specific objective for this chapter was to investigate the effects of dietary FAs in GPx1 overexpressed H9c2 cells. The overall hypothesis was that **glutathione peroxidase 1 (GPx1), which is an important mitochondrial antioxidant enzyme, is able to protect the heart following a high fat diet**. To address this hypothesis, it was shown that n-6 PUFA feeding in GPx1 overexpressed cells had increased neutral red uptake, possibly indicating increased cellular health. This is expected due to the increased ability of the cell to neutralize ROS. Furthermore, we demonstrated that GPx1 overexpression increased caspase 3/7 activity. Additionally, LA in both the control and GPx1 overexpression transfected cells had decreased caspase 3/7 activity, thereby indicating apoptotic signaling. LDH on the other hand was only altered by FA exposure, and not due to the GPx1 overexpression. Lastly, we showed that in GPx1 overexpressed cells that were exposed to LA, there was an increase in the amount of GSH. This result may have occurred by an attempt to reach redox homeostasis that would have been altered from the addition of the stressor LA. When interpreting the GSH result with caspase 3/7 activity, it is already established that GSH depletion observed in apoptotic cells and extreme deficits of GSH shifts cell death from apoptosis to necrosis [180,195]. While this result is not entirely supported in this chapter, the trend of LA in GPx1 overexpressed cells did indeed have decreased caspase 3/7 activity. In conclusion, this work has provided introductory steps to investigate the effects of dietary FA exposure in GPx1 overexpressed H9c2 cells.

Chapter 4: Conclusion

4.1 Limitations of Thesis Work

Although ROS related phenomena are universal in nature, the work described in this thesis was primarily performed in *a Mus musculus* model for the *in vivo* work and a *Rattus norvegicus* H9c2 cell culture line for *in vitro*. Thus, it is not appropriate to extrapolate results directly from this thesis to explain human phenomena[196]. More research needs to be done in humans with GPx1 gene mutations under various fat diets using research findings from this thesis as appropriate[116]. Finally, it is difficult to study isolated systems *in vivo* due to biological redundancy of antioxidant systems[84,197,198].

4.2 Significance

With substantial research investigating GPx1 and its role in CVD, there is a gap in the literature regarding experimentation accurately depicting the current western diet[114,171]. It is becoming more established that our current diets may be contributing to the onset or worsening of chronic diseases, such as heart disease[8,57,102]. When studying Keshan disease specifically, which is a form of cardiomyopathy characterized by low levels of GPx1, it is important to note these results show that these individuals may not at further risk for cardiac function problems when consuming a western diet[116,199]. While this thesis utilized murine models, it is important to investigate the human system to observe if a similar result occurs. Furthermore, this thesis draws attention to the necessity for further research to determine the role of GPx1 in cardiac health following a high n-6 PUFA diet.

4.3 Conclusion

With current research indicating that excess n-6 PUFA consumption could be contributing to development of chronic diseases we can better understand the mechanisms underlying CVD[45,57,59]. While there is evidence supporting n-6 PUFA as a cardiotoxic agent in elevated amounts, the mechanisms through which this cardiotoxicity occurs are not well understood. This thesis provides the following results, that which add to the understanding of GPx1 and cardiotoxicity following a n-6 PUFA diet:

1. In GPx1 KO mouse hearts, gene expression of GPx4, SOD2, and SOD1 was increased compared to the WT mouse hearts. Catalase gene expression, however, was decreased. This indicates that perhaps other antioxidants are in place to combat oxidative stress. Additionally, it demonstrates that biological redundancy in antioxidant systems contribute to the difficulty in investigating the role of a specific antioxidant enzyme such as GPx1. Interestingly, dietary intervention did not alter gene expression of the antioxidants in WT or GPx1 KO mouse hearts and perhaps indicates that dietary lipids are not key regulators in gene expression when compared to a reduction of an antioxidant enzyme.
2. There were no functional changes by various dietary fats or GPx1 heterozygous genetic KO as detected by echocardiogram in the heart of WT or GPx1 KO mice. While the LV mass result observed in GPx1 KO mice may imply that perhaps the small corn oil hearts are more efficient due to no reduction in cardiac output, MAP, or stroke volume.
3. There was a shift in cellular death occurring in GPx1 overexpressed cells, as observed by an increase caspase 3/7 activity. As seen in Chapter 1, mitochondrial copy number was reduced in heterozygous GPx1 KO mouse hearts. This result therefore adds to the current understanding that GPx1 plays a large role in retaining mitochondrial health. While we were unable to show any changes occurring in necrosis, there is a need to increase the n number of this assay to conclude whether there was a change.

4.4 Future Directions

This thesis is able to add to the breadth of understanding GPx1 in the heart following high fat feeding; however, several opportunities for further research have arisen to address further questions.

1. Measure glutathione synthetase and glutamyl cysteine ligase by qPCR to obtain an indication as to if synthesis of GSH is increased in the presence of LA in GPx1 overexpressed H9c2 cardiomyocytes.
2. Measure ROS in GPx1 and empty vector overexpressed cells to determine if there is a difference in ROS production, that which could explain the increase in neutral red uptake, that which is a measurement of cell viability, observed in GPx1 overexpressed cells.

3. Measure antioxidant activity in addition to gene expression levels (GPx1, SOD1, SOD2, etc.) to determine if there were functional changes beyond those occurring at a transcriptional level.

Bibliography

1. Fornage M, Gillespie C, Isasi CR, Lichtman JH, Lisabeth L. Heart Disease and Stroke Statistics - 2017 Update: *Circulation*. 2017;135(10):e146-603. doi:10.1161/CIR.0000000000000485.Heart
2. Tarride J, Lim M, Desmeules M, et al. A review of the cost of cardiovascular disease. *Can Jounrnal Cardiol*. 2009;25(6):195-202.
3. Public Health Agency of Canada. *Economic Burden of Illness in Canada, 2010*.; 2018.
4. Global Health Metrics. *Global, Regional, and National Incidence, Prevalence , and Years Lived with Disability for 328 Diseases and Injuries for 195 Countries, 1990–2016: A Systematic Analysis for the Global Burden of Disease Study 2016*. Vol 390.; 2017. doi:10.1016/S0140-6736(17)32154-2
5. Kivimäki M, Pentti J, Ferrie JE, et al. Work stress and risk of death in men and women with and without cardiometabolic disease: a multicohort study. *Lancet Diabetes Endocrinol*. 2019;6:705-713. doi:10.1016/S2213-8587(18)30140-2
6. Wang X, Ouyang Y, Liu J, et al. Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis. *BMJ*. 2014;4490:1-14. doi:10.1136/bmj.g4490
7. Yusuf S, Reddy S, Ôunpuu S, Anand S. Clinical Cardiology: New Frontiers Global Burden of Cardiovascular Diseases. *Circulation*. 2001;104:2746-2753.
8. Cordain L, Eaton SB, Sebastian A, et al. Origins and evolution of the Western diet: health implications for the 21st century. *Am J Clin Nutr*. 2005;81:341-354.
9. Mente A, de Koning L, Shannon H, Anand S. A systematic review of the evidence supporting a causal link between dietary. *Arch Intern Med*. 2009;21(3):555-563. http://archinte.highwire.org/cgi/content/abstract/169/7/659.
10. Satija A, Bhupathiraju SN, Spiegelman D, et al. Healthful and unhealthful plant-based diets and the risk of coronary heart disease in U.S. adults. *J Am Coll Cardiol*. 2017;70(4):411-422. doi:10.1016/j.jacc.2017.05.047
11. Stefan N, Häring HU, Hu FB, Schulze MB. Metabolically healthy obesity: Epidemiology, mechanisms, and clinical implications. *Lancet Diabetes Endocrinol*. 2013;1(2):152-162. doi:10.1016/S2213-8587(13)70062-7
12. Hu FB, Manson JE, Willett WC, Hu FB, Manson JE, Willett WC. Types of dietary fat and risk of coronary heart disease: A critical review types of dietary fat and risk of coronary heart disease: A critical review. *J Am Coll Nutr*. 2001;20(1):5-19. doi:10.1080/07315724.2001.10719008
13. Alberti KGMM, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome. *Circulation*. 2009;120:1640-1645. doi:10.1161/CIRCULATIONAHA.109.192644
14. Braunwald E. Cardiomyopathies. *Circ Res*. 2017;121:711-721. doi:10.1016/j.mpmed.2018.07.014
15. Maron BJ. Hypertrophic cardiomyopathy. *J Am Med Assoc*. 2002;287(10):1308-1320. doi:10.1016/B978-1-4160-3754-5.50029-9
16. Walsh R, Zinn A, Hoppel C. Mitochondrial cardiomyopathies. *Mol Mech Card Hypertrophy Fail*. 2013;3(July):587-609. doi:10.3109/9780203503249-39
17. Cabello CM, Bair WB, Lamore SD, et al. Potential impact of carbohydrate and fat intake

on pathological left ventricular hypertrophy. *Cardiovasular Res*. 2007;73(2):257-268. doi:10.1016/j.freeradbiomed.2008.10.025.The
18. Sadoshima J, Izumo S. The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu Rev Physiol*. 1997;59:551-571. doi:10.1146/annurev.physiol.59.1.551
19. Devereux RB, Nieminen MS, Rokkedal J, Harris K, Aurup P, Page P. Prognostic significance of left ventricular mass change during treatment of hypertension. *J Am Med Assoc*. 2004;292(19):2350-2356.
20. Kannel WB. Epidemiology and prevention of cardiac failure: Framingham Study insights. *Eur Heart J*. 1987;8:23-29. doi:10.1093/eurheartj/8.suppl_f.23
21. Olivetti G, Melissari M, Capasso JM, Anversa P. Cardiomyopathy of the aging human heart. *Circ Res*. 1991:1560-1568. https://www.ncbi.nlm.nih.gov/pubmed/2036710.
22. Diab DL, Yerian L, Schauer P, et al. Cardiomyopathy of aging in the mammalian heart is characterized by myocardial hypertrophy, fibrosis, and predisposition towards cardiomycote apoptosis and autophagy. *Exp Gerontol*. 2011;6(11):1249-1254. doi:10.1016/j.cgh.2008.07.016.Cytokeratin
23. Bournat JC, Brown CW. Mitochondrial dysfunction in obesity. *Curr Opin Endocrinol Diabetes Obes*. 2010;17(5):446-452. doi:10.1097/MED.0b013e32833c3026.Mitochondrial
24. Marseglia L, Manti S, Angelo GD, et al. Oxidative stress in obesity: A critical component in human diseases. *Int J Mol Sci*. 2015;16:378-400. doi:10.3390/ijms16010378
25. Ford ES, Mokdad AH, Giles WH, Brown DW. The metabolic syndrome and antioxidant concentrations. *Diabetes*. 2003;52.
26. Guenther B. Obesity and free fatty fcids (FFA). *Endocrinol Metab Clin North Am*. 2008;37(3):635-x. doi:10.1016/j.ecl.2008.06.007.Obesity
27. Sun X, Pan H, Tan H, Yu Y. High free fatty acids level related with cardiac dysfunction in obese rats. *Diabetes Res Clin Pract*. 2012;95(2):251-259. doi:10.1016/j.diabres.2011.10.028
28. Lavie CJ, Milani R V., Ventura HO. Obesity and cardiovascular disease. *J Am Coll Cardiol*. 2009;53(21):1925-1932. doi:10.1016/j.jacc.2008.12.068
29. Poirier P, Giles TD, Bray GA, et al. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss. *Circulation*. 2006;113(6):898-918. doi:10.1161/CIRCULATIONAHA.106.171016
30. Vincent J. Understanding cardiac output. 2008;3:12-14. doi:10.1186/cc6975
31. Alpert MA. Obesity cardiomyopathy: pathophysiology and evolution of the clinical syndrome. *Am J Med Sci*. 2001;321(4):225-236.
32. Buettner R, Schölmerich J, Bollheimer LC. High-fat diets: Modeling the metabolic disorders of human obesity in rodents. *Obesity*. 2007;15(4):798-808.
33. Woods SC, Seeley RJ, Rushing PA, Alessio DD, Tso P. A controlled high-fat diet induces an obese syndrome in rats. *J Nutr*. 2003;133(4):1081-1087.
34. Mamounis KJ, Yasrebi A, Roepke TA. Linoleic acid causes greater weight gain than saturated fat without hypothalamic inflammation in the male mouse. *J Nutr Biochem*. 2017;40:122-131. doi:10.1016/j.jnutbio.2016.10.016
35. Orsavova J, Misurcova L, Ambrozova JV, Vicha R. Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids. *Int J Mol Sci*. 2015;16:12871-12890. doi:10.3390/ijms160612871

36. Siri-tarino PW, Sun Q, Hu FB, Krauss RM. Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease 1 – 5. *Am J Clin Nutr*. 2010;91:535-546. doi:10.3945/ajcn.2009.27725.1
37. Menotti A, Karvonen MJ, Aravanis C, et al. the Diet and 15-Year Death Rate in the Seven Countries. *Am J Epidemiol*. 1986;124(6):903-915. http://aje.oxfordjournals.org/content/124/6/903.full.pdf+html.
38. Siri-Tarino PW, Sun Q, Hu FB, Krauss RM. Saturated fatty acids and risk of coronary heart disease: Modulation by replacement nutrients. *Curr Atheroscler Rep*. 2010;12(6):384-390. doi:10.1007/s11883-010-0131-6
39. Spady DK, Woollett LA, Dietschy JM. Regulation of plasma LDL-cholesterol levels by dietary cholesterol. *Annu Rev Nutr*. 1993;13:355-381.
40. Appel LJ, Moore TJ, Obarzanek E, et al. A clinical trial of the effects of dietary patterns on blood pressure. *N Engl J Med*. 1997;336(16):1117-1124. doi:10.1056/nejm199704173361601
41. Ramsden CE, Zamora D, Majchrzak-hong S, et al. Re-evaluation of the traditional diet-heart hypothesis: analysis of recovered data from Minnesota Coronary Experiment (1968-73). *Br Med Journalitish Med J*. 2016;353:1-17. doi:10.1136/bmj.i1246
42. DiNicolantonio JJ, Lucan SC, O'Keefe JH. The evidence for saturated fat and for sugar related to coronary heart disease. *Prog Cardiovasc Dis*. 2016;58(5):464-472. doi:10.1016/j.pcad.2015.11.006
43. Malhotra A, Redberg RF, Meier P. Saturated fat does not clog the arteries: coronary heart disease is a chronic inflammatory condition, the risk of which can be effectively reduced from healthy lifestyle interventions. *Br J Sports Med*. 2017;51(15):1111-1112. doi:10.1136/bjsports-2016-097285
44. Harris WS, Mozaffarian D, Rimm E, et al. Omega-6 fatty acids and risk for cardiovascular disease. *Circulation*. 2009;119:902-907. doi:10.1161/CIRCULATIONAHA.108.191627
45. Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*. 2002;56(8):365-379. doi:10.1016/S0753-3322(02)00253-6
46. Serhan CN. Novel pro-resolving lipid mediators in inflammation are leads for resolution physiology. *Nature*. 2014;510(7503):92-101. doi:10.1038/nature13479.Novel
47. Serhan CN, Petasis NA. Resolvins and protectins in inflammation-resolution. *Chem Rev*. 2012;111(10):5922-5943. doi:10.1021/cr100396c.Resolvins
48. Patterson E, Wall R, Fitzgerald GF, Ross RP, Stanton C. Health Implications of High Dietary Omega-6 Polyunsaturated Fatty Acids. 2012;2012. doi:10.1155/2012/539426
49. Wall R, Ross RP, Fitzgerald GF, Stanton C. Fatty acids from fish: The anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutr Rev*. 2010;68(5):280-289. doi:10.1111/j.1753-4887.2010.00287.x
50. Gilbert K, Malick M, Madingou N, Bourque-riel V, Touchette C, Rousseau G. Linoleic acid attenuates cardioprotection induced by resolvin D1. *J Nutr Biochem*. 2016;31:122-126. doi:10.1016/j.jnutbio.2015.12.016
51. Anderson EJ, Thayne KA, Harris M, et al. Do fish oil omega-3 fatty acids enhance antioxidant capacity and mitochondrial fatty acid oxidation in human atrial myocardium via PPARγ Activation? *Antioxid Redox Signal*. 2014;21(8):1156-1163. doi:10.1089/ars.2014.5888
52. Anderson EJ, Thayne KA, Harris M, Carraway K, Shaikh SR. Aldehyde stress and up-regulation of Nrf2-mediated antioxidant systems accompany functional adaptations in

cardiac mitochondria from mice fed n-3 polyunsaturated fatty acids. *Biochem J*. 2012;441(1):1-17. doi:10.1042/BJ20110626.Aldehyde
53. Schmitz G, Ecker J. Progress in Lipid Research The opposing effects of n-3 and n-6 fatty acids. *Prog Lipid Res*. 2008;47:147-155. doi:10.1016/j.plipres.2007.12.004
54. Katunga LA, Gudimella P, Jimmy TE, et al. Obesity in a model of gpx4 haploinsufficiency uncovers a causal role for lipid-derived aldehydes in human metabolic disease and cardiomyopathy. 2015;4(April):493-506. doi:10.1016/j.molmet.2015.04.001
55. Wijendran V, Hayes KC. Dietary n-6 and n-3 fatty acid balance and cardiovascular health. *Annu Rev Nutr*. 2004;24:597-615. doi:10.1146/annurev.nutr.24.012003.132106
56. Hu F. Plant-based foods diets and prevention of cardiovascular disease: an overview. *Am J Clin Nutr*. 2003;78:544S-551S. doi:10.1201/9781420005905.ch9
57. Simopoulos AP. The importance of the mmega-6/mmega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med*. 2008:674-688. doi:10.3181/0711-MR-311
58. Ornish D, Scherwitz LW, Billings JH, et al. Intensive lifestyle changes for reversal of coronary heart disease. *J Am Med Assoc*. 1998;280(23):2001-2007. http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed4&NEWS=N&AN=1998420502.
59. Ramsden CE, Zamora D, Ringel A, Davis JM, Hibbeln JR. Use of dietary linoleic acid for secondary prevention of coronary heart disease and death: Evaluation of recovered data from the Sydney Diet Heart Study and updated meta-analysis. *BMJ*. 2013;346:1-18. doi:10.1136/bmj.e8707
60. Farvid MS, Ding M, Pan A, et al. Dietary linoleic acid and risk of coronary heart disease: A systematic review and meta-analysis of prospective cohort studies. *Circulation*. 2014;130(18):1568-1578. doi:10.1161/CIRCULATIONAHA.114.010236
61. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of aging. *Nature*. 2000;408:239-247.
62. Fearon IM, Faux SP. Oxidative stress and cardiovascular disease: Novel tools give (free) radical insight. *J Mol Cell Cardiol*. 2009;47(3):372-381. doi:10.1016/j.yjmcc.2009.05.013
63. Aon MA, Stanley BA, Sivakumaran V, et al. Glutathione/thioredoxin systems modulate mitochondrial H2O2 emission: An experimental-computational study. *J Gen Physiol*. 2012;139(6):479-491. doi:10.1085/jgp.201210772
64. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, aging. *Cell*. 2005;120:483-495. doi:10.1016/j.cell.2005.02.001
65. Zhang J, Wang X, Vikash V, et al. ROS and ROS-mediated cellular signaling. *Oxid Med Cell Longev*. 2016;2016:1-18.
66. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol Rev*. 2014;94:909-950. doi:10.1152/physrev.00026.2013
67. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J*. 2009;417:1-13. doi:10.1042/BJ20081386
68. Forman HJ, Maiorino M, Ursini F. Signaling Functions of Reactive Oxygen Species. *Biochemistry*. 2010;49:835-842. doi:10.1021/bi9020378
69. Chen Q, Wang Q, Zhu J, Xiao Q, Zhang L. Reactive oxygen species: Key regulators in vascular health and diseases. *Br J Pharmacol*. 2018;175:1279-1292. doi:10.1111/bph.13828

70. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol*. 2007;39(1):44-84. doi:10.1016/j.biocel.2006.07.001
71. Weydert CJ, Cullen JJ. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nat Protoc*. 2010;5(1):51-66. doi:10.1038/nprot.2009.197
72. Manrique C, DeMarco VG, Aroor AR, et al. Obesity and insulin resistance induce early development of diastolic dysfunction in young female mice fed a western diet. *Endocrinology*. 2013;154(10):3632-3642. doi:10.1210/en.2013-1256
73. Thygesen K, Alpert JS, White HD, et al. Universal definition of myocardial infarction. *J Am Coll Cardiol*. 2007;50(22):2173-2195. doi:10.1016/j.jacc.2007.09.011
74. Yusuf S, Hawken S, Ôunpuu S, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet*. 2004;364:937-952.
75. Finkel T. Oxygen radicals and signaling. *Curr Opin Cell Biol*. 1998;10:248-253.
76. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Bioloy*. 2014;24(10):R453-R462. doi:10.1016/j.cub.2014.03.034
77. Slauch JM. How does oxidative burst of macrophages kill bacteria? Still an open question. *Mol Microbiol*. 2011;80(3):580-583. doi:10.1111/j.1365-2958.2011.07612.x.How
78. Simon H-U, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*. 2000;5:415-418.
79. Redza-dutordoir M, Averill-bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochem Biophys Acta*. 2016;1863(12):2977-2992. doi:10.1016/j.bbamcr.2016.09.012
80. Fang FC. Antimicrobial actions of reactive oxygen species. *Perspective*. 2011;2(5):1-6. doi:10.1128/mBio.00141-11.Copyright
81. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat Rev*. 2011;11(9):607-615. doi:10.1038/nri3041
82. Powers SK, Lennon SL. Analysis of cellular responses to free radicals: focus on exercise and skeletal muscle. *Proc Nutr Soceity*. 1999;58:1025-1033.
83. Radak Z, Chung HY, Koltai E, Taylor AW, Goto S. Exercise, oxidative stress and hormesis. *Ageing Res Rev*. 2008;7:34-42. doi:10.1016/j.arr.2007.04.004
84. Bouayed J, Bohn T. Exogenous antioxidants — Double-edged swords in cellular redox state health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxid Med Cell Longev*. 2010;3(4):228-237. doi:10.4161/oxim.3.4.12858
85. Mari M, Morales A, Colell A, García-Ruiz C, Fernàndez-Checa JC. Mitochondrial glutathione, a key survival antioxidant. 2009;11(11).
86. Brigelius-flohe R. Glutathione peroxidases and redox-regulated transcription factors. *Biol Chem*. 2006;387:1329-1335. doi:10.1515/BC.2006.166
87. Pietta PG. Flavonoids as antioxidants. *J Nat Prod*. 2000;63(7):1035-1042. doi:10.1021/np9904509
88. Kanti BP, Syed IR. Plant polyphenols as dietary antioxidants in human health and disease, oxidative medicine and cellular longevity. *Landes Biosci*. 2009;2(5):270-278.
89. Renaud S, De Lorgeril M. Wine, alcohol, platelets and the French paradox for coronary heart disease. *Lancet*. 1992;339:1523-1526. doi:10.1016/0140-6736(92)91277-F

90. Dubick MA, Omaye ST. Evidence for grape, wine and tea polyphenols as modulators of atherosclerosis and ischemic heart disease in humans. *J Nutraceuticals, Funct Med Foods*. 2005;3(3):67-93. doi:10.1300/j133v03n03_04
91. García-Lafuente A, Guillamón E, Villares A, Rostagno MA, Martínez JA. Flavonoids as anti-inflammatory agents: Implications in cancer and cardiovascular disease. *Inflamm Res*. 2009;58(9):537-552. doi:10.1007/s00011-009-0037-3
92. Pastore A, Federici G, Bertini E, Piemonte F. Analysis of glutathione: Implication in redox and detoxification. *Clin Chim Acta*. 2003;333(1-2):19-39. doi:10.1016/S0009-8981(03)00200-6
93. Sies H. Glutathione and its role in cellular functions. *Free Radic Biol Med*. 2000;27:916-921.
94. Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. *Biomed Pharmacother*. 2003;57(3):145-155. doi:10.1016/S0753-3322(03)00043-X
95. Imai H, Nakagawa Y. Biological significance of phospholipd hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammallian cells. *Free Radic Biol Med*. 2003;34(2):145-169.
96. Damy T, Kirsch M, Khouzami L, et al. Glutathione deficiency in cardiac patients is related to the functional status and structural cardiac abnormalities. *PLoS One*. 2009;4(3). doi:10.1371/journal.pone.0004871
97. Watanabe Y, Watanabe K, Kobayashi T, et al. Chronic depletion of glutathione exacerbates ventricular remodelling and dysfunction in the pressure-overloaded heart. *Cardiovasc Res*. 2013;97(2):282-292. doi:10.1093/cvr/cvs333
98. Labunskyy VM, Hatfield DL, Gladyshev VN, et al. Selenoproteins: Molecular pathways and pathways and physiogoical roles. *Physiol Rev*. 2014;94:739-777. doi:10.1152/physrev.00039.2013
99. Kryukox G V., Castellano S, Novoselov S V., et al. Characterization of mammalian selenoproteomes. *Science (80-)*. 2003;300:1439-1444.
100. Tinggi U. Selenium: its role as antioxidant in human health. *Environ Heal Prev Med*. 2008;13:102-108. doi:10.1007/s12199-007-0019-4
101. Handy DE, Lubos E, Yang Y, et al. Glutathione peroxidase-1 regulates mitochondrial function to modulate redox-dependent cellular responses. *J Biol Chem*. 2009;284(18):11913-11921. doi:10.1074/jbc.M900392200
102. Lubos E, Loscalzo J, Handy DE. Glutathione peroxidase-1 in health and disease: From molecular mechanisms to therapeautic opportunities. *Antioxid Redox Signal*. 2011;15(7):1957-1997. doi:10.1089/ars.2010.3586
103. Chabory, Eleanore Damon C, Lenoir A, Kauselmann G, et al. Epididymis seleno-independent glutathione peroxidase 5 maintains sperm DNA integrity in mice. *J Clin Invest*. 2009;119(7):2074-2085. doi:10.1172/JCI38940
104. Keele SW, Diener HC, Gopal HS, et al. Characterization of mammalian selenoproteomes. *Science (80-)*. 2003;300:1439-1444.
105. Peng DF, Belkhiri A, Hu TL, et al. Glutathione peroxidase 7 protects against oxidative DNA damage in oesophageal cells. *Gut*. 2012;61(9):1250-1260. doi:10.1136/gutjnl-2011-301078
106. Ho Y, Magnenat J, Bronson RT, et al. Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. *J Biol Chem*. 1997;272(26):16644-16651.

107. Chu FF, Doroshow JH, Esworthy RS. Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. *J Biol Chem*. 1993;268(4):2571-2576.
108. Ghyselinck NB, Dufaure I, Lareyre JJ, Rigaudière N, Mattéi MG, Dufaure JP. Structural organization and regulation of the gene for the androgen-dependent glutathione peroxidase-like protein specific to the mouse epididymis. *Mol Endocrinol*. 1993;7(2):258-272. doi:10.1210/mend.7.2.8469239
109. Schneider M, Förster H, Boersma A, et al. Mitochondrial glutathione peroxidase 4 disruption causes male infertility. *FASEB J*. 2009;23(9):3233-3242. doi:10.1096/fj.09-132795
110. Yant LJ, Ran Q, Rao L, et al. The selenoprotein GPx4 is essential for mouse development and protects from radiation and oxidative damage insults. *Free Radic Biol Med*. 2003;34(4):496-502. doi:10.1016/S0891-5849(02)01360-6
111. Ueta T, Inoue T, Furukawa T, et al. Glutathione peroxidase 4 is required for maturation of photoreceptor cells. *J Biol Chem*. 2012;287(10):7675-7682. doi:10.1074/jbc.M111.335174
112. Flohe L. Glutathione peroxidase: a Selenoenzyme. *FEBS Lett*. 1973;32:132-134.
113. Arthur JR. The glutathione peroxidases. *Cell Mol Life Sci*. 2000;57:1825-1835.
114. Ardanaz N, Yang XP, Cifuentes ME, et al. Lack of glutathione peroxidase 1 accelerates cardiac-specific hypertrophy and dysfunction in angiotensin II hypertension. *Hypertension*. 2010;55(1):116-123. doi:10.1161/HYPERTENSIONAHA.109.135715
115. Blankenberg S, Rupprecht HJ, Bickel C, et al. Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. *N Engl J Med*. 2003;349:1605-1613.
116. Zhang J, Wang Z, Zhang J, Zhu L, Gao X, Chen S. Association of glutathione peroxidase-1 (GPx-1) rs1050450 Pro198Leu and Pro197Leu polymorphisms with cardiovascular risk: a meta-analysis of observational studies. *J Geriatr Cardiol*. 2014;11:141-150. doi:10.3969/j.issn.1671-5411.2014.02.003
117. Beam J, Botta A, Ye J, et al. Excess linoleic acid increases collagen I / III ratio and ' stiffens ' the heart muscle following high fat diets. 2015;(1). doi:10.1074/jbc.M115.682195
118. Sears C, Ghosh S. Excess omega-6 polyunsaturated fatty acid intake is associated with negative cardiovascular, intestinal and metabolic outcomes in mice. *Can J Diabetes*. 2016;40(4):278-279. doi:10.1016/j.jcjd.2016.06.002
119. Ghosh S, Qi D, An D, et al. Brief episode of STZ-induced hyperglycemia produces cardiac abnormalities in rats fed a diet rich in n-6 PUFA. 2004;3:2518-2527. doi:10.1152/ajpheart.00480.2004.
120. Cohen GM. Caspases: Executioners of apoptosis. *Biochemistry*. 2014;16:145-152. doi:10.1016/B978-0-12-386456-7.01411-8
121. Gustafsson ÅB, Gottlieb RA. Heart mitochondria: Gates of life and death. *Cardiovasc Res*. 2008;77(2):334-343. doi:10.1093/cvr/cvm005
122. Payne AM, Zorman J, Horton M, Dubey S, Ter Meulen J, Vora KA. Caspase activation as a versatile assay platform for detection of cytotoxic bacterial toxins. *J Clin Microbiol*. 2013;51(9):2970-2976. doi:10.1128/JCM.01161-13
123. Cummings BS, Wills LP, Schnellmann RG. Measurement of Cell Death in Mammalian Cells. *Curr Protoc Pharmacol*. 2004;1:0-12. doi:10.1002/0471141755.ph1208s25.Measurement

124. Norberg E, Orrenius S, Zhivotovsky B. Mitochondrial regulation of cell death: Processing of apoptosis-inducing factor (AIF). *Biochem Biophys Res Commun*. 2010;396(1):95-100. doi:10.1016/j.bbrc.2010.02.163
125. Chan FK, Moriwaki K, Josè De Rosa M. Detection of necrosis by release of lactate dehydrogenase (LDH) activity. *Methods Mol Biol*. 2013;979:65-70. doi:10.1007/978-1-4020-6754-9_8298
126. Bowcutt AA. How dying cells alert the immune system to danger. *Nat Rev Immunol*. 2008;8(4):279-289. doi:10.1038/nri2215.How
127. Lekshmi A, Varadarajan SN, Lupitha SS, et al. A quantitative real-time approach for discriminating apoptosis and necrosis. *Cell Death Discov*. 2017;3:1-10. doi:10.1038/cddiscovery.2016.101
128. Chan FK, Shisler J, Bixby JG, et al. A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. *J Biol Chem*. 2003;278(51):51613-51621. doi:10.1074/jbc.M305633200
129. Mattes MJ. Apoptosis assays with lymphoma cell lines: Problems and pitfalls. *Br J Cancer*. 2007;96(6):928-936. doi:10.1038/sj.bjc.6603663
130. Fink SL, Cookson BT. Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells. *Infect Immun*. 2005;73(4):1907-1916. doi:10.1128/IAI.73.4.1907
131. Gouazé V, Andrieu-Abadie N, Cuvillier O, et al. Glutathione peroxidase-1 protects from CD95-induced apoptosis. *J Biol Chem*. 2002;277(45):42867-42874. doi:10.1074/jbc.M203067200
132. Peter ME, Budd RC, Desbarats J, et al. The CD95 Receptor: Apoptosis Revisited. *Cell*. 2007;129(3):447-450. doi:10.1016/j.cell.2007.04.031
133. Faucher K, Rabinovitch-Chable H, Cook-Moreau J, Barrière G, Sturtz F, Rigaud M. Overexpression of human GPX1 modifies Bax to Bcl-2 apoptotic ratio in human endothelial cells. *Mol Cell Biochem*. 2005;277(1-2):81-87. doi:10.1007/s11010-005-5075-8
134. Westphal D, Dewson G, Czabotar PE, Kluck RM. Molecular biology of Bax and Bak activation and action. *Biochim Biophys Acta - Mol Cell Res*. 2011;1813(4):521-531. doi:10.1016/j.bbamcr.2010.12.019
135. Nomura K, Imai H, Koumura T, Arai M, Nakagawa Y. Suppresses apoptosis mediated by a mitochondrial death pathway. *J Biol Chem*. 1999;274(41):29294-29302.
136. Matsushima S, Kinugawa S, Ide T, et al. Overexpression of glutathione peroxidase attenuates myocardial remodeling and preserves diastolic function in diabetic heart. *Am J Physiol Circ Physiol*. 2006;291(5):H2237-H2245. doi:10.1152/ajpheart.00427.2006
137. Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA*. 2002;287(19).
138. Chen Z, Chua CC, Gao J, et al. Prevention of ischemia/reperfusion-induced cardiac apoptosis and injury by melatonin is independent of glutathione peroxdiase 1. *J Pineal Res*. 2009;46(2):235-241. doi:10.1111/j.1600-079X.2008.00654.x
139. Crack PJ, Taylor JM, Flentjar NJ, et al. Increased infarct size and exacerbated apoptosis in the glutathione peroxidase-1 (Gpx-1) knockout mouse brain in response to ischemia/reperfusion injury. *J Neurochem*. 2001;78(6):1389-1399. doi:10.1046/j.1471-4159.2001.00535.x
140. Yoshida T, Watanabe M, Engelman DT, et al. Glutathione Peroxidase are Resistant to

Myocardial Ischemia Reperfusion Injury. *J Mol Cell Cardiol*. 1996;1767:1759-1767.
141. Lei XG, Cheng W-H, McClung JP. Metabolic Regulation and Function of Glutathione Peroxidase-1. *Annu Rev Nutr*. 2007;27(1):41-61. doi:10.1146/annurev.nutr.27.061406.093716
142. Shiomi T, Tsutsui H, Matsusaka H, et al. Overexpression of Glutathione Peroxidase Prevents Left Ventricular Remodeling and Failure after Myocardial Infarction in Mice. *Circulation*. 2004;109(4):544-549. doi:10.1161/01.CIR.0000109701.77059.E9
143. Simopoulos AP. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: Nutritional implications for chronic diseases. *Biomed Pharmacother*. 2006;60:502-507. doi:10.1016/j.biopha.2006.07.080
144. Bai Y, Shakeley RM, Attardi G. Tight control of respiration by NADH Dehydrogenase ND5 Subunit gene expression in mouse mitochondria. *Mol Cell Biol*. 2000;20(3):805-815.
145. Rubio-aliaga I. Model organisms in molecular nutrition research. *Mol Nutr Food Res*. 2012;56:844-853. doi:10.1002/mnfr.201100784
146. Chalvon-demersay T, Blachier F, Tomé D, Blais A. Animal models for the study of the relationships between diet and obesity: A focus on dietary protein and estrogen deficiency. *Front Nutr*. 2017;4:1-13. doi:10.3389/fnut.2017.00005
147. Utomo A, Jiang W, Furuta S, et al. Identification of a novel outative non-selenocysteine containing phospholipid hydroperoxide glutathione peroxidase (NPGPx) essential for alleviating oxidative stress generated from polyunsaturated fatty acids in breast cancer cells. *J Biol Chem*. 2004;279(42):43522-43529. doi:10.1074/jbc.M407141200
148. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: Minimum information for publication of wuantitative real-time PCR experiments. *Clin Chem*. 2009;55(4):611-622. doi:10.1373/clinchem.2008.112797
149. Kang JX, Wang J. A simplified method for analysis of polyunsaturated fatty acids. *BMC Biochem*. 2005;6(5):1-4. doi:10.1186/1471-2091-6-5
150. Ichihara K, Fukubayashi Y. Preparation of fatty acid methyl esters for gas-liquid chromatography. *J Lipid Res*. 2009;51(3):635-640. doi:10.1194/jlr.d001065
151. Zeb A, Ullah F. A simple spectrophotometric method for the determination of thiobarbituric acid reactive substances in fried fast foods. *J Anal Methods Chem*. 2016;2016. doi:10.1155/2016/9412767
152. Gao X-M, Dart AM, Dewar E, Jennings G, Du X-J. Serial echocardiographic assessment of left ventricular dimensions and function after myocardial infarction in mice. *Cardiovasc Res*. 2000;45(2):330-338. http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L30069498%5Cnhttp://dx.doi.org/10.1016/S0008-6363(99)00274-6%5Cnhttp://sfx.library.uu.nl/utrecht?sid=EMBASE&issn=00086363&id=doi:10.1016/S0008-6363(99)00274-6&atitle=Serial+echocardi.
153. Gao S, Ho D, Vatner DE, Vatner SF. Echocardiography in Mice. *Curr Protoc Mouse Biol*. 2011;1:71-83. doi:10.1002/9780470942390.mo100130.Echocardiography
154. Qi J, Yu J, Tan Y, et al. Mechanisms of Chinese medicine xinmailong's protection against heart failure in pressure-overloaded mice and cultured cardiomyocytes. *Sci Rep*. 2017;7(42843):1-12. doi:10.1038/srep42843
155. Zhang J, Critchley LAH, Huang L. Five algorithms that calculate cardiac output from the arterial waveform: a comparison with Doppler ultrasound. *Br J Anaesth*. 2015:392-402. doi:10.1093/bja/aev254

156. Razminia M, Trivedi A, Molnar J, et al. Validation of a new formula for mean arterial pressure calculation: The new formula Is superior to the standard formula. *Charact Cardiovasc Interv*. 2004;63:419-425. doi:10.1002/ccd.20217
157. Niki E. Lipid peroxidation: Physiological levels and dual biological effects. *Free Radic Biol Med*. 2009;47(5):469-484. doi:10.1016/j.freeradbiomed.2009.05.032
158. Morrow EH, Dowling DK, Camus MF, Wolf JBW, Morrow EH, Dowling DK. Single nucleotides in the mtDNA sequence modify mitochondrial molecular function and are associated with sex-specific effects on fertility and aging. *Curr Biol*. 2015;25(20):2717-2722. doi:10.1016/j.cub.2015.09.012
159. Sasidharan SR, Joseph JA, Anandakumar S, et al. An experimental approach for selecting appropriate rodent diets for research studies on metabolic disorders. *Biomed Res Int*. 2013;2013:1-9.
160. Warden CH, Fisler JS. Compairsons of diets used in animal models of high fat feeding. *Cell Met*. 2008;7(4):2008-2010.
161. Ghnimi S, Budilarto E, Kamal-eldin A. The new paradigm for lipid oxidation and Insights to microencapsulation of omega-3 fatty acids. *Compr Rev Food Sci Food Saf*. 2017;16:1206-1218. doi:10.1111/1541-4337.12300
162. Honma T, Tsuduki T, Sugawara S, et al. Aging decreases antioxidant effects and increases lipid peroxidation in the Apolipoprotein E deficient mouse. 2013;52(3):234-240. doi:10.3164/jcbn.12
163. Haan JB De, Witting PK, Stefanovic N, et al. Lack of the antioxidant glutathione peroxidase-1 does not increase atherosclerosis in C57BL/J6 mice fed a high-fat diet. 2006;47. doi:10.1194/jlr.M500377-JLR200
164. Fu Y, Cheng W-H, Porres JM, Ross, Deborah A, Lei WG. Knockout of cellcular glutathione peroxidase gene renders mice susceptibly to diquat-induced oxidative stress. *Free Radic Biol Med*. 1999;27(99):605-611.
165. Cheng W, Ho Y, Valentine BA, Ross DA, Combs GF, Lei XG. Cellular glutathione peroxidase is the mediator of body selenium to protect against paraquat lethality in transgenic mice. *Boichemical Mol Roles Nutr*. 1998;128:1070-1076.
166. Esposito LA, Kokoszka JE, Waymire KG, Cottrell B, Grant R, Wallace DC. Mitochondrial oxidative stress in mice lacking glutathione peroxidase-1 gene. *Free Radic Biol Med*. 2011;28(5):754-766.
167. Nowak MA, Boerlijst MC, Cooke J, Smith JM. Evolution of genetic redundancy. *Nature*. 1997;388(6638):167-170. doi:10.1038/40618
168. Hariri N, Thibault L. High-fat diet-induced obesity in animal models. *Nutr Res Rev*. 2010;23(2):270-299. doi:10.1017/S0954422410000168
169. Flórez-Vargas O, Brass A, Karystianis G, et al. Bias in the reporting of sex and age in biomedical research on mouse models. *Elife*. 2016;5:1-14. doi:10.7554/eLife.13615
170. Jackson SJ, Andrews N, Ball D, et al. Does age matter? The impact of rodent age on study outcomes. *Lab Anim*. 2017;51(2):160-169. doi:10.1177/0023677216653984
171. Thu VT, Kim HK, Ha SH. Glutathione peroxidase 1 protects mitochondria against hypoxia / reoxygenation damage in mouse hearts. *Eur J Physiol*. 2010;460:55-68. doi:10.1007/s00424-010-0811-7
172. Yue P, Jing S, Liu L, et al. Association between mitochondrial DNA copy number and cardiovascular disease : Current evidence based on a systematic review and. *PLoS One*. 2018;13(11):1-15.

173. Cikes M, Solomon SD. Beyond ejection fraction: An integrative approach for assessment of cardiac structure and function in heart failure. *Eur Heart J*. 2016;37:1642-1650. doi:10.1093/eurheartj/ehv510
174. Kerkhof XPLM, Kuznetsova XT, Ali XR, Handly XN. Left ventricular volume analysis as a basic tool to describe cardiac function. *Adv Physiol Educ*. 2019;42:130-139. doi:10.1152/advan.00140.2017
175. Phillips JF, Black M. Unreliability of M-Mode left ventricular dimensions for calculating stroke volume and cardiac output in patients without heart disease. *Chest*. 1982;81:614-619. doi:10.1378/chest.81.5.614
176. Simone G De, Devereux RB, Ganau A, et al. Chamber and stroke volume by limited & mode echocardiography and validation by Two-dimensional and Doppler echocardiography. *Am J Cardiol*. 1996;78:801-807.
177. Tirziu, Daniela MS, Manuscript A, Events E, et al. Cell Communications in the Heart. *Circulation*. 2011;122(9):47-68. doi:10.1161/CIRCULATIONAHA.108.847731.Cell
178. Yu J, Zhang HF, Wu F, et al. Insulin improves cardiomyocyte contractile function through enhancement of SERCA2a activity in simulated ischemia/reperfusion. *Acta Pharmacol Sin*. 2006;27(7):919-926. doi:10.1111/j.1745-7254.2006.00388.x
179. Repetto G, Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc*. 2008;3(7):1125-1131. doi:10.1038/nprot.2008.75
180. Lu SC. Regulation of glutathione synthesis. *Mol Aspects Med*. 2010;30(1-2):42-59. doi:10.1016/j.mam.2008.05.005.REGULATION
181. Golbidi S, Botta A, Gottfred S, Nusrat A, Laher I, Ghosh S. Glutathione administration reduces mitochondrial damage and shifts cell death from necrosis to apoptosis in ageing diabetic. *Br J Pharmacol*. 2014;171:5345-5360. doi:10.1111/bph.12847
182. Ghallab A. In vitro test systems and their limitations. *EXCLI J*. 2013;12:1024-1026. doi:10.17877/DE290R-7558
183. Zhou P, Pu WT. Recounting cardiac cellcular composition. *Circ Res*. 2016;118(3):923-930. doi:10.1016/j.clinbiochem.2015.06.023.Gut-Liver
184. Pereira SL, Ramalho-Santos J, Branco AF, Sardão VA, Oliveira PJ, Carvalho RA. Metabolic remodeling during H9c2 myoblast differentiation: Relevance for in vitro toxicity studies. *Cardiovasc Toxicol*. 2011;11(2):180-190. doi:10.1007/s12012-011-9112-4
185. Zinck A, Newen A. The H9C2 cell line and primary neonatal cardiomyocte cells show similar hypertrophic responses in vitro. *Vitr Cell Dev Biol*. 2011;47:125-131. doi:10.1007/sl
186. Nilsson S, Helou K, Walentinsson A, Szpirer C, Nerman O, Ståhl F. Rat-mouse and rat-human comparative maps based on gene homology and high-resolution zoo-FISH. *Genomics*. 2001;74(3):287-298. doi:10.1006/geno.2001.6550
187. Xiong Y, Liu X, Lee CP, Chua BHL, Ho YS. Attenuation of doxorubicin-induced contractile and mitochondrial dysfunction in mouse heart by cellular glutathione peroxidase. *Free Radic Biol Med*. 2006;41(1):46-55. doi:10.1016/j.freeradbiomed.2006.02.024
188. Van Tonder A, Joubert AM, Cromarty AD. Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC Res Notes*. 2015;8(1):1-10. doi:10.1186/s13104-015-1000-8

189. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem*. 2000;267(17):5421-5426. doi:10.1046/j.1432-1327.2000.01606.x
190. Zalata AA, Lammer Tijn N, Christophe A, Comhaire FH. The correlates and alleged biochemical background of the resazurin reduction test in semen. *Int J Androl*. 1998;21(5):289-294. doi:10.1046/j.1365-2605.1998.00126.x
191. Lamkanfi M, Declercq W, Kalai M, Saelens X, Vandenabeele P. Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death Differ*. 2002;9(4):358-361. doi:10.1038/sj.cdd.4400989
192. Lamkanfi M, Kanneganti TD. Caspase-7: A protease involved in apoptosis and inflammation. *Int J Biochem Cell Biol*. 2010;42(1):21-24. doi:10.1016/j.biocel.2009.09.013
193. Wu G, Fang Y-Z, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. *J Nutr*. 2004;134(3):489-492. doi:10.1093/jn/134.3.489
194. Akerboom TPM, Bilzen M, Sies H. The relationship of biliary glutathione disulfide efflux and intracellular glutathione disulfide content in perfused rat liver. *J Biol Chem*. 1982;257(8):4248-4252.
195. Hall AG. The role of glutathione in the regulation of apoptosis. *Eur J Clin Invest*. 1999;29(3):238-245. doi:10.1046/j.1365-2362.1999.00447.x
196. Shanks N, Greek R, Greek J. Are animal models predictive for humans? *Philos Ethics, Humanit Med*. 2009;4(1):1-20. doi:10.1186/1747-5341-4-2
197. Mates JM, Perez-Gomez C, Nunez De Castro I. Antioxidant enzymes and human diseases. *Clin Biochem*. 1999;32(8):595-603.
198. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. *World Allergy Organ J*. 2012;5(1):9-19. doi:10.1097/WOX.0b013e3182439613
199. Reich HJ, Hondal RJ. Why nature chose selenium. *ACS Chem Biol*. 2016;11:821-841. doi:10.1021/acschembio.6b00031