GLUTATHIONE DYSREGULATION, CARDIAC OXIDATIVE STRESS, AND INFLAMMATION DURING N-6 POLYUNSATURATED FATTY ACID OVERLOAD

by

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Glutathione Dysregulation, Cardiac Oxidative Stress, and Inflammation during n-6 Polyunsaturated Fatty Acid Overload

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Abstract

Historically dietary saturated fats were blamed for cardiovascular disease (CVD). However, lowering dietary saturated fats did not curb CVD rates and instead detrimental roles of n-6 polyunsaturated fatty acid (n-6 PUFA) which was used to substitute saturated fats have come to the forefront. My objective was to identify mechanisms leading to dietary n-6 PUFA induced CVDs using mice and cell models. Here isocaloric diets rich in n-6 PUFA or monounsaturated fatty acids (MUFA) were used in vivo or cardiac cells like cardiomyocytes and fibroblasts were incubated with n-6 PUFA or MUFA in vitro. We established that n-6 PUFA reduced glutathione (GSH), promoted oxidative stress and impaired mitochondrial function. n-6 PUFA diets also increased pro-inflammatory cytokines and impaired GSH synthesis in vivo. Removal of a primary pro-inflammatory stimulus by using mice deficient in monocyte chemotactic protein 1 (MCP-1) restored GSH and lowered inflammation in n-6 PUFA-fed MCP-1/- mice (chapter 2). Although inflammatory biomarkers were high in n-6 PUFA incubated cells, treatment with LPS lowered murine macrophage function suggesting a dysregulated immune response. This dysregulated immune response was also reversed by increasing GSH in macrophages. These data indicate that n-6 PUFA increases inflammatory biomarkers but impairs macrophage function due to GSH depletion (chapter 3). Finally, as cell death is a major contributor to CVD, my final chapter 4 showed that under cardiac stress induced by beta-adrenergic agonist, isoproterenol, n-6 PUFA promotes necrosis, increases in cytochrome P450-induced metabolites and a reduction in DNA repair genes. Overall, these results show the key roles of GSH dysregulation in n-6 PUFA induced inflammation, which could be key novel mediator of PUFA-specific cardiotoxicity in the Western world.
Preface

Information on fatty acids and oxidative stress in the Introduction section was modified Beam, J., [Botta, A.], Barendregt, R., Ghosh, S. (2014) Redox Signaling and the Heart. In 'Systems of Biology of Free Radicals and Antioxidants'. Laher, I. (Ed.), pp. 1498-1513, Springer Verlag, Germany. Figure 1.1 is a modified version of a figure first created by Dr. Ghosh, with the version used in this thesis being solely made by me. The publications associated with each chapter are listed below:

Chapter 2:

Table 2.1 and 2.2: Setting up the high fat diet in mice models that alters cardiac distribution of fatty acids. Published in Beam, J.*, Botta, A*. et al. (2015) Excess linoleic acid increases collagen I/III ratio and 'stiffens' the heart muscle following high fat diets. The Journal of Biological Chemistry 290(38): 23371-23384. Joint First authors

Figure 2.1: Published in Botta et al. (2013) Short Term Exercise Induces PGC-1α, Ameliorates Inflammation and Increases Mitochondrial Membrane Proteins but Fails to Increase Respiratory Enzymes in Diabetic Hearts. PloS ONE 8(8): e70248.

Figure 2.2: Preliminary data for Figure 3 in Beam, J.*, Botta, A*. et al. (2015) Excess linoleic acid increases collagen I/III ratio and 'stiffens' the heart muscle following high fat diets. The Journal of Biological Chemistry 290(38): 23371-23384. Joint First authors

Chapter 3:

Chapter 4:

Mechanisms explaining n-6 PUFA mediated cardiotoxicity: focus on inflammation and cell death. Botta, A., Ghosh, S. (In prep)

**Table 4.2:** Basal heart function data published in Beam, J.*, Botta, A*. et al. (2015) Excess linoleic acid increases collagen I/III ratio and 'stiffens' the heart muscle following high fat diets. The Journal of Biological Chemistry 290(38): 23371-23384. Joint First authors

*Salmonella* experiments conducted in chapter 3 were done in collaboration with Dr. Deanna Gibson and were performed with the help of Dr. Yee Kwan Chan. Echocardiography presented in chapter 4 was done in collaboration with Dr. Kathleen McLeod and the exams were performed by Dr. Hesham Soliman. The analysis of qPCR arrays shown in Figure 4.1 was done in collaboration with Dr. Deanna Gibson and was conducted by Nijiati Abulizi using Ingenuity Pathway Analysis Software from Qiagen. All animal experiments conducted in this thesis was reviewed and approved by the UBC Animal Care Committee.
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<td>alpha linolenic acid</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>aminopeptidase</td>
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<td>DAPI</td>
<td>4',6'-Diamidino-2'-phenylindole dihydrochloride</td>
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<td>DCF</td>
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<td>DHET</td>
<td>dihydroxyeicosatrienoic acid</td>
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<td>DIC</td>
<td>dicarboxylate carrier</td>
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<td>deionised water</td>
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<td>DMEM</td>
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<td>Dnaja1</td>
<td>DnaJ heat shock protein family (Hsp40) member A1</td>
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<td>DSBs</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EET</td>
<td>epoxyeicosatrienoic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ETC</td>
<td>electron transport chain</td>
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<td>FBS</td>
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<td>FID</td>
<td>flame ionization detector</td>
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<td>Fmo4</td>
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<td>glutathione peroxidase</td>
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<td>glutathione reductase</td>
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GS glutathione synthetase
GSH glutathione
GSNO S-nitrosoglutathione
GSSG glutathione disulfide
GST glutathione S-transferase
Gstm1 glutathione S-transferase mu 1
GTPase guanosine tri phosphatase
H2DCFDA 2′7′-dichlorodihydrofluorescein diacetate
H2O2 hydrogen peroxide
HETE hydroxyeicosatetraenoic acid
HR homologous recombination
Hmox1 heme oxygenase 1
Hmox2 heme oxygenase 2
Hsp heat shock proteins
Hspa4 heat shock protein family A (Hsp70) member 4
Hspa5 heat shock protein family A (Hsp70) member 5
Hspa8 heat shock protein family A (Hsp70) member 8
Hspa11 heat shock protein family A (Hsp70) member 1 like
Hspb1 heat shock protein family B (small) member 1
Hspd1 heat shock protein family D (Hsp60) member 1
Hspe1 Heat Shock Protein Family E (Hsp10) Member 1
IFNG interferon gamma
IL-1alpha interleukin-1 alpha
IL-6 interleukin-6
IL-10 interleukin-10
IL-12 interleukin-12
IPGTT intraperitoneal glucose tolerance testing
ISO isoproterenol
JC1 566-tetrachloro-11-33-tetraethylbenzimidazolcarbocyanine iodide
KC chemokine C-X-C motif ligand 1
LA linoleic acid
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<td>lipopolysaccharide</td>
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<td>MOI</td>
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<td>SFA</td>
<td>saturated fatty acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>Sod1</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>Sod2</td>
<td>Superoxide dismutase 2</td>
</tr>
<tr>
<td>SPI</td>
<td><em>Salmonella</em> pathogenicity island</td>
</tr>
<tr>
<td>SSBs</td>
<td>single stranded breaks (DNA)</td>
</tr>
<tr>
<td>ST</td>
<td><em>Salmonella enterica</em> Typhimurium</td>
</tr>
<tr>
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<td>streptozotocin</td>
</tr>
<tr>
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<td>tissue growth factor</td>
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<tr>
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<td>transforming growth factor beta 3</td>
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<tr>
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<td>tumor necrosis factor alpha</td>
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<tr>
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</tr>
<tr>
<td>XRCC2</td>
<td>X-ray repair cross complementing 2</td>
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Dedication

This thesis is dedicated to the original Dr. Botta, you will forever be missed.
Chapter 1: Introduction

Since the beginning of the industrial revolution, there have been continual advances in science and medicine towards cures for various ailments impinging on society. Two of these ailments, which have now been studied for decades, are diabetes mellitus and cardiovascular disease (CVD). Both diabetes mellitus and CVD represent a significant economic and clinical burden annually, especially in Western countries. In 2015, 9.1 million Canadians suffered from diabetes or prediabetes. This number is expected to increase to 11.4 million by 2025. In Canada, the total economic cost of diagnosed diabetes was 12.2 billion in 2005 and is expected to increase by another 4.7 billion by 2020. With respect to CVDs, ischemic heart disease is the second leading cause of death in Canada resulting in over 48,000 deaths in 2012. Currently, CVD represents an annual cost of 12 billion dollars in Canada. This number is expected to increase as 90% of Canadians over the age of 20 have at least one risk factor for CVD.

1.1 The structure and function of the heart muscle

The cardiovascular system is an organ system that circulates blood and transports nutrients, oxygen, carbon dioxide and hormones throughout the body. The primary components include the heart and the circulatory system, which is comprised of the blood, lymph, lymphatic fluid, and blood vessels. The heart, as it is constantly contracting and relaxing, requires a continuous supply of energy. While the heart is capable of using energy sources such as lactate, ketones and amino acids, the primary sources utilized are glucose and
fatty acids. Under normal physiological conditions, fatty acids are the preferred energy source. Up to 60-70% of the acetyl-CoA created in the heart is derived from fatty oxidation, whereas the remaining 30-40% of acetyl-CoA comes from glucose.

The heart is an obligate aerobic organ, meaning that a constant supply of oxygen is required for function. At rest, the mammalian heart on average consumes between 8-15ml O₂/min/100g of tissue, which is 3-5 times more oxygen than is required by the brain. When exercising this value can increase to 70ml O₂/min/100g of heart tissue. While the majority of mammalian hearts function in a similar manner, differences in size and metabolic rate can give rise to small discrepancies between species.

While there are similarities in the structure between mice and human hearts, their variation in size gives rise to several key differences. In particular, the human heart has an average rate of 70 beats per minute. In contrast, the mouse heart has an average rate of around 600 beats per minute. In a human, it takes approximately 1 minute for the blood to fully circulate the body. In a mouse, because of their small size, blood to fully circulates in 15 seconds. Over the human lifespan (75-85 years), the heart will beat approximately 2.5 billion times. In contrast, over the lifespan of a mouse (2-3 years), the heart will beat approximately 700 million times.

The heart comprises a total of three layers. The outermost layer is known as the epicardium, followed by the myocardium, with the innermost being the endocardium. The primary function of the epicardium is for protection; therefore it is mainly composed of connective tissue. The middle layer, the myocardium, contains the muscle cells of the heart, and of the three layers, is the thickest. The final layer, the endocardium, is the layer which lines the chambers of the heart, and is composed of a single layer of endothelial cells.
1.1.1 Cardiac cell types

Within the cardiovascular system, there are several predominant cell types, which can be found within the different layers of the heart. These major cell types include smooth muscle, endothelial, cardiomyocytes and resident immune cells. While not found directly in the heart, smooth muscle cells are found on the coronary arteries and veins and play a major role in the movement of blood into and out of the heart. Smooth muscle cells have a single nucleus and are spindle-shaped. Their particular shape allows them to be closely interconnected and to contract simultaneously. Their main function is to contract and to extend ducts, organs and sacs. Therefore, they play an important role in the movement of blood throughout the body.

Like smooth muscle cells, endothelial cells are found in the coronary arteries and veins but are also present in the endocardium of the heart. Of all the different cell types, they are considered to be the most numerous non-cardiomyocyte type, comprising 60% of all cells in the heart. The primary function of these cells is to provide a lining; as such they form a thin, one-cell thick layer covering the blood and lymphatic vessels. Research has shown that depending on location, that there are differences in the regulation of these cells. For example, endocardial endothelium cells may have different morphology and function than vascular endothelium cells. Dysfunction in the endothelial cells is associated with an increased occurrence of negative cardiovascular events.

Of all the different types of cells in the heart recent data suggests that in the heart, next to endothelial cells, cardiomyocytes are the most abundant cell type. Cardiomyocytes are the muscle cells of the heart and therefore their primary function is contraction. Each
cardiomyocyte is composed of myofibrils, which contain contractile units known as sacromeres. Sacromeres are composed of the contractile proteins actin and myosin. Along with other proteins, troponins and tropomyosin, these proteins have an integral role in cardiac regulation. As their primary function is contraction, cardiomyocytes require large amounts of adenosine triphosphate (ATP). In order to fulfill this increased requirement for ATP, mitochondria comprise 30-40% of the intracellular volume. Within cardiomyocytes there are also large stores of calcium which are essential for regulating mitochondria. Specifically calcium is required for the activation of mitochondria to produce ATP. Calcium is released from the sarcoplasmic reticulum for contraction of cardiac muscle. As well, by sequestering calcium, cardiomyocytes help to prevent calcium overload which can lead to the production of reactive oxygen species (ROS) and/or apoptosis.

After cardiomyocytes and endothelial cells, fibroblasts are the next numerous cell type in the heart. They are spindle shaped and contain multiple cytoplasmic extensions. When activated, fibroblasts can take on a stellate shape. Along with cardiomyocytes, fibroblasts are also found in the myocardium, specifically within the connective tissue where they play a crucial role in ensuring proper function of cardiomyocytes. This function is carried out by producing mitogens, similar to tissue growth factors (TGFs), which lead to the development of cardiomyocytes and remodeling. One of the primary functions of fibroblasts is maintaining the extracellular matrix (ECM). As the main cell type involved in the production of the ECM, fibroblasts are responsible for the production of growth factors and other mediators which help produce and remodel this matrix.

Unlike, cardiomyocytes and fibroblasts, the existence and role of resident cardiac immune cells is a recent discovery. Resident cardiac immune cells include mast cells and
macrophages. In the heart, approximately 5-10% of all non-cardiomyocyte cells are hematopoietic-derived cells. In the normal resting heart, the majority of the immune cells are macrophages. These cells are predominately found in the epicardium surrounding the endothelial cells, but are also found in the interstitial space between the cardiomyocytes. Besides macrophages, other immune cells, such as mast cells, dendritic cells, B cells, and T cells are also present, but at lower levels. To date, in the resting normal heart, neutrophils and monocytes have not been found.

1.2 Cardiovascular disease

CVD is a broad term encompassing diseases that occur in the heart or blood vessels. This includes coronary artery diseases including myocardial infarction, stroke, cardiomyopathy and other diseases. There are several underlying risk factors that have been shown to contribute to the development and progression of CVD. These risk factors include high blood pressure, lack of exercise, poor diet, obesity, diabetes, smoking and high blood cholesterol.

1.3 Role of inflammation in cardiovascular disease

No matter the cause, a common hallmark of any type of CVD is inflammation. Currently the exact role of inflammation in the development and progression of CVDs is not fully understood. Chronic inflammation is considered a significant risk factor for the development of CVD, yet the mechanisms behind its development and progression, with
respect to CVD are unclear\textsuperscript{29,30}. An example of an inflammatory CVD is atherosclerosis. Atherosclerosis is a chronic condition whereby lesions comprising immune cells and the surrounding tissue form in the arteries and release pro-inflammatory cytokines. Eventually, these lesions or plaques can rupture leading to clotting and development of other CVDs \textsuperscript{31}. One striking characteristic of atherosclerosis is the accumulation of macrophages within the plaque. These macrophages internalize and become loaded with oxidized lipoprotein particles, rendering them immobile and turning them into foam cells. Foam cells originate from M2 macrophages. After entering the area in response to inflammatory signals they become laden with lipids after trying to engulf and destroy the lipid material, giving them their characteristic foamy appearance. While not inherently dangerous foam cells can become the center of atherosclerotic plaques, leading to a narrowing of the arteries \textsuperscript{32}.

Besides atherosclerosis, increased levels of inflammatory biomarkers are associated with heart failure. In elderly patients without a previous history of myocardial infarction, increased levels of IL-6 were associated with an increased risk of congestive heart failure \textsuperscript{33}. In a separate study, increased levels of tumor necrosis factor alpha (TNFα) in patients after a myocardial infarction greatly increased their risk for subsequent vascular events \textsuperscript{34}.

Another condition with an inflammatory component is obesity. Studies have shown that obese patients have an elevated level of inflammation. This is a direct result of increased secretion of pro-inflammatory mediators from adipocytes \textsuperscript{35}. Recent evidence has shown that obesity is also a risk factor for the development of CVD \textsuperscript{36}. As mentioned, while it is recognized that inflammation is an important risk factor for the development of CVDs the exact role it plays in the CVD development remains unclear.
1.3.1 Role of obesity in cardiovascular disease

Obesity is defined as having a body mass index (BMI) greater than 30 kg/m². However, in determining risk of CVD the waist to hip ratio has been shown to be a more accurate indicator of determining risk of CVD than BMI 37,38. Nevertheless, BMIs greater than 30, patients are at a significant risk for early development of atherosclerosis 39, myocardial infarction 37 and heart failure 40. Some of the main obesity related risk factors, which also contribute to the development of heart disease, are hypertension, dyslipidemia, impaired heart function and the development of insulin resistance40-42. As well the presence of chronic inflammation, due to increased basal levels of cytokines, also heightens the patients risk for development of CVD 43.

1.3.1.1 Cardiovascular disease and obesity in Canada

In 2014 more than 40.0% of Canadians were overweight and 20.2% were obese 44. Obesity, particularly abdominal obesity, has been recognized as a major risk factor for the development of CVD 40,45. As well, development of obesity during childhood, has been shown to be related to increased risk of CVD during adulthood 46. Typically on average it is recommended that humans consume around 2,000 kilocalories a day, depending on activity level. However, statistics show that the majority of Canadians are consuming approximately 500 more kilocalories daily then what is required, with the majority of this increase coming from higher intakes of carbohydrate (11% increase) and fat (18% increase) 47. With respect to the composition of this increase in fat, over the last four decades intake of saturated fatty
acids (SFA) has remained relatively constant due to concerns over levels of cholesterol. Instead this increase in fats is primarily due to increased consumption of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) \(^{44}\). In order to understand the causes for such a rise in MUFA and PUFA fat consumption, we need to appreciate the history of CVD research.

1.3.2 History of lipids and cardiovascular disease research

Since the early 1900s, scientists have been conducting experiments to determine the effect of certain dietary components on the development of CVD. One of these components was cholesterol. One of the first recorded experiments involving cholesterol and the development of heart disease occurred in 1908. That experiment, conducted in rabbits, provided evidence for the role of cholesterol in the development of atherosclerosis \(^{48}\). In the years that followed, other researchers confirmed the role of cholesterol in exacerbating the development of atherosclerosis. Therefore, by the early 1950s, society started looking for ways to reduce cholesterol consumption in an attempt to reduce the number of cases of heart disease \(^{49-51}\).

Cholesterol is a lipid molecule synthesized by all animals and is structurally important in cell membranes as it increases membrane fluidity and provides structural support. Besides its structural role in cell membranes, it is also a precursor for the synthesis of steroid hormones and bile. However, at the start of the 19th century, cholesterol’s role in the development of atherosclerosis was beginning to unfold \(^{52}\). An investigation of the plaques formed during atherosclerosis revealed that they contained high amounts of
cholesterol. Numerous animal studies were then conducted that showed dietary cholesterol induced both hypercholesterolemia and atherosclerosis\textsuperscript{53}. Furthermore, it was found that individuals with higher levels of serum cholesterol were more likely to develop atherosclerotic plaques\textsuperscript{53}. Finally, it was observed that intake of cholesterol subsequently led to an increase in total plasma cholesterol levels\textsuperscript{52}.

In a landmark study, using data from seven countries (United States, Finland, the Netherlands, Italy, Yugoslavia, Greece and Japan), Dr. Ancel Keys showed that the risk of cardiovascular events was directly related to the level of total serum cholesterol\textsuperscript{54,55}. It is primarily from the research of Dr. Keys that the ‘diet heart hypothesis’ was formulated. This hypothesis stated that a diet high in cholesterol and fats would lead to increased serum cholesterol levels and this would then lead to the development of atherosclerosis and other CVDs\textsuperscript{56}.

Therefore, as research mounted on the role of cholesterol in heart disease progression, researchers searched for cholesterol free alternatives. As it was thought that diets rich in animal fat were responsible for increased cholesterol, a push was made to switch to low cholesterol diets composed of a higher amount of carbohydrates and fats from plant based sources\textsuperscript{57,58}. While all animal cells produce cholesterol, plant cells either contain none, or minute quantities of cholesterol\textsuperscript{59}. Animal cells contain high levels of SFA within the plasma membranes. As such cholesterol is needed to increase fluidity at low temperatures, and at high temperatures it increases the stability of the membranes. Therefore if there is a higher intake of unsaturated fatty acids, than less cholesterol is needed in the plasma membranes\textsuperscript{60}.

It was this research along with the formation of a bipartisan, non-legislative select committee on Nutrition and Human Needs by Senator George McGovern that led to changes
in nutrition policy in the United States that are still observed today\textsuperscript{61,62}. Between 1976 and 2005 in Canada, the consumption of different types of fat was altered. In 1976, Canadians were consuming 86.49 g of total fat whereas in 2005 this number had increased to 102.49 g per day. Over this time period, the amount of SFA remained consistent therefore, as mentioned earlier, this increase in daily fat consumption was primarily due to increases in MUFA and PUFA. During this 30 year time period, the amount of MUFA consumed increased by 22\%, whereas the amount of PUFA consumed increased by 54\% \textsuperscript{44}.

1.4 Dietary fatty acids

Within our diet, the fatty acids we consume can be divided into several classifications. The two main classifications are that of saturated and unsaturated fatty acids, and are based on the number of double bonds. SFA contain no double bonds whereas unsaturated fatty acids contain one or more double bonds\textsuperscript{63}.

The presence of double bonds can drastically alter the physical characteristics of the fats\textsuperscript{64}. SFA are waxy solids at room temperature. While most fat sources contain an assortment of differing fatty acids, the most common sources of saturated fat are from red meat and dairy products, such as butter. The two most common SFA found in animal products are palmitic acid (C16:0) and stearic acid (C18:0). In contrast to animals, plants may be rich in either medium or long chain fatty acids. For example, coconut oil is composed primarily of the medium chains lauric (C12:0) and myristic (C14:0) acids. Whereas palm oil contains longer chains, which similar to animal fats, predominantly contains palmitic acid\textsuperscript{63}. 
There are two main types of unsaturated fatty acids: MUFA and PUFA. MUFAs contain only a single double bond, an example being oleic acid (C18:1n9), the main component of olive oil. PUFA can be divided into two broad classes based on the position of the double bond. PUFAs with double bonds starting at the third carbon from the end of the carbon chain are termed n-3 PUFAs. Whereas PUFAs with the double bond starting at the sixth carbon from the end of the chain are known as n-6 PUFAs.

1.4.1 History of type of dietary fats and cardiovascular disease

Since the early 1950s, research has been conducted on the differing effects of dietary fats on heart disease. Initially, as mentioned above, it was thought that because of their cholesterol content SFA were responsible for the development of CVD. In an attempt to reduce the prevalence of CVD, plant based oils were advocated as an alternative to cholesterol containing animal fats. However, since the inclusion of plant based oils in the Western diet, research surrounding their effects on CVD has been inconclusive. Of the plant-based oils, there are two broad classes used widely in our diet, MUFA and n-6 PUFA. While n-3 PUFAs are important from a dietary standpoint, they are present at much lower levels than MUFA and n-6 PUFA. Both MUFA and PUFA are derived from plants, but they have markedly different effects on heart health. For example, initially it was thought that n-6 PUFA were cardioprotective. However, recent evidence has shown that they may in fact be cardiotoxic.

Beginning as early as the 1960s and 1970s, the use of n-6 PUFA as a "heart healthy" alternative was widely reported. Controlled clinical trials using unsaturated fatty acids
showed evidence of cardioprotective effects, but this was mainly because reduction in the amount of serum cholesterol levels was found\textsuperscript{70,71}. Early on, it was discovered that n-6 PUFAs do indeed help lower the levels of circulating cholesterol\textsuperscript{72}. However, in studies this lowering of cholesterol did not always translated into not cardioprotective effects. A study published in 1965 found that although n-6 PUFA did indeed lower cholesterol levels in ischemic heart disease patients, after a two year follow up n-6 PUFA caused a reduced survival rate\textsuperscript{73}.

Currently, n-6 PUFAs are a subject of controversy. Conflict exists between researchers over their effects on the heart, and whether or not present dietary recommendations match the available scientific evidence. Of all the different types of fatty acids, PUFAs are prone to oxidation, specifically peroxidation, as they contain multiple double bonds\textsuperscript{74}. Recently, a meta-analysis has suggested that the harmful effects of n-6 PUFA peroxidation may outweigh the positive effects of serum cholesterol reduction\textsuperscript{75}.

In contrast, MUFAs have generally been shown to be cardioprotective\textsuperscript{76}. Unlike the other types of dietary fatty acids, to date there has been little controversy about the effects of MUFA on heart disease risk. As MUFAs only have a single double bond they are less susceptible to peroxidation than n-6 PUFA\textsuperscript{77}. Even during the 1980s, when the debate over the role of saturated fat in heart disease raged on, MUFAs were considered to be a "heart healthy" alternative; as similar to n-6 PUFA they led to a reduction in plasma cholesterol and low density lipoprotein (LDL)\textsuperscript{78}. When replacing SFA with either carbohydrate or MUFA in the diet, MUFA was found to have less adverse effects such as the occurrence of hyperglycemia. This difference is primarily due to the fact that unlike carbohydrates, MUFA intake did not cause a increase in blood glucose and insulin\textsuperscript{79}. Along with MUFA, generally
n-3 PUFA is thought to be cardioprotective. However some research in other tissues has shown that n-3 PUFA, due to a lowering of immune responses, may be detrimental during infection\textsuperscript{80}.

1.4.2 Impact of dietary fatty acids on cardiovascular disease

As mentioned above, literature describing the effects of dietary fatty acids on the heart has been mixed. Below is a summary of the current state of the field regarding the effects of MUFA, n-3 PUFA and n-6 PUFA on CVD.

1.4.2.1 Monounsaturated fatty acids

A large scale meta-analysis conducted in 2012 found that there were no reported adverse effects of MUFA on the heart\textsuperscript{76}. While not directly related to CVD, MUFAs were also found to exert a hypoglycemic effect in type 2 diabetic patients\textsuperscript{81}.

The Mediterranean diet is based on the 1960s dietary patterns of individuals in Crete, Greece and southern Italy\textsuperscript{82}. At the time, individuals in this region consumed high amounts of olive oil, legumes, fruit, vegetables and cereals. This was combined with a high consumption of fish, moderate consumption of dairy and wine, and a low consumption of other meat products. While individuals in this area tend to have higher BMIs, there is no corresponding increase in CVD. One of the explanations for this phenomenon was that the increased consumption of MUFAs (from olive oil; OA) and n-3 PUFAs (from fish), coupled with a lower consumption of SFA, was cardioprotective\textsuperscript{83,84}. 
Numerous studies investigating the effects of the Mediterranean diet on heart disease have been conducted\textsuperscript{85-87}. In comparison to traditional western diets, the Mediterranean diet has been shown to lead to a reduction in CVD risk\textsuperscript{88}. For example, in a study of close to 75,000 women without a history of CVD food frequency questionnaires, 6 times over a period of 20 years, were used to examine the composition of the women's diet over time\textsuperscript{87}. After a 20-year follow-up, women who followed the Mediterranean diet were at a much lower risk for the development of CVD.\textsuperscript{87} In a meta-analysis, looking at the effects of adherence to the Mediterranean diet and health status, it was found that the higher a person's adherence to the diet, the better their health status. As well, increased adherence led to decreased all-cause mortality and decreased CVD mortality. Besides cardiovascular rate, increased adherence was also found to lead to a reduction in the occurrence of Parkinson's disease and cancer\textsuperscript{88}.

1.4.2.2 n-3 polyunsaturated fatty acids

As mentioned above the Mediterranean diet, besides consisting of high amounts of MUFA, contains higher levels of n-3 PUFA than the Western diet. In the Western diet, the main source of n-3 PUFA is from canola and soy bean oil\textsuperscript{89}. Both of these oils contain alpha linoleic acid (ALA), which is then converted into longer chain fatty acids such as eicosapentaenoic acid (EPA: C20:5n-3) and docosahexaenoic acid (DHA)\textsuperscript{90}. In contrast to plant based oils, which are rich in ALA, fish are rich in EPA and DHA, and are the main dietary source for these two fatty acids\textsuperscript{89}. 
Due to the low consumption of fish in the western diet the main source of long chain n-3 PUFA is through supplementation. n-3 PUFAs are generally considered to be anti-inflammatory. Both EPA and DHA reduce the production of pro-inflammatory prostaglandins and thromboxanes which are derived from the n-6 PUFA arachidonic acid (ARA). Therefore an increased level of n-3 PUFA can result in an anti-inflammatory milieu. Several studies have reported that fish in the diet or fish oil supplementation reduces the risk of CVD. In addition consumption of long-chain omega-3 PUFAs from fish oil has been shown to lower blood pressure, thrombosis, cholesterol levels, triglycerides and reduce the occurrence of arrhythmias. Furthermore addition of n-3 PUFA has been shown to lead to reduce the negative effects, such as reducing inflammation, of the n-6 PUFA rich Western diet.

1.4.2.3 n-6 polyunsaturated fatty acids

n-6 PUFAs are consumed in the North American diet primarily from meats and vegetable oils such as corn, safflower, maize, rapeseed and soybean oils. Interestingly, n-6 PUFAs have been shown to exhibit both positive and negative effects on CVD. Beneficial effects were reported when 5% of daily energy intake from SFAs was replaced by n-6 PUFAs, namely linoleic acid (LA). The study showed a risk reduction of 26% for CVD-linked deaths, a risk reduction of 24% for cardiovascular events and a decrease in circulating LDL. Further research on n-6 PUFAs in the diet have shown lowered plasma concentrations of total and LDL cholesterol, prevention in the development of
atherosclerosis \(^{103}\), and anti-arrhythmic properties \(^{104}\). Collectively, these studies gave evidence that the replacement of SFA with PUFAs was beneficial \(^{100,101,103,104}\).

In recent years it has been continuously reported that n-6 PUFA may also have detrimental effects \(^{67,69,75,105}\). In a meta-analysis of clinical trials which utilized specific interventions with a n-6 PUFA rich diet, n-6 PUFAs failed to exert beneficial effects, and instead showed adverse effects and increased the risk of cardiac disease \(^{67,69,75}\). An increase in dietary LA is commonly thought to be linked to inflammatory and thrombotic events \(^{106}\).

Additionally, a high n-6, low n-3 PUFA diet has been linked to several pro-inflammatory conditions such as insulin resistance \(^{107-109}\), atherosclerosis \(^{110,111}\), colorectal cancer \(^{112,113}\), pancreatic cancer \(^{114}\) and inflammatory bowel disease \(^{115,116}\). With respect to using n-6 PUFAs as a secondary prevention, a reanalysis of the Sydney diet heart study has shown that when given to patients following a cardiovascular event, an increase in all-cause, CVD and coronary disease mortality are seen in comparison to those not given any intervention \(^{75}\). As well, recently re-analyzed data from the Minnesota heart study has shown that intervention with n-6 PUFA (LA), does not prevent CVD mortality and instead leads to an increase in the rate of mortality compared to controls \(^{69}\). One potential explanation for the detrimental effects of n-6 PUFA is that of increased oxidative stress \(^{75}\).

LA is the precursor for ARA, which produces potent inflammatory and arrhythmogenic molecules such as prostaglandins I2 (PGI2) and prostaglandin E2 (PGE2) \(^{117}\), and thromboxanes, such as thromboxane A2 (TXA2) \(^{118}\). Therefore, an increase in n-6 PUFAs could lead to adverse health effects and increase the risk of cardiac disease and other inflammatory diseases through increased cytokine production. However, currently the
research on the effects of n-6 PUFA is mixed. As such the mechanisms of n-6 PUFA induced cardiac toxicity remain unclear.

1.5 Oxidative stress

Oxidative stress is the result of an imbalance between antioxidant defense systems and the production of pro-oxidant species, including reactive oxygen and nitrogen species. Although oxygen is required for all aerobic organisms, excess pro-oxidants can be harmful to those organisms. As an example, approximately 0.4-4% of the oxygen used by mitochondria for normal cellular function, is converted into reactive oxygen species (ROS)\textsuperscript{119,120}. ROS encompasses a wide variety of different species of molecules. Examples of ROS include superoxide (O$_2^-$) and the hydroxyl radical (‘OH). Over the years, research showed that damage caused by ROS can result in many human diseases. ROS is an underlying cause of CVD\textsuperscript{121} as well as obesity, aging and multiple diabetic complications\textsuperscript{122}.

1.5.1 Detrimental effects of reactive oxygen species

O$_2^-$ can interact with sulfur iron clusters causing the release of free iron, or can be converted by the enzyme superoxide dismutase (SOD) to hydrogen peroxide. When hydrogen peroxide interacts with free iron, it produces the highly toxic ‘OH radical that can damage DNA, proteins, lipids and carbohydrates. When the ‘OH interacts with membrane lipids, it produces peroxyl radicals that can damage membranes, proteins, and enzymes\textsuperscript{123}. 
To prevent this damage from occurring, hydrogen peroxide is converted by catalase and certain peroxidases to water. The reactions for each of these enzymes are shown below\textsuperscript{124}.

\begin{align*}
\textbf{Superoxide Dismutase:} & \quad \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \quad \rightarrow \quad \text{H}_2\text{O}_2 + \text{O}_2 \\
\textbf{Catalase:} & \quad \text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \quad \rightarrow \quad 2\text{H}_2\text{O} + \text{O}_2 \\
\textbf{Glutathione Peroxidase:} & \quad \text{H}_2\text{O}_2 + 2\text{GSH} \quad \rightarrow \quad 2\text{H}_2\text{O} + \text{GSSG}
\end{align*}

In order to prevent ROS induced cellular damage, there are several different defense systems present in living organisms. One of the major defense mechanisms is that of antioxidants. While ROS is often put in a negative light, ROS play an essential role in cellular systems. For example, while ROS can have harmful effects at high levels, it is also required for proper functioning of the immune system\textsuperscript{9}. The positive side of ROS will be explored in a later section of this introduction.

1.5.2 Antioxidants

Antioxidants are chemical molecules that can transfer or accept electrons from other substances, such as ROS. They can prevent oxidative stress through neutralization reactions with ROS. Antioxidants can be subdivided into either exogenous or endogenous\textsuperscript{125}. Exogenous antioxidants are supplied through the diet from antioxidant-rich foods and supplements. In contrast, endogenous antioxidants are synthesized \textit{de novo} inside the organism\textsuperscript{126}. 
1.5.2.1 Exogenous antioxidants

Exogenous antioxidants and cofactors include vitamins such as vitamin A, C and E, minerals such as zinc and selenium, and enzymes such as coenzyme Q10. Of particular interest to this thesis is the report that addition of coenzyme Q10 to rats fed an n-6 PUFA rich diet prevented the detrimental effects of n-6 PUFAs. As well, a recent in vitro study looking at placental cell function in pregnant type 1 diabetic women found that redox status was increased and proliferation of cells was decreased with administration of either n-3 or n-6 PUFA. However addition of either vitamin C/E with n-3 or n-6 PUFA restored cell proliferation and decreased redox status in these cells. These results indicate that antioxidants are able to reverse the increased oxidative stress caused by n-3 and n-6 PUFA.

1.5.2.2 Endogenous antioxidants

Similar to exogenous antioxidants, endogenous antioxidants are found in the cell membrane, cytosol and plasma. They are further subdivided into non-enzymatic and enzymatic antioxidants. Non-enzymatic antioxidants include glutathione (GSH), and enzymatic antioxidants include superoxide dismutase (SOD), catalase and GSH peroxidase (GPX). When removing O$_2^-$, in humans and most mammals, SOD is required to convert it into H$_2$O$_2$. There are three isoforms of SOD: SOD1, SOD2 and SOD3. SOD1 is a soluble enzyme antioxidant which along with SOD2 is found primarily in the cytoplasm and the intermembrane space of mitochondria. Unlike SOD1 and SOD2, SOD3 is primarily found in the extracellular matrix. SOD1 and SOD3 contain copper and zinc functional motifs.
whereas SOD2 contains magnesium, and is often referred to as MnSOD (Fig. 1.1)\textsuperscript{131}. Once O$_2^-$ is converted to hydrogen peroxide it must be removed by either catalases or peroxidases, and the type of enzyme used is tissue specific.

In order to neutralize the hydrogen peroxide generated from SOD, two endogenous antioxidant systems exist, catalase and GPX. Although both function in a similar fashion, converting hydrogen peroxide into water and oxygen, GPX requires GSH whereas catalase does not\textsuperscript{132,133}. GPX uses GSH to remove the O$_2^-$, and in this process the GSH is converted to glutathione disulfide (GSSG, Fig. 1.1)\textsuperscript{121}. In the heart, of these two endogenous antioxidant systems, GPX is found in much higher abundance than catalase\textsuperscript{134}. Therefore within the heart, GSH remains the most effective antioxidant against H$_2$O$_2$.

1.5.3 Glutathione

GSH, $\gamma$-L-glutamyl-L-cysteinylglycine, is a tripeptide molecule composed of three amino acids: glutamate, cysteine and glycine. The bond between glutamate and cysteine is considered a gamma peptide bond, and not a usual alpha peptide bond. This gamma peptide bond prevents GSH from being readily hydrolyzed by peptidases thereby reducing hydrolysis and increasing the stability of GSH\textsuperscript{135}.

1.5.3.1 Regulation and biosynthesis of glutathione

Despite the crucial role of GSH in protecting against mitochondrial oxidative stress, mitochondria cannot synthesize GSH \textit{de novo}\textsuperscript{136}. As such they rely on transporters, such as
dicarboxylate carrier (DIC) and 2-oxalocarboxylate carrier (OGC), to import GSH from the cytosol \(^{136}\). In mammals GSH \textit{de novo} synthesis is a two-step process with cysteine being considered the limiting substrate \(^{137}\). An overview of GSH homeostasis is shown in Fig 1.1 below.

The first, and rate-limiting step of synthesis, is the formation of the \(\gamma\)-glutamylcysteine bond. This step is catalysed by glutamate-cysteine ligase (GCL). Two genes encode for GCL, one for the catalytic site (\textit{Gclc}) and one for the modifier subunit (\textit{Gclm}, Fig 1.1). Of the two genes, it has been found that \textit{Gclc} is essential for synthesis. Knockouts of \textit{Gclm} on the hand, still possess a low level of GSH biosynthesis \(^{138}\). High levels of GSH have been shown to regulate and cause feedback inhibition on GCL \(^{139}\).

The second step of GSH synthesis involves the addition of glycine to \(\gamma\)-glutamyl-cysteine (Fig. 1.1). This step is catalysed by glutathione synthetase (GS). Unlike GCL, which undergoes feedback inhibition with high levels of GSH, GS does not experience this feedback inhibition \(^{140}\).

Cysteine on its own is unstable in the extracellular environment. In its free-state, cysteine rapidly undergoes autooxidation, forming the dimer cystine and ROS as a by-product. To prevent this from occurring, GSH is used as the main method of storage for cysteine \textit{in vivo} \(^{141}\). This process is carried out by gamma-glutamyl transpeptidase (GGT) and ultimately leads to the formation of cysteinyl-glycine, which can further be broken down by aminopeptidase (AP) into free cysteine and glycine as needed (Fig. 1.1) \(^{141}\). As GSH is the main storage form of cysteine, its regulation within the cell is extremely important.
Figure 1.1 Overview of the glutathione homeostasis in the cell. Summary of key pathways of glutathione homeostasis within the cell. The grey circle represents the mitochondria and the double square represents the cellular membrane. Abbreviations: AP, Aminopeptidase; Cys, Cysteine; DIC, dicarboxylate carrier; ETC, Electron transport chain; GCLC, Glutamate-Cysteine Ligase Catalytic Subunit; GCLM, Glutamate-Cysteine Ligase Modifier Subunit; Glu, Glutamine; Gly, Glycine; GSH, Glutathione; GR, Glutathione Reductase; GS, Glutathione Synthetase; GSSG, Glutathione Disulfide; GST, Glutathione S-Transferase; GTT, Gamma-Glutamyl Transpeptidase; $\text{H}_2\text{O}_2$, Hydrogen Peroxide; MnSOD; Maganese Superoxide Dismutase; NADPH, Nicotinamide Adenine Dinucleotide Phosphate Hydrogen; OGC, 2-oxoglutarate; O$_2$, Oxygen; ROOR', Organic Peroxide.

1.5.3.2 Antioxidant functions of glutathione

GSH is able to function has an antioxidant via two distinct mechanisms, direct and indirect. First, GSH directly exhibits antioxidant properties in conjunction with GPX. To
convert H₂O₂ to water, two molecules of GSH are converted by GPX to the oxidized form of GSH, GSSG. This GSSG can then be recycled back into the reduced form GSH by glutathione reductase using NADPH as an electron donor (Fig. 1.1)¹⁴¹. Besides directly functioning as an antioxidant, GSH can also be used indirectly as an antioxidant. Organic peroxides can be reduced through glutathione S-transferase (GST)¹⁴¹. Using GSH, GST can bind these organic peroxides and they can be removed from the cell for excretion via transporters such as DIC and OGC (Fig. 1.1)¹⁴².

1.5.3.3 Evidence from knockout animals

GSH is an extremely important molecule, as such efforts to produce knockouts are often unsuccessful¹⁴¹. The first step of GSH synthesis is controlled by two separate genes, Gclc and Gclm. When knockouts of Gclm are studied, compared to wild type controls, these animals are much more susceptible to myocardial ischemia-reperfusion injury¹⁴³. As well, in hearts experiencing pressure overload, compared to wild type controls homozygous knockout of Gclm exacerbated dysfunction, increased fibrosis and decreased survival¹⁴⁴. In contrast to Gclm, where homozygous knockouts can be generated, homozygous knockouts of Gclc are embryonic lethal. Therefore, studies must be conducted in animals which are heterozygous for the knockout [Gclc(+/−)]¹⁴⁵. Tissue specific knockouts of Gclc have also been investigated. In particular, a hepatocyte specific knockout of Gclc leads to liver failure and malfunction of the mitochondria¹⁴⁶. Taken together, these previous studies again confirm the vital role of Gclc in the synthesis of GSH.
The next step in the biosynthesis of GSH is controlled by Gs. Again like Gclc, homozygous knockouts of Gs are embryonically lethal, whereas heterozygote's are viable with normal GSH levels and no discernible phenotype. GPX is the predominant antioxidant system found in the heart. GPX uses reducing equivalents from GSH, and in the process, converts the reduced GSH to its oxidized form, GSSG. To regain function as an antioxidant, GSSG is converted back into the active form of GSH through GSH reductase (GR), using NADPH as a cofactor. Homozygous knockouts of Gpx1 are viable, but demonstrate increased susceptibility to oxidative stress and myocardial ischemia reperfusion injury. With respect to Gr, a strain of knockout mice known as Gr1aNeu are available with decreased levels of GR activity. While no cardiac specific information is available, these animals are more susceptible to bacterial infection and proximal tubule injury than wild type controls.

1.5.3.4 Therapeutic uses

As mentioned, GSH is a tripeptide, thus it must be given intravenously, as oral administration leads to its premature breakdown and inactivation. Intravenous GSH treatment has been investigated for its potential use alongside chemotherapy treatments as an antioxidant. It was also previously used in Parkinson's disease as a potential treatment as well.

One downside of using intravenous injections for GSH is impracticality, as such; other methods for increasing GSH have been investigated. One such option used clinically is that of N-acetylcysteine (NAC). NAC is a form of cysteine to which an acetyl group has been
added. In GSH synthesis, cysteine is considered the limiting substrate\textsuperscript{137}. Therefore by providing an additional source of cysteine, NAC leads to an increase in GSH levels \textit{in vivo}. As well treatment with NAC has been shown to prevent/reduce oxidative stress in patients with non-insulin dependent diabetes\textsuperscript{158}. In patients with kidney disease treatment with NAC reduces oxidative stress, and leads to an improvement in kidney function\textsuperscript{159,160}. Furthermore, it has been shown to reduce the number of cardiovascular events that occur in patients with end stage renal failure\textsuperscript{161}.

1.5.3.5 Glutathione and cardiac oxidative stress

Hearts from both obese humans and animals have reduced GSH and GPX\textsuperscript{162,163}, but the cause of such a decline remains obscure. As mentioned earlier, mitochondria cannot synthesize GSH \textit{de novo}\textsuperscript{136}. As such they rely on transporters, such as DIC and OGC to import GSH from the cytosol (Fig 1.1)\textsuperscript{136}. Cardiac GSH homeostasis is dependent on 3 major factors: the activity of enzymes glutamate-cysteine ligase (GCL) and glutathione synthase (GS), the regeneration of GSH from GSSG, and the utilization of GSH by GPX (Fig. 1.1)\textsuperscript{164,165}. Impairment in any of these 3 factors in isolation or in combination can lead to a significant decrease of GSH in the heart.

1.5.4 Oxidative stress and macrophages

While ROS can be detrimental to cells, it also plays a vital role in the immune system. In the case of phagocytic cells such as macrophages, the production of ROS is used as a
method to kill invading pathogens \textsuperscript{166}. Unlike other cell types, where the addition of antioxidants reduces the amount of ROS, the opposite effect is seen in macrophages. A previous study showed that the addition of N-acetylcysteine to macrophages increased the production of ROS, restoring their function \textsuperscript{167}.

On the membrane of macrophages, and other phagocytic cells, a NADPH oxidase multi-subunit complex is assembled. This complex is composed of six separate components. The first component is a Rho family guanosine triphosphatase (GTPase), usually Rac1 or Rac2 \textsuperscript{168}. The other five components are known as "phox units" for phagocytic oxidase and include p22phox, p40phox, p47phox and p67phox. The fifth phox component gp91phox, is a heme containing subunit that has four different isoforms, depending on where the complex is expressed \textsuperscript{169}. Within the heart, NADPH Oxidase (Nox) 4 is the predominant isoform, and in macrophages, Nox2 is the predominant isoform. After the complex is assembled on the membrane, electrons are subsequently pumped into the compartment leading to the formation of the superoxide anion. The high concentration of $\text{O}_2^-$ anion kills the bacteria, thereby inactivating the pathogen \textsuperscript{169}.

Although most bacteria are killed by high concentrations of superoxide, there are some bacteria which can survive within macrophages. Examples of such bacteria are the \textit{Salmonella}. \textit{Salmonella enterica} subspecies enterica serovar Typhimurium, abbreviated \textit{Salmonella} Typhimurium (ST), can cause Typhoid disease in mice \textsuperscript{170}. Once in the host, \textit{Salmonella} invade the mucosa and replicate in macrophages. However, unlike other bacteria which are killed by macrophage ROS production, \textit{Salmonella} species are able to survive and replicate inside the macrophage \textsuperscript{171}. 
In order to survive inside the macrophage there are two separate systems utilized. The first of these systems includes effectors and a type III secretion system which is encoded on the *Salmonella* pathogenicity island (SPI) 1. The bacteria insert effectors into the host cell, which leads to subsequent disruption in normal cellular function. This alteration to the host membrane also allows ST to enter the cell. Once inside the cell, the bacteria is able to avoid lysosomal fusion by using a second type III secretion system known as SPI-2. In a laboratory setting, aside from using ST as a model of infection in macrophages, lipopolysaccaride (LPS), a major constituent of gram negative cell membranes, can also be used to simulate infection conditions.

1.6 Deoxyribonucleic acid damage pathways

In the cell, deoxyribonucleic acid (DNA) constantly undergoes changes due to cellular processes, such as transcription or damaging agents. When DNA damage occurs, it can involve covalent changes in DNA structure, base-pair mismatches, or result in the formation of loops and bubbles. This damage can then lead to the activation of biochemical pathways that help alleviate the stress, including DNA repair. If the cell is unable to repair the damage incurred, the cell is forced to undergo cell death. Classically, there are two distinct mechanisms by which cell death can occur, apoptosis and necrosis. However, more recently a third mechanism of cell death, known as necroptosis, has been described.
1.6.1 Types of DNA damage

There are many types of DNA damage that result from oxidative stress. These include double-stranded DNA breaks, single-stranded DNA breaks and oxidative DNA damage. For each of these different types of damage, the cell possesses specific repair enzymes capable of fixing it. However, if the damage becomes so extensive that the repair enzymes are unable to repair the damage, then the cell will undergo either apoptosis or necrosis.  

Of the different types of DNA damage, double-stranded DNA breaks (DSBs) are considered to be of the most detrimental. A single DSB can cause the cell to undergo apoptosis. Combined with single stranded DNA breaks, this type of damage are forms of detrimental backbone modifications. In particular, these breaks are result of ROS, ionizing radiation including x-rays and high UV or other DNA-damaging reagents such as the chemotherapy agents cisplatin and nitrogen mustard. Within cells there are two main mechanisms for repairing DSBs, homologous recombination (HR) between sister DNA molecules or rapid non-homologous neighbor joining (NHNJ). Two of the main genes which encode repair enzymes used in double stranded DNA breaks are X-ray repair cross complementing 2 (XRCC2) and Rad50 double strand break repair protein (Rad50).

Like DSBs, single stranded DNA breaks (SSBs) are a type of backbone damage. Typically, these breaks are considered to be less dangerous than DSBs. This particular form of damage occurs as a result of damaging agents, or can also result as an intermediate step during base and nucleotide excision repair processes. If the break is a result of oxidative damage then often the sugar residue remains at the site of the break. The main gene
encoding a repair enzyme for the repair of SSBs is X-ray repair cross complementing 1 (XRCC1).

With respect to oxidative stress, one of the main types of DNA damage to occur is to nucleotide bases. In particular, one of the most common targets is guanine. DNA damage to guanine is considered to be the main oxidative DNA-damage product, which unless removed can lead to the formation of mutations \(^{179,180}\). These mutations include alteration of base pair binding from the standard DNA base pairs A:T to C:C or G:C to T:A. The main repair enzyme is 8-Oxoguanine glycosylase (OGG1), which is used in the base excision repair process. Base excision repair is a mechanism which removes small non-helix-distorting base legions \(^{181}\).

1.6.2 Apoptosis, necrosis and necroptosis

Apoptosis is often referred to as programmed cell death, and is the main method by which aged cells are removed \(^{182}\). Within organisms there are two major pathways which lead to cell death, apoptosis and necrosis \(^{183}\). Apoptosis occurs both during development and aging as a mechanism to maintain the populations of cells in tissues. As well, it can be used as a defense mechanism by the immune system to protect the organism from infections.

Apoptosis commonly occurs with a characteristic change of nuclear morphology, which involves condensation of chromatin, known as pyknosis, leading to the fragmentation of the nucleus known as karyorrhexis \(^{184}\). Within apoptosis there are three distinct apoptotic pathways: extrinsic (death receptor) pathways, intrinsic (mitochondrial) pathways and T-cell mediated cytotoxicity and perforin-granzyme-dependent killing pathways. In contrast to
apoptosis, necrosis is the mechanism used during acute or extreme injury. It does not involve any regular DNA or protein degradation pattern, and is followed by swelling of the cytoplasm and mitochondrial matrix before the cell ruptures. In contrast to both apoptosis and necrosis, necroptosis is a different mechanism of cell death, which falls in-between the other two types. It is often referred to as programmed necrosis. Since its discovery as a mechanism of cell death, it has been implicated in cell survival and inflammation. Interestingly, apoptosis, inflammation and necroptosis can be triggered from the same stimulus, TNFα. However, it is the downstream pathways which become activated that determine cell fate.

1.7 Hypothesis and Aims

As mentioned above, there has been an increase in the levels of n-6 PUFA in the western diet. This increase was an attempt to reduce the levels of CVD. However, instead levels of CVD have continued to rise. As described earlier recent research has suggested that n-6 PUFA may in fact be cardiotoxic. Therefore the increase in n-6 PUFA in the western diet could explain the continued increase in CVD. However, the mechanisms of this cardiotoxicity are not well understood. Although previous research has demonstrated that n-6 PUFA hearts have reduced levels of GSH; the effects of n-6 PUFA in a diet-induced model of obesity, in the absence of other comorbidities remains unclear. Therefore the overall goal of my thesis was to investigate the specific cardiac effects of n-6 PUFA. As well I sought to determine the cellular mechanisms of damage and repair which may be altered after n-6 PUFA feeding.
The unifying hypothesis of this research is that *Inherent defects in GSH homeostasis in diet-induced obesity aggravate cardiac OXS leading to changes in inflammation and DNA damage.*

In this thesis the following three specific aims, designed to elucidate the hypothesis, were investigated:

The first specific hypothesis for this thesis (addressed in chapter 2) is *linoleic acid modifies redox status and biomarkers of inflammation in the heart.*

To address this hypothesis the following objectives were evaluated:

1. Determine if NIH-3T3 fibroblasts and H9c2 cardiomyocytes have mitochondrial impairment after exposure to increased ROS.
2. Determine if n-6 PUFA induces responses similar to that of observed after exposure to ROS in NIH-3T3 fibroblasts.
3. Determine, in the absence of diabetes, if n-6 PUFA diet-induced obesity alters GSH homeostasis and inflammatory biomarkers.
   a. Determine if the observed changes in GSH homeostasis and inflammatory biomarkers are mediated by MCP-1

The second specific hypothesis for this thesis (addressed in chapter 3) is *GSH homeostasis mediates linoleic acid induced macrophage impairment.*

To address this hypothesis the following objectives were conducted:

1. Determine how LA alters GSH and ROS in Raw 264.7 murine macrophages after exposure to inflammatory stimuli.
   a. Investigate how GSH and ROS are altered in LA treated cells after LPS stimulation.
b. Determine if LA induced effects can be reversed with n-3 PUFA supplementation.

c. Investigate if infection with ST produces responses similar to those observed after LPS stimulation.

2. As macrophages produce ROS using NADPH as a cofactor, determine the effects of alteration of NADPH on ROS production in Raw 264.7 macrophages.

   a. Investigate if overexpression of NADPH oxidase alters GSH and ROS levels after stimulation/infection in LA treated cells.

3. Determine the effects of modulation of GSH on ROS production in LA treated Raw 264.7 macrophages after LPS stimulation or ST infection.

4. Investigate if incubation with LA in human macrophages leads to changes similar to those observed in murine macrophages.

5. Investigate in vivo if inflammatory stimulus (cardiac challenge using ISO) alters levels of inflammatory biomarkers.

   a. Investigate if increasing GSH reverses n-6 PUFA induced increases in inflammatory biomarkers.

The third specific hypothesis for this thesis (addressed in chapter 4) is in the heart linoleic acid increases cell death and decreases repair pathway responses.

To address this hypothesis the following objectives were conducted:

1. Determine if a cardiac challenge (using ISO) alters cardiac function in n-6 PUFA fed animals.

2. Investigate which pathways are altered after a cardiac challenge in n-6 PUFA fed animals compared to MUFA fed animals.
3. Determine if cardiac challenge alters the mechanisms of cell death in n-6 PUFA fed animals.
   a. Investigate if a cardiac challenge alters the amount of SSBs and DSBs.
   b. Investigate how cardiac challenge affects expression of DNA damage and anti- and pro-apoptotic genes.
4. As LA is the parent molecule for ARA, determine if cardiotoxicity is a result of increased toxic ARA metabolites.
Chapter 2: Linoleic acid modifies cardiac inflammation through monocyte chemoattractant protein-1 expression

2.1 Overview

Due to the removal of SFA in our diet, there has been an increase in consumption of unsaturated fats, such as n-6 polyunsaturated fatty acids (n-6 PUFA). Despite this shift, rates of CVD have continued to rise, indicating that n-6 PUFA may be cardiotoxic. The mechanism of this proposed cardiotoxicity however, remains unclear. Previous research has shown that n-6 PUFA can induce systemic inflammation and decrease cardiac GSH\textsuperscript{188,189}.

Therefore, we aim to investigate how cardiac inflammation is modulated with n-6 PUFA \textit{in vitro} and \textit{in vivo}. We first show that under high levels of oxidative stress, increasing GSH restores mitochondrial potential. As well, we show that LA decreases GSH, ROS, and impairs mitochondrial membrane potential in fibroblasts To determine if similar conditions exist \textit{in vivo}, we demonstrate that a corn oil (CO) diet (LA rich) leads to a decrease in GSH, GSH synthesis and antioxidant genes in the heart. As well, a CO diet increases serum cytokines, including monocyte chemotactic protein 1 (MCP-1). This data indicates that diets rich in n-6 PUFA increase biomarkers of inflammation. By eliminating MCP-1 expression, using MCP-1-/- mice, we observe a reversal of the n-6 PUFA effects. Taken together, these data indicate that diets rich in n-6 PUFA increase serum chemokines and impair GSH synthesis, which is in part regulated through MCP-1.
2.2 Background

In the last several decades, North America has seen a shift in the consumption of dietary fats; that is, SFA were replaced with unsaturated fatty acids \textsuperscript{190}. This change was initiated over concerns that SFAs may cause elevated levels of circulating LDL, a major CVD risk factor \textsuperscript{191}. The major class of unsaturated fatty acids that replaced SFA were n-6 PUFAs, obtained from vegetable oils like corn, sunflower and safflower oils. Besides deliberate use, dietary n-6 PUFA remains elevated in the through the usage of vegetable oil seeds as a feed source for farmed animals \textsuperscript{192}. Thus, consumption of eggs, meat, butter etc. derived from these animal further elevates consumption n-6 PUFA \textsuperscript{193}.

Despite this shift away from SFA, the rates of CVDs and other inflammatory diseases have continued to rise. In particular, several meta-analysis studies have shown that in fact SFA do not lead to an increase in heart disease \textsuperscript{194-196}. Instead, such studies suggest that increased consumption of n-6 PUFA may lead to the development of CVD and obesity \textsuperscript{197-199}. Specifically in the heart, further studies have shown that n-6 PUFA can lead to an increase in cell death \textsuperscript{188,200}, increased mitochondrial oxidative stress \textsuperscript{201} and inflammation \textsuperscript{188,202}. However, the mechanisms behind these processes remain unclear.

Our lab has previously demonstrated that aberrations in GSH homeostasis leads to cardiac lipotoxicity in diet-induced obesity \textsuperscript{203}. We have also recently concluded that in 8-month old exercised diabetic hearts, which demonstrate both inflammation and lipotoxicity, the enzymes for GSH biosynthesis (GCL and GS) and GSH recycling (NADPH/NADP ratio, GR) are also impaired \textsuperscript{204,205}. When exogenous GSH was administered in the latter study, cardiac GSH homeostasis was restored and oxidative damage decreased in aged exercised
diabetic hearts. Furthermore, previous research has shown that feeding diets rich in n-6 PUFA to rats, also reduced GSH levels. In streptozotocin (STZ) induced diabetes, diets supplemented with n-6 PUFA resulted in a reduction of cardiac GSH and an increase in GSSG, leading to an overall decrease in the GSH/GSSG ratio. In a separate rat study, it was also found that n-6 PUFA feeding led to a reduction in GSH/GSSG ratios in erythrocytes. However, the mechanisms and consequences of this reduction in GSH remain unclear. As n-6 PUFA has potential to increase inflammation, we sought to investigate how cardiac inflammation is modulated with n-6 PUFA, which has previously been shown to cause a loss of GSH.

In this chapter, we first show that under conditions of oxidative stress, increasing GSH improves mitochondrial health. We then demonstrate that LA decreases both ROS and GSH levels and impairs mitochondrial membrane potential in fibroblasts. In addition, we demonstrate that in vivo, a corn oil (CO) diet (LA rich) leads to a decrease in GSH synthesis genes and an increase in serum cytokines. Increases in levels of cytokines are indicative of inflammation. One of the elevated cytokines is MCP-1, which is involved in the recruitment and proliferation of macrophages. MCP-1 binds to the C-C chemokine receptor type 2 (CCR2) receptor. Signaling through the receptor results in transcription of monocyte chemotactic protein-induced protein (MCP-IP) that leads to increased inflammation, oxidative stress and apoptosis. We demonstrate that elimination of MCP-1 by using MCP-1-/- mice reverses the observed CO diet-induced effects. We conclude that diets rich in n-6 PUFA, at least partially through the MCP-1 pathway, increase serum chemokines and impair GSH synthesis.
2.3 Materials and Methods

2.3.1 In vitro experiments

NIH/3T3 (CRL-1658) mouse fibroblasts and H9c2 (CRL-1446) rat myoblasts were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS) at 37°C under 5% CO₂. H9c2 cell line was differentiated into mature cardiomyocyte over 4 days with daily addition of 0.1 μM all-trans retinoic acid.

2.3.1.1 Fatty acid preparation

100mM stock solutions of oleic acid (OA), and LA (Sigma Aldrich, St. Louis, MO, USA) were prepared by dissolving fatty acids in 70% ethanol. These solutions were heated to 37°C to ensure dissolution of the fatty acid. The 100 mM fatty acid solutions were then diluted in 8% bovine serum albumin (BSA) in phosphate buffered saline (PBS) to make 5 mM working solutions. Conjugation was completed at 37°C with shaking for 4 hours. Finally, the 5 mM stock solutions were sterile filtered into aliquots and stored at -70°C. For LA supplemented EPA and DHA (LED), the LA stock was diluted to 4.5mM using 8% BSA and 0.25mM of each EPA and DHA was added. For all experiments, after addition of fatty acids (0.25, or 0.5 mM) cells were incubated for a minimum of 18 hours before conducting cellular assays as described below.
2.3.1.2 Overexpression of Gclc

Augmentation of GSH in fibroblasts and cardiomyocytes was achieved by overexpressing the glutamate cysteine ligase catalytic subunit (Gclc). GCLC is the rate-limiting enzyme for GSH biosynthesis. Overexpression of the catalytic subunit of glutamyl cysteine ligase (mouse Gclc, Origene, Rockville, MD, USA; rat Gclc; Transomic Technologies, Huntsville, AL, USA) in both NIH-3T3 and H9c2 was achieved using Attractene transfection reagent (Qiagen, Toronto, ON, Canada). For both cell lines 5000 cells per well were plated in 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 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 room temperature for 20 minutes. After addition of the transfection solution cells were incubated for a minimum of 6 hours at 37°C under 5% CO₂. Then 100 µl antibiotic free media containing 20% FBS was added to each well. Cells were then incubated for 48 hours to ensure overexpression of Gclc.

In order to ensure that overexpression of Gclc had occurred ribonucleic acid (RNA) was extracted from the cells 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 or overnight at 4°C. 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 H₂O. The samples were then incubated at 65°C for 10 minutes. Synthesis of cDNA and qPCR were preformed as described below in section 2.3.2.3. It was found that incubation with Gclc overexpression plasmid led to an average fold increase of 6.53 in NIH-3T3 and an 8.23 fold increase in H9C2 cells.

2.3.1.3 Induction of cell death using hydrogen peroxide

After overexpression of Gclc, as described above, cells were left to recover for 48 hours. After this time, cells were then treated with varying concentrations of hydrogen peroxide (H₂O₂; Sigma-Aldrich, St. Louis, MO, USA). For NIH-3T3 concentrations of 0, 500 and 1000 µM H₂O₂ were used whereas 0, 100 and 500 µM H₂O₂ was used for H9c2 cells.
2.3.1.4 Cellular assays

2.3.1.4.1 Mitochondrial membrane potential

Mitochondrial membrane potential was measured using 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide (JC1)\(^{209}\), a dye that accumulates as red aggregates in the nucleus of healthy cells but preferentially forms green monomers in the cytosol of apoptotic and necrotic cells\(^{210}\). As a positive control for apoptosis, some plated cells were first treated with 1 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP) which affects protein synthesis and leads to uncoupling of the proton gradient, leading to apoptosis\(^{210}\). All cells were then washed with PBS. Working solutions of JC1 (100 μM in PBS) were then added to each well and incubated for 30 minutes followed by two washes with PBS to remove the dye. Afterwards, red fluorescence was read at 525 nm excitation and 580/640 nm emission and green fluorescence at 490 nm excitation and 510/570 nm emission using a GloMax Multi+ detection system (Promega, Madison, WI, USA). A ratio of the fluorescence was determined, with higher numbers indicating increased mitochondrial membrane potential. The calculation used for determining the ratio is shown below:

\[
\text{JC1} = \frac{\text{red fluorescence}}{\text{green fluorescence}}
\]
2.3.1.4.2 Reactive oxygen species

For the estimation of ROS, H9c2 and NIH-3T3 fibroblasts were incubated with 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA; Sigma Aldrich, St. Louis, MO, USA). The acetate groups of H2DCFDA are cleaved (due to intracellular esterases and oxidation) producing the highly fluorescent 2',7'-dichlorofluorescein (DCF) molecule. Cells were washed with PBS and incubated with 20 µM H2DCFDA in PBS for 30 min. Fluorescence was measured at 490 nm excitation and 510/570 nm emission using a GloMax Multi+ detection system (Promega, Madison, WI, USA). For each well the fluorescence was calculated and normalized as follows:

\[
\text{ROS (Normalized to BSA control)} = \frac{\text{Well}}{\text{Average Florescence of BSA control}}
\]

2.3.1.4.3 Glutathione

Cellular GSH was measured using a ortho-phthalaldehyde assay. Cells were lysed in a lysis buffer containing 3% Triton X-100 and 50mM potassium phosphate buffer pH 7.5. 75 µl of lysate was placed into two separate wells. 75 µl of 1M potassium phosphate buffer (pH 7.0) was added. One well was treated with 7.5mM N-ethylmaleimide for 5 mins (A) while the other remained untreated (B). Then 250 µl 1M potassium phosphate was added to each well. Then 500 µl 0.1 M potassium phosphate was added to each well. Finally all wells were then incubated with 0.5 mg/ml ortho-phthalaldehyde (Sigma Aldrich, St. Louis, MO,
USA) for 30 mins. Fluorescence was measured at 365nm excitation and 410/460nm emission using a GloMax Multi+ detection system (Promega, Madison, WI, USA).

\[ \text{Amount of GSH} = \text{Well B} - \text{Well A} \]

\[ \text{GSH relative to BSA} = (\text{GSH in well}) - (\text{Average GSH in BSA Control}) \]

2.3.1.4.4 Mitochondrial redox status and cell viability

For the determination of mitochondrial redox status, which is often used as an indication or cell viability, a Resazurin assay to determine mitochondrial redox potential was used. Resazurin is a dye that is converted to the fluorescent resofurin when it is reduced in the mitochondria. Stock resazurin die (Biotium, Fremont, CA, USA) was diluted 10x into media. Cells were then incubated with this solution for 3-4 hours at 37°C and 5% CO₂. Fluorescence was measured at 525nm excitation and 580/640 nm emission using a GloMax Multi+ detection system (Promega, Madison, WI, USA).

\[ \text{Resazurin (normalized to BSA control)} = (\text{Well}) - (\text{Average of BSA Control}) \]

As a way to directly measure cell viability in certain experiments, an lactate dehydrogenase (LDH) release assay was used. Upon lysis, cells release a stable cytosolic enzyme known as lactate dehydrogenase (LDH). The enzyme activity of LDH can be determined to quantify the level of LDH in each sample. Increased cell death leads to increased levels of LDH. Levels of LDH release were determined using the Cytoscan LDH
Assay (G-Biosciences, St. Louis, MO, USA). Briefly 50 µl of media from each of the treatments was added to the assay plate. As well a positive control (complete cell lysis) and an untreated sample were also added to the assay plate. To each well 50 µl of substrate mix was added and incubated at room temperature for 30 min. 50 µl of stop solution was then added and absorbance at 490 nm was measured using a GloMax Multi+ detection system (Promega, Madison, WI, USA). The % cell death was determined as follows:

\[
\text{LDH release (cell death)} = \frac{\text{Experimental} - \text{Untreated Control}}{\text{Positive Control}}
\]

2.3.2 In vivo experiments

Male C57BL/6 or MCP-1 (-/-) mice were weaned at 3 weeks of age onto a high-fat isocaloric, iso-nitrogenous diets with 40% coming from fats and fed for 5 weeks. High-fat diets contained 20% w/w of various dietary oils. These diets were prepared by Harlan Teklad by combing the rodent basal diet mix (catalog# TD.88232) with either olive oil (OO), corn oil (CO) or corn oil plus fish oil (COF; n-3 PUFA supplemented n-6 PUFA diet). For n-3 PUFA supplementation, the American Heart Association guidelines were utilized (for humans, 0.5–1.8 g of long chain n-3 PUFA per day). This value was obtained by converting the average consumption of n-3 PUFA among humans into a proportion of what mice would eat based on the high fat diet provided. Therefore, 1% fish oil (w/w) was added to 19% (w/w) corn oil to offset the high n-6 PUFA diet. The final diet (basal plus oil) contained 13.81 kJ/g of energy composed of 21.2% protein by weight (19.0% by energy), 44.7% carbohydrate by
weight (40.1% by energy), and 20.2% fat by weight (40.8% by energy). Composition and nutritional information for the diets are given below in Table 2.1 and Table 2.2. Food and water were provided *ad libitum*.

During the last week of feeding (week 5), to determine if animals were normoglycemic for intraperitoneal glucose tolerance (IPGTT) testing was conducted. The animals were fasted for 5 h and blood glucose was measured (time 0). Mice were then injected intraperitoneally with 1 g/kg glucose, followed by blood glucose measurements at 15, 30, 60 and 120 min. After 5 weeks of feeding, mice were then scarified and hearts and serum were collected. For hearts, the apex of the heart was removed using a razor blade and fixed in 10% formalin for use in immunohistochemistry. The remaining heart was divided into two sections with the section containing the left ventricle being preserved in RNA later for later RNA extraction. The final section was flash frozen in liquid nitrogen for use in determining GSH and for enzyme-linked immunosorbent assays (ELISA) (chapter 4).
Table 2.1 Composition of high fat diets.
Diet consisted of basal mix (Teklad, #88232) plus additional oil as indicated. CO, corn oil; OO, olive oil

<table>
<thead>
<tr>
<th>Formula</th>
<th>OO Composition (g/kg)</th>
<th>CO Composition (g/kg)</th>
<th>COF composition (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>240.0</td>
<td>240.0</td>
<td>240.0</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>3.6</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>298.7</td>
<td>298.7</td>
<td>298.7</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>75.0</td>
<td>75.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>75.0</td>
<td>75.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral Mix AIN-76 (170915)</td>
<td>42.0</td>
<td>42.0</td>
<td>42.0</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>3.6</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Vitamin Mix (Teklad 40060)</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Oil</td>
<td>190.0 (olive oil)</td>
<td>190.0 (corn oil)</td>
<td>180 (corn oil)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 (fish oil)</td>
</tr>
</tbody>
</table>

Table 2.2 Percent composition of diets.
Final diet composition of mouse diets

<table>
<thead>
<tr>
<th></th>
<th>% by weight</th>
<th>% by energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>44.7</td>
<td>40.1</td>
</tr>
<tr>
<td>Protein</td>
<td>21.2</td>
<td>19.0</td>
</tr>
<tr>
<td>Fat</td>
<td>20.2</td>
<td>40.8</td>
</tr>
</tbody>
</table>

ENERGY DENSITY /g of finished diet: 13.81 kJ/g
2.3.2.1 Cytokine response in vivo

Multiplexing MILLPLEX Mouse Cytokine/Chemokine kit (Millipore) using the Luminex™ 100 system analysis of cytokines, chemokines and growth factors was performed by Eve Technologies Corp. (Calgary, AB, Canada) using a Bio-Plex™ 200 system (Bio-Rad Laboratories Inc, Hercules, CA, USA). In total 31 cytokines were measured (Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1alpha, MIP-1beta, MIP-2, RANTES, TNFalpha, VEGF). Results were expressed as pg/ml of plasma with detection limits for each cytokine indicated in the figure.

2.3.2.2 Determination of GSH

Levels of GSH in the hearts of OO and CO fed animals was determined using a HT glutathione assay kit (Trevigen, Gaithersburg, MD, USA). Briefly 30 mg of heart tissue was homogenized using radioimmunoprecipitation assay buffer (RIPA buffer). The total protein content of the samples was determined using a Bradford assay (Bio-Rad Laboratories Inc, Hercules, CA, USA). Samples were then deproteinated using 5% metaphosphoric acid. 25 µl of a 30x dilution of each sample was added to the assay plate. Wells for GSH standard using the provided GSSG were also prepared. Then 75ul of reaction mix was added to each of the wells and immediately placed in the GloMax Multi+ detection system (Promega, Madison, WI, USA). The absorbance at 414 nm was measured.
every 2 minutes for 30 minutes using a GloMax Multi+ detection system (Promega, Madison, WI, USA). Following completion the time point in which no wells had reached saturation was determined. Total GSH for each well was determined by comparing sample wells to the standard. Samples were normalized as follows:

\[
\text{Total GSH per mg protein} = \frac{\text{Total GSH (GSH/ml)}}{\text{Protein (mg/ml)}}
\]

2.3.2.3 Quantitative PCR

Total RNA was extracted from frozen left-ventricle heart sections using the Fibrous tissue RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufactures instructions. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad Laboratories Inc, Hercules, CA, USA). Integrated DNA Technologies synthesized all desired mRNA primers (Table 2.3). Quantitative polymerase chain reactions (qPCR) were performed on a Bio-Rad CFX Manager 2.0 machine using Sso Fast Eva Green Supermix (Bio-Rad Laboratories Inc, Hercules, CA, USA). All samples were done in duplicate (volume= 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 preformed. Quantification of gene expression was carried out using CFX Manager Software version 3.0.1224.1015. Primer efficiencies were obtained using LinREG software. 18s rRNA was used as the reference gene for all samples. All primers were designed using NCBI primerblast 213. For all genes, unless indicated, primers
were designed to be intron spanning and matched with all protein coding mRNA transcripts. The number/sequence of protein coding sequences was determined using ENSEMBL and sequences were aligned using Clustal Omega to determine areas of similarity for primer design.

**Table 2.3 Primer sequences used for the quantification of mRNA levels by real-time PCR.** Primers used to determine gene expression; both the forward primer (F) and reverse (R) primers are indicated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5'-3')</th>
<th>Product Size</th>
<th>Primer Efficiency</th>
<th>Gene Bank Reference #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>F: AGAAGCCTAAGAACGCAATTC R: ATCCATCCAGCGTTGATTAC</td>
<td>152 bp</td>
<td>91.4%</td>
<td>NM_009804.2</td>
</tr>
<tr>
<td>Gclc</td>
<td>F: ACATCTACCACGCAGTCAAG R: CATGTACTCCACCTCGTCAC</td>
<td>68 bp</td>
<td>88.7%</td>
<td>NM_010295.2</td>
</tr>
<tr>
<td>Gclm</td>
<td>F: CGGATTTTAGTCAGGGAGTTTC R: TTTCATCGGGATTTATCTTTC</td>
<td>78 bp</td>
<td>98.3%</td>
<td>NM_008129.4</td>
</tr>
<tr>
<td>Gr</td>
<td>F: ACGTGATTACGGCTTTTC R: CTTGATGACATGCCAACTG</td>
<td>56 bp</td>
<td>82.8%</td>
<td>NM_010344.4</td>
</tr>
<tr>
<td>Gs</td>
<td>F: CTCAGCTAATGCCTGGGTGTTAG R: CTTGATGACATGCCAACTG</td>
<td>91 bp</td>
<td>95.3%</td>
<td>NM_008180.2</td>
</tr>
<tr>
<td>Gpx1</td>
<td>F: GTTCGGACACCAGGAGAGAA R: GCCTTCTCACCATTCCTTTC</td>
<td>126 bp</td>
<td>93.7%</td>
<td>NM_00132952 8.1</td>
</tr>
<tr>
<td>Sod1</td>
<td>F: GGCTTCTGCTTGTGCTCTCT R: TTCACCGCTTGCCCTCTG</td>
<td>147 bp</td>
<td>97.2%</td>
<td>NM_011434.1</td>
</tr>
<tr>
<td>Sod3</td>
<td>F: AACTTCACCAGGAGAGAG R: AGGTTCCTCTGCACCTGTCA</td>
<td>54 bp</td>
<td>98.4%</td>
<td>NM_011435.3</td>
</tr>
</tbody>
</table>
2.3.2.4 Statistical analysis

Results are expressed as mean ± SEM. For fatty acid analysis (chapter 3), a two-way analysis of variance (ANOVA) was used with Bonferroni post hoc comparisons. For all other data, analysis of two samples t-tests were performed. For analysis of three or more groups a one-way ANOVA tests with Tukey post-hoc analysis were used to determine the differences between groups mean values. This method was chosen as Tukey tests control for type 1 and type 2 error, without a major reduction in power which occurs with Bonferroni post-hoc analysis. All groups had an n of between 3-6, as indicated in each figure. The level of statistical significance was set at \( P < 0.05 \). In certain figures multiple comparisons were made. There has been considerable debate within the literature as to if correction for multiple comparisons is necessary. To determine the effects of multiple comparisons, and to account for the increase in type 1 error, a correction of the p values was conducted using a Bonferroni-Holms sequential correction factor calculator in excel. The results of this analysis are shown in Appendix 2.

For all graphs statistical difference is denoted by differing letters. For example, samples labelled with A are not statistically different from each other. If there is a different letter, then the samples are statistically different from each other. For example, three samples with the letters A, B, and C, respectively, are statistically different from each other. GraphPad Prism 4.0 was used for plotting and analysis (GraphPad Software Inc., San Diego, CA, USA).
2.4 Results

2.4.1 Increased reactive oxygen species and depletion of glutathione leads to mitochondrial impairment in fibroblasts and cardiomyocytes.

Within the heart, two major cell types predominate: fibroblasts and cardiomyocytes. The addition of 1000 µM H$_2$O$_2$ to NIH-3T3 fibroblasts led to a decrease in GSH levels. As well addition of 500 µM and 1000 µM H$_2$O$_2$ led to a significant decrease in mitochondrial membrane potential - an indicator of mitochondrial function (Fig. 2.1a). This decrease in mitochondrial membrane potential could be the result of increased cell death. As the concentration of H$_2$O$_2$ increases, so does the amount of LDH release indicating increased levels of cell death (Fig. 2.1a). To increase levels of GSH, overexpression of Gclc was performed (Fig. 2.1a). Overexpressing Gclc led to an increase in GSH in all conditions, which indicates that overexpression of Gclc was successful. At either 0 µM or 500 µM overexpression of Gclc did not alter mitochondrial membrane potential in comparison to the corresponding PTCN control. However at 1000 µM H$_2$O$_2$ overexpression of Gclc increased mitochondrial membrane potential to levels observed with incubation of 500 µM H$_2$O$_2$, thereby partially restoring mitochondrial function (Fig. 2.1a). No changes in LDH were observed between Gclc overexpression and the corresponding PTCN control. Therefore the increase in membrane potential at 1000 µM is not due to differences in cell death. Similar to NIH-3T3 fibroblasts, addition of increasing amounts of hydrogen peroxide to H9C2 cardiomyocytes led to a decrease in mitochondrial membrane potential. In contrast to NIH-3T3, addition of H$_2$O$_2$ increased GSH levels (Fig. 2.1b). As well increasing concentrations of
H$_2$O$_2$ did not lead to increased levels of LDH release. Similar to fibroblasts, overexpression of Gclc was performed (Fig. 2.1b), which led to a decrease in mitochondrial membrane potential at 0 μM H$_2$O$_2$, no change at 100 μM and an increase at 500 μM compared to PTCN controls. Therefore like in NIH-3T3 incubation with higher concentrations of H$_2$O$_2$ (1000 μM in NIH-3T3 and 500 μM in H9C2) rescued H$_2$O$_2$ induced mitochondrial dysfunction (Fig. 2.1b) $^{221,222}$. 
Figure 2.1 Effects of modulation of ROS on GSH levels, mitochondrial membrane potential and cell viability in fibroblasts and cardiomyocytes.

Effect of 14 h H$_2$O$_2$ treatment on a) NIH-3T3 fibroblasts or b) H9C2 cardiomyocytes overexpressing Gclc or empty vectors (PTCN). Fibroblasts and cardiomyocytes were analysed for GSH content using o-phthalaldehyde assay, mitochondrial membrane potential with JC-1 dye, or cell viability using an LDH release assay. Data are normalized to PTCN 0 µM H$_2$O$_2$ and shown as arbitrary units of fluorescence. All data was analyzed using one-way ANOVA with Tukey’s post hoc analysis with different letters indicating significant difference (p<0.05); n=4. Abbreviations: AU, arbitrary units; Gclc, with overexpression of glutamate cysteine ligase catalytic subunit; GSH, glutathione; LDH, lactate dehydrogenase; H$_2$O$_2$, hydrogen peroxide  PTCN, overexpression with empty vector;
2.4.1 Linoleic acid attenuates glutathione and reactive oxygen species and decreases mitochondrial membrane potential in NIH-3T3 fibroblasts

In order to determine if similar responses are observed when incubated with fatty acids, NIH-3T3 fibroblasts were treated with 0.25 mM of oleic acid (OA; MUFA), linoleic acid (LA, n-6 PUFA) or bovine serum albumin (BSA) as a control. Some cells were treated with transforming growth factor beta 3 (TGFB3) as an inducer of fibroblast activity. It is well known that TGFB3 leads to the production of collagen after injury in the heart, which may lead to fibrosis. In comparison with untreated cells, treatment with TGFB3 did not alter ROS or mitochondrial membrane potential in any fatty acid treatment (Fig. 2.2). Treatment with TGFB3 did lower GSH levels in cells incubated with BSA. No difference in GSH levels was observed in OA or LA incubated cells. Treatment with OA led to an increase in ROS compared to BSA controls. Treatment with OA did not alter GSH or mitochondrial potential in comparison to BSA and LA. After treatment with LA, there was a decrease in ROS and GSH in both TGFB3 treated and untreated cells (Fig. 2.2). As well, incubation with LA led to a significant decrease in mitochondrial membrane potential under TGFB3 treated and untreated cells conditions. This decrease in mitochondrial membrane potential in LA is not the result of a decrease in mitochondrial redox status, as there was no difference in Resazurin between OA and LA treated cells (Fig. 2.2). This indicates that incubation with LA alters the function of mitochondria in NIH-3T3 fibroblasts (Fig.2.2).
Figure 2.2 *n*-6 PUFA decreases mitochondrial membrane potential and GSH in fibroblasts. Fibroblasts were analyzed for GSH content using o-phthalaldehyde assay, ROS using DCFDA, mitochondrial membrane potential with the ratiometric JC-1 dye, and mitochondrial redox status using a resazurin assay. Fibroblasts were either incubated with transforming growth factor beta 3 (TGFB3) or were control wells. For ROS, GSH, and mitochondrial redox status data was normalized to BSA UTX and shown as arbitrary units of fluorescence. For JC-1 data is shown as a ratio of red/green florescence. All data was analyzed using one-way ANOVA with Tukey's post hoc analysis with different letters indicating significant difference (p<0.05); n=4. Abbreviations: AU, arbitrary units; BSA, bovine serum albumin; GSH, glutathione; LA, linoleic acid; OA, oleic acid; ROS, reactive oxygen species; TGFB3; transforming growth factor beta 3.
2.4.2 A diet rich in linoleic acid decreases glutathione and increases serum chemokines

In order to determine if the results found *in vitro* were consistent with that found in the intact heart muscle we fed a corn oil (CO; n-6 PUFA rich) diet. After 5 weeks in feeding there was no change in body weight or in IPGTT between OO and CO fed animals (Fig. 2.3a,b). However, 5 weeks of feeding led to attenuation in GSH levels and synthesis genes: *Gclc*, *Gclm*, *Gs*, *Gr*, and *Gpx1* (Fig. 2.3c,d). Feeding of a CO diet also led to attenuation in other antioxidant genes: *Catalase*, *Sod1*, and *Sod3* (Fig. 2.3c). As GSH and/or ROS can trigger the immune system, we investigated how feeding of either olive oil (OO; MUFA rich) or CO diet altered secretion of serum cytokines and chemokines. Compared to OO feeding, the CO diet led to a significant increase in several key chemokines (MCP-1, CCL11, KC) which are involved in macrophage recruitment and proliferation (Fig. 2.4a) but not cytokines (Fig. 2.4b)
Figure 2.3 *n*-6 PUFA reduces total GSH and GSH synthesis gene levels.

a) Intraperitoneal glucose tolerance test (IPGTT) and b) Body weight for OO and CO animals after 5 weeks of feeding. c) Total GSH levels (ng/mg protein) with the detection limited indicated by a dotted line. Data was normalized to total protein levels d) qPCR of glutathione synthesis and antioxidant genes relative to OO control. All data was analyzed using t-tests with different letters indicating significant difference (p<0.05); n=4. Abbreviations: CO, Corn oil; OO, Olive oil; GCLC, Glutamate-Cysteine Ligase Catalytic Site; GCLM, Glutamate-Cysteine Ligase Modifier Subunit; GR, Glutatione Reductase; GS, Glutathione Synthetase; GSH, Glutathione; GPX1, Glutathione Peroxidase 1; SOD1, Superoxide Dismutase 1; SOD3, Superoxide Dismutase 3.
**Figure 2.4 n-6 PUFA alters chemokines, but not cytokines.**

a) pro-inflammatory macrophage/monocyte trafficking chemokines and b) cytokines analyzed in plasma using a MILLPLEX Mouse Cytokine/Chemokine kit and quantified in pg/ml. Detection limits are indicated using a dotted line. All data was analyzed using t-tests with different letters indicating significant difference (p<0.05); n=4. Abbreviations: CCL4, Chemokine (C-C motif) Ligand 4; CCL11, Chemokine (C-C motif) Ligand 11; CO, Corn oil; CXCL1, Chemokine (C-X-C motif) Ligand 1; G-CSF, Granulocyte-Colony Stimulating Factor; IFNγ, Interferon Gamma; IL-1α, Interleukin-1 alpha; IL-6, Interleukin-6; IL-10, Interleukin-10; MCP-1, Monocyte Chemotactic Protein 1; OO, Olive oil; TNFα, Tumor Necrosis Factor Alpha; IL-1α.
2.4.3 Monocyte chemotactic protein-1 modulates serum chemokines and glutathione synthesis genes in corn oil fed animals

An n-6 PUFA rich diet specifically increases some chemokines (Fig. 2.4a), which are involved in macrophage recruitment and proliferation $^{225,226}$. We hypothesized a role of innate immunity in modulating such a response. In this regard, MCP-1 expression was shown to be induced by LA in endothelial cells $^{227,228}$, in cultured vascular smooth muscle cells $^{229}$ and in human umbilical endothelial cells. MCP-1 is one of the primary chemokines which regulates migration and infiltration of macrophages into the tissues, including the heart $^{207}$. As levels of MCP-1 increased in serum after n-6 PUFA feeding, we next sought to determine the role of MCP-1 in modulating both immune responses and GSH levels. Therefore, MCP-1 deficient (MCP-1 +/-) mice were fed an OO or CO high fat diet. Compared to OO, removal of the MCP-1 led to a decrease of CCL11 in CO fed animals. No difference in CCL4 and CXCL1 was observed between OO and CO fed animals after removal of MCP-1. Previously it was shown that under normal conditions there was a decrease in GSH, GSH synthesis genes and antioxidant genes in CO fed animals compared to OO (Fig. 2.3). In contrast MCP-1 knockout led to an increase in Sod1, Sod3, and GSH synthesis genes compared to OO MCP1 (-/-) (Fig. 2.6b). However GSH levels (Fig. 2.6a) and the expression of Catalase was similar in both OO and CO fed animals (Fig. 2.6b). This indicates that MCP-1, or the cytokines released under its influence, might be responsible for the diet-induced modulation of GSH homeostasis.
**Figure 2.5** *MCP-1 knockout alters chemokine responses.*

a) pro-inflammatory macrophage/monocyte trafficking chemokines in control or MCP-1 knock out animals analyzed in plasma using a MILLPLEX Mouse Cytokine/Chemokine kit and quantified in pg/ml. Data are shown as a percentage change compared to c57/bl6 cytokines from Fig. 2.4. All data was analyzed using t-tests with different letters indicating significant difference (p<0.05); n=5. Abbreviations: CCL4, Chemokine (C-C motif) Ligand 4; CCL11, Chemokine (C-C motif) Ligand 11; CO, Corn oil; CXCL1, Chemokine (C-X-C motif) Ligand 1; MCP-1, monocyte chemotactic protein 1; ND, Not Detected; OO, Olive oil.
Figure 2.6 MCP-1 deletion restores corn oil induced reduction in GSH genes in the heart.
a) Total GSH levels (ng/mg protein). Detection limit is indicated using a dotted line. Data was normalized to total protein levels b) qPCR of glutathione synthesis and antioxidant genes relative to OO control. Data was analyzed using t-test with different letters indicating significant difference (p<0.05); n=5. Abbreviations: CO, Corn oil; Glutamate-Cysteine Ligase Catalytic Site; GCLM, Glutamate-Cysteine Ligase Modifier Subunit; GR, Glutathione Reductase; GS, Glutathione Synthetase; GSH, Gluthathione; GCLC, GPX1, Glutathione Peroxidase 1; OO, Olive oil; SOD1, Superoxide Dismutase 1; SOD3, Superoxide Dismutase 3.
2.5 Discussion

Obesity often presents itself in conjunction with metabolic syndrome. Determining the specific effects of diet on the immune system during obesity is challenging due to the presence of confounding co-morbidities such as insulin resistance or hyperglycemia \(^2\), which themselves can alter inflammatory processes. Moreover, despite elevated inflammation, both obesity and metabolic syndrome demonstrate impaired immune responses and delayed wound healing \(^2\). To understand the specific impacts of diet and ascertain the impact of obesity on inflammatory processes, we investigated the role of specific dietary fatty acids on modulating immune responses \textit{in vitro} and \textit{in vivo}.

As an alternative to primary cell culture methods, NIH-3T3 fibroblasts, established from primary mouse embryonic fibroblast cells, are often used as an \textit{in vitro} cell model \(^4\). In cells increasing ROS can initiate and propagate inflammation \(^7\). In the heart, mitochondrial ROS leads to the release of pro-inflammatory cytokines \(^8,9\). Of the cell types in the heart, cardiomyocytes are the most abundant cell type. As an \textit{in vitro} model, differentiated H9c2 have a much higher density of mitochondria than fibroblasts. As a consequence of high rates of aerobic respiration, cardiomyocytes release higher ROS. Therefore in cardiomyocytes maintenance of antioxidant levels to combat oxidative stress is extremely important. GSH is a crucial antioxidant for combating oxidative stress, and its status is important for ensuring proper immune responses depending on the tissue type \(^0\). Previous literature has shown that GSH levels are critical for maintaining proper mitochondrial function \(^3\). Mitochondria are unable to synthesize GSH, and thus are required to transport it from the cytosol \(^4\). As a major producer of ROS, a byproduct of cellular respiration, mitochondria require the antioxidant capabilities of GSH and associated enzymes.
Therefore, depletion of GSH has been shown to lead to an impairment in mitochondrial function.

In order to investigate the relationship between exogenous ROS insult, GSH, and mitochondrial function, both NIH-3T3 fibroblasts and H9c2 cardiomyocytes were incubated with varying concentrations of H$_2$O$_2$. In fibroblasts, 1000 µM H$_2$O$_2$ led to a decrease in GSH levels, and impairment in mitochondrial membrane potential (Fig. 2.1). However, while increasing ROS (at 500 µM H$_2$O$_2$) in cardiomyocytes did lead to impairment in mitochondrial function, an increase in GSH was observed. Despite this, further increasing levels of GSH (through overexpression of Gclc) did lead to improvement of GSH after incubation with 500 µM H$_2$O$_2$ in cardiomyocytes and after incubation with 1000 µM in fibroblasts (Fig. 2.1).

One explanation of the decrease in mitochondrial function is that of toxicity. As such toxicity was determined using an LDH release assay. In NIH-3T3 fibroblasts increasing H$_2$O$_2$ concentration did lead to increased toxicity. However, at the 1000 µM concentration for PTCN and Gclc there was no difference in toxicity. Therefore we can conclude that the differences in mitochondrial function between PTCN and Gclc at this concentration are not due to differences in toxicity. Contrary to fibroblasts, increased H$_2$O$_2$ did not result in higher levels of toxicity. However, overexpression of Gclc did lead to increased toxicity at 500 µM. However, despite this toxicity there was still an increase in mitochondrial function observed with Gclc overexpression at this concentration. Taken together these results indicate that at high levels of ROS exposure increased levels of GSH are able to reverse ROS induced damage in cardiac cells.

In order to determine the effects of fatty acid on ROS and GSH levels, NIH-3T3 fibroblasts were incubated with either OA or LA. Incubation with LA led to decreased ROS
and GSH (Fig. 2.2). Not surprisingly LA incubated fibroblasts also displayed impairment in mitochondrial function, which when compared to OA, was independent of changes in mitochondrial redox status. Therefore this indicates that incubation with LA may be cardiotoxic (Fig 2.2).

Mitochondrial dysfunction in an obese type 2 diabetic can play a critical role in the complications of diabetes and metabolic syndrome. Therefore, understanding the mechanisms associated with mitochondrial dysfunction and how it affects GSH status and inflammation during diet-induced obesity alone is of utmost importance. For this reason, mice were fed a high fat diet for 5 weeks, which led to significant weight gain in the absence of diabetes (Fig. 2.3a,b). Similar to in vitro results, feeding a CO (LA-rich) diet reduced GSH levels and also reduced expression of GSH synthesis and other antioxidant genes, which indicates that the diet may be cardiotoxic (Fig 2.3).

These results of reduced GSH following high fat feeding of a n-6 PUFA rich diet are consistent with previously published studies. In particular, n-6 PUFA rich diets have been shown to lead to alterations in inflammatory status. Specifically, n-6 PUFA diets lead to the creation of a pro-inflammatory environment. Similar to these previous studies, we observed that mice fed an n-6 PUFA rich diet increase in several pro-inflammatory chemokines including MCP-1, CCL11 and CXCL1, all of which can play a role in the recruitment of immune cells (Fig. 2.4).

One of the primary chemokines involved in proliferation, differentiation and migration of macrophages is MCP-1. Previous research has shown that MCP-1 levels were increased after treatment with LA in both smooth muscle cells and endothelial cells. As it is known that MCP-1 increases during feeding with n-6 PUFA, we
endeavored to investigate if MCP-1 was involved in the modulation of the immune response during n-6 PUFA feeding. Therefore, we fed a corn oil rich diet to MCP-1 deficient (MCP-1-/-) mice. Indeed, after feeding n-6 PUFA, we found that it led to an alteration in the levels of chemokines, with decreased levels of CCL11 and no change in levels of CCL4 when compared to OO fed animals (Fig. 2.5). As well, MCP-1 deficient mice displayed an increase in GSH synthesis genes in CO, and a decrease in OO, the opposite trend of what was observed in wild type animals (Fig. 2.6). Taken together these results indicate that removal of MCP-1 is cardioprotective in animals fed a diet rich in n-6 PUFA.

With respect to statistical analysis of the data within this thesis, there is considerable debate surrounding the use of multiple comparisons in situations where one or more t-test or ANOVA was used. The use of multiple t-tests/ANOVAs can increase the probability of a type 1 error, which means that it increases the rate of the occurrence of false positives. In order to account for this possibility the p-values for figures where multiple comparisons were conducted in chapters 2, 3, and 4 were corrected using a Holms-Bonferroni sequential correction \(^2\). However, while this does reduce the occurrence of type 1 errors, it can increase the occurrence of type 2 errors (occurrence of false negatives) \(^2\). As such several sources argue against the use of correction factors in situations with low n number \(^2\). With respect to the data presented in this thesis, the correction factor did not affect the statistical significance of p-values which were less than 0.001. However, depending on the value of the other p values in the analysis certain p-values above 0.001 were found to be non-significance. However, because of the low sample size, the values reported in this thesis are that of the original p-values, whereas the corrected values are given in Appendix B.
2.6 Chapter Conclusion

In summary as mentioned in section 1.7 the specific hypothesis for this chapter was **linoleic acid modifies redox status and biomarkers of inflammation in the heart**. To answer this hypothesis we have shown that at highest levels of ROS fibroblasts and cardiomyocytes have impaired mitochondrial function which is improved after overexpression of Gclc (Objective 1). Furthermore we show that similar to increasing ROS levels addition of LA to NIH-3T3 cells reduces GSH and impairs mitochondrial function (Objective 2). Also, *in vivo* we demonstrated that n-6 PUFA feeding increased pro-inflammatory chemokines reduces GSH and leads to impairment of GSH synthesis genes (Objective 3). As well, we found that MCP-1 is indeed modulating GSH, as MCP-1 deficient animals have impairment in GSH synthesis genes (Objective 4). Therefore, we conclude that n-6 PUFA modifies redox status and increases biomarkers of inflammation in the heart which is modulated by MCP-1 during n-6 PUFA feeding.
Chapter 3: Glutathione homeostasis is key in maintaining macrophage function during inflammation or infection in vivo and in vitro.

3.1 Overview

Obesity has previously been linked to impaired immune responses and delayed wound healing\textsuperscript{231-233}. One cell type which can be impacted by immune system is that of macrophages. In macrophages the inflammatory response is essential\textsuperscript{253}. In chapter 2, MCP-1 was shown to be a regulator of GSH depletion during n-6 PUFA feeding. Since MCP-1 is involved in the recruitment of macrophages, we investigated the characteristic ROS burst of macrophages following inflammation in the presence of various fatty acids. Here we show that in contrast to MUFA, only feeding of n-6 PUFA increases pro-inflammatory status in control animals. Using RAW 264.7 macrophages, and using OO, CO, and COF fat fed mice, we further demonstrate that following inflammation, n-6 PUFA impairs O$_2^-$ and cytokine production, both of which were directly related to a loss of GSH. We further demonstrate that this impairment in ROS in n-6 PUFA treated macrophages can be rescued by either addition of n-3 PUFA or by increasing GSH through pharmacological or gene manipulation techniques. In order to determine if similar responses are found in human macrophages, we incubated primary human macrophages with fatty acids and showed that there is similar impairment in ROS and GSH following exposure to n-6 PUFA and an inflammatory stimulus. Taken together, these data indicate that n-6 PUFA, through depletion of GSH selectively impairs macrophages function.
3.2 Background

It is now well established that CVD is linked to aberrant inflammation, which in turn is linked to abdominal obesity\textsuperscript{254,255}. Despite a pro-inflammatory milieu, obesity and metabolic syndrome are related to impaired immune responses and delayed wound healing\textsuperscript{231,232,256}. Among mediators of inflammation, macrophages derived from monocytes are an important part of the inflammatory response\textsuperscript{257}. However, the role of macrophages under obesogenic conditions and how these conditions alter macrophages ability to produce ROS during infection and inflammation has yet to be fully determined.

Among various factors, a high calorie diet is a notable cause for rising obesity rates. We recently reported that Canadians on average are consuming 11\% more energy with higher carbohydrate (11\% increase) and fat (18\% increase) intakes, than four decades ago, with most of this increase coming from PUFA\textsuperscript{44}. In Canada due to higher consumption of vegetable oils like sunflower, safflower and corn oil, intake of linoleic acid (LA, the primary dietary n-6 PUFA) has soared. LA is an essential fatty acid with daily requirements between 0.5\%\textsuperscript{258} to 2\% energy\textsuperscript{259}. Canadians however consume \sim 7\% of their daily energy intake from LA and other n-6 PUFA\textsuperscript{260,261}. The impact of this elevated n-6 PUFA consumption on macrophage function especially in the context of the heart muscle remains unclear.

It is well recognized that n-6 PUFA increases general pro-inflammatory status\textsuperscript{105,225} whereas n-3 PUFA and MUFA exhibit the opposite effects\textsuperscript{105,262}. One of the major ways fatty acids regulate inflammation is by promoting the production of cytokines. Cytokines can induce pro- or anti-inflammatory characteristics depending on the target immune cell. Regarding macrophages, colony stimulating factors (CSF) can either recruit or prevent
recruitment of macrophages, which can be induced by n-6 PUFA \(^{93}\). For example, n-6 PUFA can also activate monocyte and granulocyte-monocyte CSF (M-CSF and GM-CSF respectively) to promote the differentiation of macrophages \(^{263}\).

We investigated the characteristics of macrophages following inflammation in the presence of various fatty acids. Using RAW 264.7 macrophages we show that following inflammation n-6 PUFA impairs ROS. We further demonstrate that this impairment in ROS as a result of n-6 PUFA treatment can be rescued by addition of either n-3 PUFA or by increasing GSH through pharmacological or gene manipulation techniques. We further show in human differentiated macrophages that similar impairment in ROS and GSH occurs following exposure to n-6 PUFA and inflammatory stimulus. Furthermore, after 5 weeks of feeding, we demonstrate that n-6 PUFA, not MUFA, increases pro-inflammatory status in control animals but surprisingly impairs such responses when challenged with inflammatory stimuli. Both \textit{in vivo} and \textit{in vitro} results were directly related to a loss of GSH. Therefore, we conclude that n-6 PUFA selectively impairs the function of macrophages during infection through depletion of GSH.

3.3 Materials and Methods

3.3.1 In vivo experiments

Feeding experiments were conducted as previously described in section 2.3.3 using OO, CO and COF diets. To cause mild cardiac damage after 5 weeks of feeding, mice were given intraperitoneal injection with a saline control or isoproterenol (ISO), a non-selective
beta-adrenergic agonist, twice at 100mg/kg, 24 hours apart. 24 hours after the last injection, the animals were sacrificed and serum was collected (Fig. 3.2)

To manipulate GSH on a high n-6 PUFA diet, a subset of mice on the corn oil diet were treated with either 10g/L N-acetyl cysteine (NAC; boosts GSH, Sigma Aldrich, St. Louis, MO, USA) in drinking water for 14 days or 30mM buthionine sulfoximine (BSO; lowers GSH, Sigma Aldrich. St. Louis, MO, USA) in drinking water for 14 days (Fig. 3.1) \[264,265\].

![Figure 3.1 Timeline of in vivo experiments.](image)

Timeline of experimental procedures. Abbreviations: BSO, bithionine sulfoximine; CO, Corn oil; NAC, N-acetyl cysteine; ISO, isoproterenol; OO, Olive oil.

3.3.2 Fatty acid analysis

To determine the fatty acid content of high fat diets, to see how feeding of different diets altered composition, evaluation of fatty acids was done using a combined extraction and methylation protocol \[266\]. Briefly, 30mg of tissue was added to 1.2ml hexane and 1.2ml boron trifluoride-methanol solution. Samples were then heated to between 90- 110°C for 1 hour. Next, 1ml water was added to the samples, centrifuged for 1 min. The top layer, containing
the extracted and methylated fatty acids was then removed. Fatty acid methyl esters were then 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 (% fatty acid) was determined for each fatty acid in that sample.

3.3.3 Cytokine Analysis

Cytokine analysis was performed as described in section 2.3.2.1.

3.3.4 Cell culture and treatments

A mouse macrophage cell line RAW 264.7, was purchased from American Type Culture Collection (ATCC; TIB-71). As well, human peripheral blood mononuclear cells (PBMC) were obtained cryopreserved (Zenbio, Research Triangle Park, NC, USA). RAW 264.7 cells were maintained in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin (Corning, Corning, NY, USA) for a final concentration of 200 IU penicillin and 200 µg/ml streptomycin, at 37°C in a humidified atmosphere of 5% CO2. Re-suspended PBMCs were placed in flasks and incubated for 3 days with Roswell Park Memorial Institute (RPMI) 1640 media (Hyclone, Logan, UT, USA) supplemented with 10% FBS and 2% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO2. After 3 days, media was supplemented with 50U/mL
granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ, USA) and incubated for 6 days to ensure differentiation of monocytes into macrophages. In both RAW 264.7 (n=4-6) and human macrophage experiments (n=3), cells were first plated, left overnight and then treated with 0.25 or 0.5 µM of either BSA, OA, LA, or LA+EPA+DHA (LED) for 18 hours. Thereafter, cells were stimulated with either 1ug/ml lipopolysaccaride (LPS; Sigma Aldrich, St. Louis, MO, USA) or infected with live *Salmonella enterica* Typhimurium (ST). ST was inoculated to wells at a multiplicity of infection (MOI, the theoretical number of bacteria for each cell) of between 10-50. Total bacteria were counted from serial dilution of stock inoculum. Wells were then incubated for 16 hours.

In a subset of experiments, to increase cellular GSH, cells were co-incubated with fatty acids and 0.25mM NAC for 18 hours. After the 18h incubation period LPS stimulation or ST infection occurred, following the above procedure.

3.3.4.1 Plasmids and siRNA

Overexpression of *Gclc* and *Gs*, were done using plasmids (Transomic, Huntsville, AL, USA) and Attractene transfection reagent (Qiagen, Hilden, Germany). Overexpression of *Gclc* and *Gs* were conducted as previously described in section 2.3.1.2. Overexpression of p22phox (*Cyba*) was done similar to *Gclc* and *Gs* using plasmids (Transomic, Huntsville, AL, USA) and Attractive transfection regent (Qiagen, Hilden, Germany). The method of transfection was the same as that described in section 2.3.1.2. After verification of
transfection, the average fold increase of GCLC in Raw 264.7 was 12.34 and 10.35 for Gs. For p22phox overexpression the average fold increase was 7.45.

3.3.4.2 Fatty acid preparation

Fatty acid solutions were prepared as described in section 2.3.1.1.

3.3.4.3 Cellular Assays

Cellular assays (ROS, GSH, Resazurin) were performed as previously described in section 2.3.1.4.

3.3.4.3.1 NADP/NADPH

The ratio of NADP/NADPH was measured using a kit following manufacturer's instructions (Sigma Aldrich, St. Louis, MO, USA). Briefly cells were washed with ice cold PBS and then extracted using the provided NADP/NADPH Extraction. Cells were then placed on ice for 10 minutes to ensure full lysis. Samples were then centrifuged at 10,000 g for 10 min to remove cellular debris. For total NADP+NADPH content 25 µl of sample was placed into the well. For NADPH content, the sample was heated at 60°C for 30 min to decompose NADP, leaving only NADPH. 25 µl of sample was then placed in a well. To all wells 50 µl of master mix containing NADP cycling buffer and NADP cycling enzyme mix was added. Wells were incubated for 5 min at room temperature. 5 µl of NADPH developer
was added and after 2 hours incubation absorbance was measured at 450 nm. The values of NADPH and total NADPH+NADP were calculated by comparison to a NADPH standard. The ratio of NADP/NADPH was calculated as follows:

\[
\text{ratio NADP/NADPH} = \frac{\text{NADP}_{\text{total}} - \text{NADPH}}{\text{NADPH}}
\]

3.3.4.4 Statistical Analysis

Statistical Analyses were preformed as previously described in section 2.3.2.4.

3.4 Results

3.4.1 Linoleic acid decreases GSH and ROS after LPS stimulation

In order to determine the effects of fatty acids on macrophages, murine RAW 264.7 macrophages were incubated with 0.25 mM or 0.50 mM OA and LA. As a control cells were incubated with 0.5 mM BSA. To simulate infection conditions cells were stimulated with 1ug/ml LPS. In control unstimulated conditions (-LPS), incubation with LA led to an increased ROS response in comparison to BSA and OA. However in LA treated cells, after stimulation with LPS there was no further increase in ROS response (Fig. 3.2a). This is contrary to BSA and OA, where a significant increase in ROS was observed after stimulation. Thus this indicates that incubation with LA reduces the ROS response of macrophages to inflammatory stimuli (Fig. 3.2a). Incubation with LA also led to a significant
decrease in GSH in both unstimulated and stimulated cells that was not observed after incubation with BSA or OA at either concentration (Fig. 3.2b). Previous research has shown that coincubation of n-3 PUFA with n-6 PUFA can reverse n-6 PUFA induced effects and lead to increases in GSH $^{94,95}$. Indeed addition of n-3 PUFA to n-6 PUFA (LED) led to a significant increase in GSH over LA (Fig. 3.2e). While treatment with LED did not reduce unstimulated ROS levels, incubation with 0.25 mM LED did lead to a significant increase in ROS compared to LA (Fig. 3.2d). With respect to mitochondrial redox status, which can be used as an indicator of cell viability, incubation with 0.25mM did not cause a difference in status between OA and LA treated macrophages (Fig. 3.2c). Therefore the observed differences in ROS and GSH are not a result of differences in mitochondrial redox status at this concentration. As incubation with 0.5 mM fatty acid did cause differences in mitochondrial redox status a concentration of 0.25 mM was chosen to be used for subsequent experiments.
Figure 3.2 Linoleic acid incubation of Raw 264.7 macrophages decreases stimulated ROS and baseline GSH and is rescued by co-incubation with n-3 PUFA.

a) ROS and b) GSH level c) mitochondrial redox status in Raw 264.7 macrophages with or without 1µg/ml LPS stimulation for OA and LA treated cells (0.25 or 0.50 mM) compared to 0.50 mM BSA control. d) ROS and e) GSH level f) mitochondrial redox status in Raw 264.7 macrophages with or without 1µg/ml LPS stimulation for LA and LA+EPA+DHA (LED) treated cells (0.25 or 0.50 mM). For ROS, GSH, and mitochondrial redox status data was normalized to BSA - LPS and shown as arbitrary units of fluorescence. All data was analyzed using one-way ANOVA with Tukey's post hoc analysis with different letters indicating significant difference (p<0.05); n=5. Abbreviations: AU, arbitrary units; BSA, Bovine Serum Albumin; GSH, Glutathione; LA, Linoleic Acid; LED, Linoleic Acid + Ecosapentaenoic Acid + Docosahexaenoic Acid; LPS, lipopolysaccaride; OA, Oleic Acid; ROS, Reactive Oxygen Species.

3.4.2 Bacterial infection leads to attenuated ROS in LA treated cells

LPS is produced by gram-negative bacteria. Therefore, to extend our observations to real-life interactions, we inquired whether infection of macrophages with a gram-negative bacterium would exhibit differences depending on FA treatment. After fatty acid incubation,
we infected RAW 264.7 macrophages with ST. Similar to results with LPS, ST infected cells treated with LA showed a decrease in ROS and GSH (Fig. 3.3a,b). Contrary to the results obtained with LPS, incubation with LED did not result in an increase in ROS, however there was a significant increase in GSH in both control and ST infected macrophages compared to LA alone (Fig. 3.3a,b).

Figure 3.3 Salmonella Typhimurium infection of Raw 264.7 macrophages causes depletion in ROS production and increasing invasion frequency after treatment with linoleic acid. a) ROS and b) GSH levels with 0.25mM concentrations of OA, linoleic acid LA, LA+EPA+DHA (LED) or a BSA control with or without ST infection. For ROS and GSH, data was normalized to BSA - ST and shown as arbitrary units of fluorescence. All data was analyzed using one-way ANOVA with Tukey's post hoc analysis with different letters indicating significant difference (p<0.05); n=4. Abbreviations: AU, arbitrary units; BSA, Bovine Serum Albumin; GSH, Glutathione; LA, Linoleic Acid; LED, Linoleic Acid + Ecosapentaenoic Acid + Docosahexaenoic Acid; OA, Oleic Acid; ROS, Reactive Oxygen Species; ST, Salmonella Typhimurium.
3.4.3 Increasing NADPH rescues reactive oxygen species depletion in linoleic acid treated RAW 264.7 macrophages

The main producer of ROS in macrophages is through the use of NADPH oxidase. This process requires the use of NADPH, which is also used as a cofactor for the reduction of GSH. In order to see if the depletion in GSH was related to depletion in NADPH, RAW 264.7 macrophages were treated with 0.05 mM or 0.1mM of NADPH sodium salt (Fig. 3.4). To ensure that the NADPH sodium salt was taken up by the cells the ratio of NADP/NADPH was determined. The addition of 0.05 mM NADPH did not lead to an increase in the NADP/NADPH ratio, in comparison to the corresponding 0 mM control. However, addition of 0.1mM NADPH did lead to an increase in the ratio across all treatments in comparison to the corresponding 0 mM control (Fig. 3.4a,b). With respect to ROS, addition of NADPH increased control levels of ROS in BSA treated cells (Fig. 3.4c). However, NADPH did not increase control levels ROS in OA and LA treated cells. After stimulation with LPS, in BSA treated cells there was a significant decrease in ROS with 0.1mM NADPH. However incubation with NADPH in OA and LA +LPS cells led to a significant increase in ROS compared to 0 mM NADPH controls (Fig. 3.4d). Therefore these results indicate that incubation with NADPH is able restore ROS levels in LPS stimulated LA treated cells.
Figure 3.4 *Increasing NADPH rescues depletion in ROS.*

a) and b) NADPH or c) and d) ROS levels for either control or stimulated with 1μg/ml LPS in RAW 264.7 macrophages incubated with 0.25mM oleic acid (OA), linoleic acid (LA) or a bovine serum albumin (BSA) control. For ROS, data was normalized to 0 mM NADPH control and shown as arbitrary units of fluorescence. For NADP/NADPH data is shown as a ratio of NADP to NADPH. All data was analyzed using one-way ANOVA with Tukey's post hoc analysis with different letters indicating significant difference (p<0.05); n=4.

Abbreviations: AU, arbitrary units; BSA, Bovine Serum Albumin; LA, Linoleic Acid; LPS, lipopolysaccharide; OA, Oleic Acid; NADP, Nicotinamide Adenine Dinucleotide Phosphate; NADPH, Nicotinamide Adenine Dinucleotide Phosphate Hypdrogen; ROS, Reactive Oxygen Species.

As mentioned earlier in macrophages ROS is produced through NADPH oxidase. After assembly of the NADPH oxidase complex on the membrane, electrons are pumped into the compartment leading to the formation of the ROS. One of the components of this complex is that of p22phox encoded by the *Cyba* gene. In order to see how NADPH
oxidase over expression affected both ROS and GSH levels in macrophages, overexpression of p22phox was conducted. In all conditions overexpression of p22phox led to a decrease in ROS production (Fig. 3.5b). Interestingly, overexpression of p22phox led to an increase in GSH levels in LA treated cells (Fig. 3.5).

Figure 3.5 Overexpression of NADPH Oxidase does not increase ROS production in fatty acid treated cells.

a) ROS or b) GSH levels for either control or overexpression of NADPH oxidase (p22phox) with either no stimulation (Unstimulated), 1µg/ml LPS (+LPS) or infection with ST (+ST) in cells incubated with 0.25mM oleic acid (OA), linoleic acid (LA), or a bovine serum albumin (BSA) control. For ROS and GSH data was normalized to unstimulated PTCN control and shown as arbitrary units of fluorescence. All data was analyzed using one-way ANOVA with Tukey's post hoc analysis with different letters indicating significant difference (p<0.05); n=4. Abbreviations: AU, arbitrary units, BSA, Bovine Serum Albumin; GSH, Glutathione; LA, Linoleic Acid LPS, Lipopolysaccaride; OA, Oleic Acid; ROS, Reactive Oxygen Species; ST, Salmonella Typhimurium.
3.4.4 Increasing GSH rescues LA induced depletion in ROS and GSH

As shown in figure 3.2 and 3.3 addition of n-3 PUFA increased levels of GSH in LA treated cells either unstimulated or stimulated with LPS or infected with ST. This increase in GSH corresponded with an increase in ROS after treatment with 0.25mM LED compared to LA alone. To determine if the difference between LA, OA or LED was due to GSH specifically, we adopted both pharmacological (NAC) and genetic approaches (overexpression of Gs and Gclc). NAC, is the acetylated form of cysteine which has previously been shown to increase levels of GSH \(^{268}\). Addition of 25 µM NAC, like addition of n-3 PUFA, was able to restore both ROS production and GSH levels in RAW cells incubated with LA. However, ROS and GSH were increased across all treatments (Fig. 3.6a,b). As there is also an increase in mitochondrial redox status, as such it is possible that the observed increase in BSA, OA and LA, after treatment with NAC is due to increased cells viability (Fig 3.6c).
Figure 3.6 Increasing GSH through increased cysteine rescues depletion in ROS and GSH.
a) ROS, b) GSH levels and c) mitochondrial redox status in control (0µM) or with addition of 25µM NAC added to cells incubated with 0.25mM OA, LA, or a BSA control with or without 1µg/ml LPS. For ROS GSH and mitochondrial redox status data was normalized to unstimulated PTCN control and shown as arbitrary units of fluorescence. All data was analyzed using one-way ANOVA with Tukey's post hoc analysis with different letters indicating significant difference (p<0.05); n=4. Abbreviations: AU, arbitrary units; BSA, Bovine Serum Albumin; GSH, Glutathione; LA, Linoleic Acid; LPS, Lipopolysaccaride; NAC, N-acetyl cysteine; OA, Oleic Acid; ROS, Reactive Oxygen Species.

NAC is often used to increase levels of GSH, but can also have non-GSH related effects. Thus we wanted to confirm that it was indeed increased GSH levels leading to reversal of ROS depletion in LA treated cells. To do this, over expression of GSH synthesis genes, Gclc and Gs, were performed in cells and stimulated with either LPS or ST infection. With respect to the control data, the results were consistent with data in Fig. 3.2. In regards to each stimulus, GSH levels were significantly increased over control for BSA, OA and LA treated cells (Fig. 3.7a,b). Furthermore, overexpression of either Gclc or Gs was able to increase ROS to levels similar to that of the BSA control in BSA, OA and LA treated cells (Fig. 3.7a,b). In terms of mitochondrial redox status overexpression of Gs, Gclc and Gclc+Gs led to a significant reduction in mitochondrial redox status in both OA and LA treated
cells. However, no difference in viability was observed between OA and LA. Therefore the observed effects of increased ROS in LA to levels similar to OA are not due to differences in viability. Therefore these results show that increased levels of GSH lead to increased ROS. As such, decreased of GSH could explain the decrease in ROS observed in LA stimulated cells.

Figure 3.7 Overexpression of GSH synthesis genes rescues depletion in ROS and GSH. ROS, GSH levels and mitochondrial redox status for either a) 1µg/ml LPS or b) ST infection with either a control containing transfection reagent or overexpression of Gclc, Gs or Gclc + Gs. For ROS, GSH and mitochondrial redox status was normalized to unstimulated PTCN control and shown as arbitrary units of fluorescence. All data was analyzed using one-way ANOVA with Tukey's post hoc analysis with different letters indicating significant difference (p<0.05); n=5. Abbreviations: AU, arbitrary units; BSA, Bovine Serum Albumin; GCLC, glutamate cysteine ligase catalytic subunit GCLC; GS, glutathione synthetase; GSH, Glutathione; LA, Linoleic Acid; LPS, Lipopolysaccaride; OA, Oleic Acid; ROS, Reactive Oxygen Species; ST, Salmonella Typhimurium.
3.4.5 LA depletes GSH and attenuates ROS in primary human macrophages

To explore whether the differences seen in macrophage function is also translatable to humans, we utilized human peripheral blood mononucleated cells (PBMC). PBMCs were differentiated into macrophages and were treated with 0.25µM FA and stimulated with LPS (4 hours) or ST (16 hours). Similar to murine macrophages, stimulation with LPS or ST did not lead to an increase in ROS production in LA treated cells compared to BSA and OA stimulated and infected cells (Fig. 3.8a). In unstimulated controls treatment with OA led to an increase in ROS, whereas there was no difference in ROS in LA treated cells (Fig. 3.8a) which is different than what was observed in murine macrophages (Fig. 3.2). Consistent with the murine data in LA treated cells, incubation with NAC led to an increase in ROS in unstimulated and LPS stimulated cells. After stimulation with ST incubation with NAC increased ROS production in LA and BSA, but led to a decrease in OA treated cells. Similar to murine macrophages, incubation with LA led to a reduction in GSH in unstimulated, LPS stimulated and ST infected cells. With respect to OA treated cells no difference in GSH was observed in unstimulated or stimulated cells when compared to BSA controls. Incubation with NAC caused a restoration of GSH in both basal and stimulated conditions (Fig. 3.8b). Therefore these results indicate that similar to mice, incubation with LA leads to decreased GSH in stimulated and unstimulated cells and leads to decreased ROS production after stimulation in human PBMCs.
Figure 3.8 Linoleic acid incubation of human differentiated macrophages decreases stimulated ROS and baseline GSH and is rescued by co-incubation with NAC.

a) ROS and b) GSH levels in human differentiated macrophages in control (0µM) or with addition of 25µM N-acetyl cysteine added to cells incubated with 0.25mM OA, LA, or a BSA control with addition of 1µg/ml LPS or ST infection. For ROS and GSH data was normalized to 0 µM NAC unstimulated control and shown as arbitrary units of fluorescence. All data was analyzed using one-way ANOVA with Tukey's post hoc analysis with different letters indicating significant difference (p<0.05); n=3. Abbreviations: AU, arbitrary units; BSA, Bovine Serum Albumin; GSH, Glutathione; LA, Linoleic Acid; LPS, Lipopolysaccharide; OA, Oleic Acid; NAC, N-acetyl Cysteine; ROS, Reactive Oxygen Species; ST, Salmonella Typhimurium.
3.4.6 Diet high in n-6 PUFA trigger alterations in cardiac fatty acid content and plasma chemokines and cytokine levels

To investigate the effects of high fat diets on systemic inflammation, we utilized C57BL/6 mice fed various diets, followed by isoproterenol (ISO) treatments to induce acute inflammation. To investigate if LA produces similar effects on murine macrophages in vivo, mice were fed a corn oil (CO; high n-6 PUFA) or a diet high in olive oil (OO; high MUFA). After 5 weeks of feeding, selected FAs in the hearts are given are given in Table 3.1 below (full table in Appendix 1). As expected feeding of a CO diet significantly increased the amount of LA (C18:2n-6), whereas feeding of OO significant increased the amount of OA (C18:1) compared to CO fed animals (Table 3.1). Animals fed a CO diet also had decreased levels of palmitic acid (C16:0) and increased levels of the n-3 PUFA cis-11,14,17-eicosatrienoic acid and DHA.
Table 3.1 Fatty acid composition of high fat fed hearts

Fatty acid analysis of hearts using GC-FID for c57/bl6 animals fed OO or CO. Data was analyzed using a 2-way ANOVA *p<0.05 compared to OO; n=4. Abbreviations: CO, Corn Oil; OO, Olive Oil.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Fatty acid (common name)</th>
<th>OO</th>
<th>CO</th>
</tr>
</thead>
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<tr>
<td></td>
<td><strong>Saturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12:0</td>
<td>Lauric Acid</td>
<td>0.52 ± 0.18</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>C14:0</td>
<td>Myristic Acid</td>
<td>1.09 ± 0.53</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic Acid</td>
<td>20.74 ± 0.71</td>
<td>18.14 ± 0.24 *</td>
</tr>
<tr>
<td>C18:0</td>
<td>Stearic Acid</td>
<td>20.75 ± 0.06</td>
<td>21.74 ± 0.57</td>
</tr>
<tr>
<td></td>
<td><strong>Monounsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1</td>
<td>Palmitoleic Acid</td>
<td>0.37 ± 0.08</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>C18:1</td>
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<td>24.92 ± 0.67</td>
<td>12.63 ± 0.4 *</td>
</tr>
<tr>
<td>C20:1</td>
<td>Cis-11-eicosenoic Acid</td>
<td>0.31 ± 0.02</td>
<td>0.27 ± 0.004</td>
</tr>
<tr>
<td></td>
<td><strong>n-3 Polyunsaturated Fatty Acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:3 n-3</td>
<td>α-linolenic Acid</td>
<td>0.42 ± 0.01</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>C20:3 n-3</td>
<td>Cis-11,14,17-eicosatrienoic Acid</td>
<td>7.61 ± 0.25</td>
<td>8.18 ± 0.34 *</td>
</tr>
<tr>
<td>C20:5 n-3</td>
<td>EPA</td>
<td>0.01 ± 0.004</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>C22:5 n-3</td>
<td>Docosapentaenoic acid n-3</td>
<td>0.32 ± 0.02</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>DHA</td>
<td>3.91 ± 0.37</td>
<td>5.56 ± 0.15 *</td>
</tr>
<tr>
<td></td>
<td><strong>n-6 Polyunsaturated Fatty acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>Linoleic Acid</td>
<td>12.43 ± 0.56</td>
<td>27.96 ± 0.85 *</td>
</tr>
<tr>
<td>C18:3 n-6</td>
<td>γ-Linolenic Acid</td>
<td>0.03 ± 0.003</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>C20:2n-6</td>
<td>Cis-11,14-eicosadienoic acid</td>
<td>0.27 ± 0.01</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>C20:3 n-6</td>
<td>Cis-8,11,14-eicosadienoic acid</td>
<td>0.928 ± 0.025</td>
<td>0.825 ± 0.019</td>
</tr>
<tr>
<td>C20:4 n-6</td>
<td>Arachidonic Acid</td>
<td>0.005 ± 0.003</td>
<td>0.005 ± 0.003</td>
</tr>
<tr>
<td>C22:5 n-6</td>
<td>Docosapentaenoic acid n-6</td>
<td>0.835 ± 0.006</td>
<td>1.668 ± 0.082 *</td>
</tr>
</tbody>
</table>

5 weeks of feeding was sufficient to cause an attenuation of anti-inflammatory G-CSF 270,271 in CO fed animals compared to olive OO fed controls. No change in the pro-inflammatory M-CSF 272 was observed with either diet (Fig. 3.9a). To mimic stimulation
practiced *in vitro*, animals were treated with ISO to induce a general inflammatory environment as well as to induce mild cardiac damage. After treatment, there was either no change (G-CSF) or a decrease (M-CSF) in colony stimulating factors when compared to untreated controls across all diets (Fig. 3.9a).

In contrast, chemokines involved in monocyte and macrophage trafficking in LA-rich CO fed animals exhibited either the same (MIP-2) or higher levels (MCP-1 and KC) in control animals not treated with ISO. Following treatment with ISO, however, there was no further increase in chemokine levels in corn oil treated animals. This indicates that macrophage responses are attenuated under inflammation in corn oil fed mice. In contrast, OO fed animals displayed an increase in MCP-1 and KC following ISO treatments (Fig. 3.9b). The lack of response from corn oil treated animals was reversed with fish oil supplementation (n-3 PUFA). It restored chemokines MCP-1 and KC to levels similar to that of OO fed animals (Fig. 3.9b).
Figure 3.9 Feeding of diet high in n-6 PUFA increases pro-inflamatory and decreases anti-inflamatory cytokines and chemokines.

a) Circulating colony stimulating factors and b) pro-inflamatory macrophage/monocyte trafficking chemokines analyzed in plasma using a MILLPLEX Mouse Cytokine/Chemokine kit and quantified in pg/ml. Detection limits for each cytokine are indicated using a dotted line. All data was analyzed using one-way ANOVA with Tukey's post hoc analysis with different letters indicating significant difference (p<0.05); n=4. Abbreviations: CO, Corn oil; COF, Corn oil+Fish oil; G-CSF, Granulocyte-colony stimulating factor; ISO, Isoproterenol; KC, Chemokine (C-X-C motif) ligand 1; M-CSF, Monocyte stimulating factor; MCP-1, Monocyte chemotactic protein 1; MIP-2, Macrophage inflammatory protein 2; OO, Olive oil.

3.4.7 GSH is responsible for modulating macrophage inflammatory responses in vivo

To identify if GSH modulation is indeed behind an effect of macrophage function, a subset of animals on the OO diet were treated with BSO and a subset on the CO diet were treated with NAC in the drinking water. As shown earlier in chapter 2, CO fed animals had decreased GSH and GSH synthesis genes which did not occur in OO. Therefore, it is possible
that the differences in cytokine levels observed in CO fed animals are due to depletion of GSH (Fig. 2.4). As such, CO fed animals was supplemented with NAC to increase their GSH. As well to determine if high levels of GSH are responsible for the cytokine profile observed in OO (Fig. 2.4), OO fed animals were treated with BSO to decrease their levels of GSH. Compared to control, addition of BSO to OO fed animals caused a significant decrease in G-CSF and M-CSF cytokines. Conversely, addition of NAC to CO fed animals caused an increase in G-CSF and M-CSF cytokines over LA controls, and also higher than OO controls (Fig. 3.10a). Furthermore, addition of BSO to OO caused a decrease in cytokines MIP-1β and MIP-2. MIP-1β was not detected in Co or CO+NAC animals; furthermore MIP-1β was also below the detectable rand for OO+BSO. As well, addition of NAC caused a decrease in MIP-2 cytokine levels (Fig. 3.10b). These results indicate that the addition of NAC can reverse CO induced cytokine differences, whereas the addition of BSO to OO mimics CO alone effects. Therefore, it appears that the changes observed in cytokines after CO diet are a result of alternations in GSH levels.
Figure 3.10 GSH is responsible for modulating macrophage inflammatory responses in n-6 PUFA and MUFA rich diets.
a) Circulating colony stimulating factors and b) pro-inflammatory macrophage/monocyte trafficking chemokines analyzed in plasma using a MILLPLEX Mouse Cytokine/Chemokine kit and quantified in pg/ml. Animals were fed either a CO or OO diet and a subset of animals were supplemented for 14 days with either NAC (CO fed) to increase GSH or BSO (OO fed) to decrease GSH. Detection limits for each cytokine are indicated using a dotted line. All data was analyzed using one-way ANOVA with Tukey's post hoc analysis with different letters indicating significant difference (p<0.05); n=4. Abbreviations: BSO, buthionine sulfoximine; CO, Corn oil; G-CSF, Granulocyte-colony stimulating factor; GSH, glutathione; MCP-1, Monocyte chemotactic protein 1; KC, Chemokine (C-X-C motif) ligand 1; M-CSF, Monocyte stimulating factor; MIP-2, Macrophage inflammatory protein 2; NAC, N-acetyl cysteine; OO, Olive oil.
3.5 Discussion

The relationship between dietary fatty acids and immune system function and regulation is complex. Studying the relationship in vivo is often confounded by the presence of comorbidities such as diabetes, obesity, and metabolic syndrome, which make studying the molecular mechanisms difficult. By using in vitro cell culture techniques and a short feeding time in vivo, the goal of this study was to look at the effect of MUFA and PUFA on macrophage function in the absence of confounding diseases. OA and LA were chosen due to their prevalence in the western diet. Furthermore, concentrations were chosen to minimize the effects on cell death and were below or within the range for serum free fatty acid concentrations of 0.3-0.6mM in the fasted state (Fig. 3.2).

Previous literature has shown that depletion in GSH can alter the function of the macrophages. Research on the effects of the glutathione-redox system in RAW 264.7 macrophages showed that GSH controls pro-inflammatory IL-12 synthesis in macrophages. Higher levels of GSH lead to increased IL-12, whereas lower levels of GSH, and high levels of GSSH, lead to decreased IL-12. Similar results have also been found in human THP-1 macrophages and in human alveolar macrophages. In 2004, Song et al. looked at the effects of ethyl pyruvate and NAC on LPS stimulated Raw 265.7 macrophages. They found that both ethyl pyruvate and NAC prevents LPS induced nitric oxide and IL-6 production, but through different mechanisms. The main mechanism attributed to NAC was that of increased GSH. In our studies, we found that after incubation with LA and stimulation with LPS there was decreased levels of ROS, which prompted us to investigate intracellular GSH. Indeed, incubation of murine macrophages with LA decreases basal and
LPS stimulated levels of GSH (Fig. 3.2). Although LPS is useful as a method of stimulation, it is not indicative of an *in vivo* situation \(^{174,280,281}\). Nonetheless, literature has shown that incubation with LPS or ST infection in Raw 264.7 cause similar modulations in gene expressions \(^{282}\). Therefore, to verify the effects of LPS and to determine the impact of fatty acids on ability to fight infection, ST infection was also conducted.

In direct contrast to increased GSH levels through NAC, it has also been shown that depletion of GSH by BSO in peritoneal macrophages caused a 60% reduction in the release of nitric oxide, thereby reducing their ability to fight off the protozoan parasite *Leishmania major*. In our studies, we found that in LA treated cells, LPS or infection with ST led to a reduction in both GSH and ROS. However, addition of fatty acids EPA and DHA, known to increase GSH, led to a partial restoration of GSH and ROS in LPS treated (Fig. 3.2) and a partial restoration of GSH in ST infected (Fig. 3.3) cells. One possible explanation for the differences in GSH and ROS is that of differences in mitochondrial redox status or cell viability. As resazurin is able to be converted by bacteria \(^{283}\), and bacteria also release LDH \(^{284}\), no viability/status assays were conducted for ST infected cells. For unstimulated and LPS stimulated cells no difference in viability after incubation with 0.25 µM OA or LA was observed. Therefore the differences in GSH and ROS at this concentration are not a result of differences in viability.

One potential reason for why GSH levels are important to macrophage function is its ability to react with nitric oxide to form S-nitrosoglutathione (GSNO). In particular, GSNO has been shown to be toxic to ST. Therefore, a reduction in GSH would lead to a reduction in GSNO and could result in a decreased ability of the macrophage to resist ST infection \(^{285}\). Other studies have shown that macrophages prevent replication of ST by increased ROS
production through NADPH oxidase\textsuperscript{281}. Treatment of cells with NAC, leads to increased levels of GSH which in turn leads to increased production of ROS\textsuperscript{167}. Therefore, depletion of GSH, as seen after treatment with LA, may explain the inability of those cells to produce ROS (Fig. 3.3).

In this study, the ratio of GSH to GSSG was not determined; therefore, it is possible that the GSH depletion is the result of an increase in the amount of GSSG. NADPH is required for the conversion of GSSG to GSH, as well as it is required for ROS production from NADPH oxidase\textsuperscript{286,287}. Therefore, in order to test if GSH and ROS depletion were a result of decreased NADPH, cells were incubated with NADPH (Fig. 3.4). Increased NADPH did restore ROS levels in LA treated cells. To investigate the relationship between NADPH oxidase and GSH, a component of NADPH oxidase, p22phox was over expressed in cells. Overexpression did not alter ROS or GSH levels (Fig. 3.5). This indicates that NOX is not a limiting factor; rather it is GSH which is responsible for modulating ROS release in RAW 264.7 macrophages.

Fish oil has been shown to increase GSH levels\textsuperscript{288}. In this study, we show that addition of fish oil components, EPA and DHA, rescues ROS attenuation with stimulation in LPS stimulated cells (Fig. 3.2 and 3.3). Furthermore, we show that fish oil does indeed partially restore GSH levels in LA treated cells (Fig. 3.2 and 3.3). To demonstrate that intracellular GSH is a critical component of macrophage function, which is impaired by LA, we specifically increased GSH either through NAC (3.6) or by overexpression of glutathione synthesis genes GSS and GCLC alone or together (Fig. 3.7).

In mice under oxidative stress, injection of NAC improved function of peritoneal macrophages following LPS treatment\textsuperscript{167}. With respect to dosing, concentrations of NAC up
to 3mM led to a significant increase in the level of GSH in RAW 264.7 macrophages which could be beneficial. However, beyond this dose, NAC led to an increase in GSSG \(^{276}\) and was proven to be toxic. In the pharmacological experiments presented here, a dose of 0.25mM NAC was used and, consistent with previous literature, was also shown to increase the level of glutathione (Fig. 3.6) \(^{279,289}\). Similar responses were found with genetic overexpression of either GSS or GCLC alone (Fig. 3.7). However, genetic overexpression of GCLC and GSS together led to a decrease in GSH levels (Fig. 3.7). This indicates, that similar to increased addition of NAC, high levels of GSH synthesis genes are toxic and lead to decreased GSH levels. Both NAC and overexpression of GSS or GCLC are sufficient to rescue ROS depletion (Fig 3.6 and 3.7).

While we demonstrate that murine macrophages have depleted ROS production after incubation with LA, and decreased GSH, these results are mouse specific. There are many examples throughout the literature of regulatory mechanisms in mice that do not translate to humans \(^{290}\). However, in the case of macrophages, we find that ROS depletion after incubation with LA does occur in human macrophages (Fig. 3.8). Therefore, this mechanism may be conserved as it is present not only in mice but in humans.

To follow up our results in an \textit{in vivo} situation, we fed various fat diets to young mice. In our feeding of n-6 PUFA to young mice, our results were similar to previous studies where feeding of n-6 PUFA showed an increase in pro-inflammatory mediators \(^{228,291}\). Furthermore, our data also confirms that addition of fish oil to a high n-6 PUFA diet can reduce expression of these pro-inflammatory mediators (Fig. 3.9) \(^{292-294}\). As depleted ROS renders the macrophage less functional \(^{167,281}\), increased cytokine levels could be indicative of a feedback response to recruit and differentiate more macrophages due to decreased
function. Moreover, to substantiate our results, we manipulated GSH \textit{in vivo} in these animals (Fig. 3.10). We show that by either decreasing GSH in a MUFA rich diet or increasing glutathione in an n-6 PUFA rich diet, we are able to modulate the responses of colony stimulating factors and chemokines involved in macrophage recruitment.

3.6 Chapter Conclusion

In summary, as mentioned in section 1.7 the specific hypothesis for this chapter was \textit{GSH homeostasis mediates linoleic acid induced macrophage impairment}. To answer this hypothesis we demonstrate that in Raw 264.7 murine macrophages (Objective 1) and isolated human macrophages (Objective 4), n-6 PUFA inhibits macrophage function by decreasing ROS production and decreasing GSH. This decrease in ROS and GSH can be rescued by increasing cellular GSH levels (Objective 3). As well, as NADPH is used as a cofactor we demonstrate that incubation with NADPH restores ROS in LA treated cells (Objective 2). Our \textit{in vivo} data further shows that n-6 PUFA causes an increase in pro-inflammatory mediators. These responses in colony stimulating factors (G-CSF and M-CSF) can be partially restored to levels found in MUFA fed animals by augmenting GSH levels through pharmacological agents or genes that elevate GSH (Objective 5). Therefore, I conclude that the reduction in macrophage function seen in LA treated cells, is a direct result of changes in GSH homeostasis.
Chapter 4: Determine DNA damage and repair responses to n-6 PUFA in the heart

4.1 Overview

Cells routinely endure DNA damage during normal cellular processes. However, if the cell is unable to repair this damage, it can lead to cell death through mechanisms such as apoptosis and necrosis. Using high fat fed animals and ISO as a cardiac challenge, we show that in CO + ISO there is a decrease in cardiac output, which is not observed in OO + ISO treated animals. Using qPCR arrays, we determine that the effected pathways included antioxidant enzymes, heat shock proteins and cytochrome p450 genes, all of which are involved in DNA repair. We then show that n-6 PUFA +ISO leads to differences in DNA damage. In particular, control CO fed animals had higher levels of SSBs than OO fed animals. However, after ISO treatment, n-6 PUFA fed animals had higher levels of DSBs, compared to CO control. In contrast there was no difference in DSBs between OO control and OO + ISO animals. This indicates a switch from apoptosis to necrosis pathways in CO fed animals following ISO treatment. Furthermore, we show that this switching is directly correlated with a decrease in double stranded DNA repair genes and oxidative stress repair genes. Finally, one major function of cytochrome P450 is the metabolism of ARA. Therefore, we characterized the levels of 20-Hydroxyeicosatetraenoic acid (20-HETE) as well as 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), which are cardiotoxic and cardioprotective metabolites of ARA, respectively. We then demonstrate that CO fed animals have higher levels of 20-HETE than OO fed animals. However, after ISO treatment, while
there is an increase in 14,15-DHET in OO fed animals, this was not observed in CO animals. Taken together, these data indicate that n-6 PUFA alters the type of DNA damage occurring in the cell, and also suggests that it is cardiotoxic due to increased production of toxic metabolites produced through ARA metabolism.

4.2 Background

DNA is constantly undergoing changes due to cellular processes, such as transcription, or from exposure to damaging mutagens. DNA damage involves covalent changes in its structure, base-pair mismatches, or the formation of loops and bubbles. This impairment leads to activation of biochemical pathways that help with damage removal, including DNA repair and cell death\textsuperscript{175}. If the cell is unable to repair the damage incurred, the cell dies. There are three distinct mechanisms by which this can occur: apoptosis, necrosis and necroptosis\textsuperscript{182}.

Apoptosis is often referred to as programmed cell death, and is the main method by which aged cells are removed\textsuperscript{182}. Unlike apoptosis, necrosis is the mechanism used following acute or extreme injury, and in contrast to apoptosis does not involve any regular DNA or protein degradation pattern. It is marked by swelling of the cytoplasm and mitochondrial matrix before the cell subsequently ruptures\textsuperscript{185}. Apoptosis commonly occurs with a characteristic change of nuclear morphology, involving condensation of chromatin, known as pyknosis, which leads to fragmentation of the nucleus, known as karyorrhexis\textsuperscript{184}. In contrast to apoptosis and necrosis, necroptosis is a mechanism of cell death falling in-
between the other two types of cell death. It is often referred to as programmed necrosis.

Although much is known regarding the mechanisms by which DNA is repaired, the effect of different dietary fatty acids on DNA damage or the repair in the mammalian heart remains unclear. One family of enzymes which have been shown to be involved in ARA metabolism is the cytochrome P450 (Cyp) family. In the process of ARA metabolism, Cyp genes lead to the production of two major classes of molecules, hydroxyeicosatetraenoic acids (HETE) and epoxyeicosatrienoic acids (EET). The major HETE produced by Cyp is 20-HETE, however small amounts of 16-HETE, 17-HETE, 18-HETE, and 19-HETE are also produced. Cyp metabolism of ARA leads to the production of four regioisomers of EETs. This occurs because epoxidation can happen at any of the four double bonds in ARA. The EETs produced are 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. However, one of the most prominent forms of EETs is the 14,15-EET. Once taken up by cells, the EETs can be hydrolyzed via soluble epoxide hydrolases into the more stable and less reactive dihydroxyeicosatrienoic acid (DHET) forms.

Of the HETEs, the most widely produced 20-HETE has been found to be a vasodilator. Of the EETs, 14,15-EET has been shown to have beneficial effects during CVD and is considered to be cardioprotective. However, 14,15-EET is rapidly hydrolyzed by soluble epoxide hydrolases into the more stable 14,15-dihydroxyeicosatrienoic acid. In general, EETs are considered to be cardioprotective. In contrast, HETEs are considered to be cardiotoxic.

As shown earlier cells/animals incubated/fed with n-6 PUFA have differences in mitochondrial membrane potential (chapter 2) and differences in immune responses (chapter
3). Therefore we sought first to determine if these alterations lead to any functional changes in the heart muscle. We then showed that after n-6 PUFA feeding and cardiac challenge with ISO, there is a decrease in cardiac output, not observed in OO fed ISO treated animals. Next we aimed to determine which pathways might be altered under these conditions. Therefore, we conducted qPCR gene arrays. These arrays indicated that there was a trend of decrease expression in heat shock proteins, specifically HSP70 and trend of increased expression in cytochrome p450 genes, which are known to be involved in ARA metabolism and DNA repair. Indeed, n-6 PUFA feeding plus ISO did lead to differences in DNA damage. Control CO fed animals had higher levels of SSBs than OO fed animals. However, after ISO treatment, n-6 PUFA fed animals had higher levels of DSBs compared to CO controls, indicating a switch from apoptosis to necrosis. Furthermore, we show that this switching is directly correlated with an attenuation of double stranded DNA repair genes and oxidative stress repair genes. Finally, due to higher levels of LA, CO fed animals are likely to have increased levels of ARA. To determine how this could affect downstream products of ARA metabolism we determined the levels of 20-HETE as well as 20 and 14,15-DHET, which are cardiotoxic and cardioprotective metabolites, respectively. We demonstrate that CO fed animals have higher levels of 20-HETE than OO fed animals. However after ISO treatment, while there is an increase in 14,15-DHET in OO fed animals, this was not observed in CO animals. Taken together, these data indicate that n-6 PUFA alters the type of cellular DNA damage, and due to increased production of toxic metabolites from ARA metabolism, is cardiotoxic.
4.3 Materials and Methods

4.3.1 In vivo experiments

Male C57BL/6 mice were weaned at 3 weeks of age onto high-fat isocaloric, iso-nitrogenous diets with 40% energy, or a normal chow diet with 9% energy from fats, as described earlier in 2.3.2.

4.3.1.1 qPCR

Tissue preparation and methods for qPCR were preformed as described earlier in section 2.3.2.3. Primers used for quantification are listed below in table 4.1.
Table 4.1 Primer sequences used for the quantification of mRNA levels by real-time PCR.

Primers used for gene expression; forward (F) and reverse (R) primers are indicated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>Product Size</th>
<th>Primer efficiency</th>
<th>Gene Bank Reference #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>F: GGACGAGCTTGG GAGCG R: AAAAGGCCCTGTCTTCATGA</td>
<td>62 bp</td>
<td>96.5</td>
<td>NM_007527.3</td>
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<tr>
<td>Bcl2</td>
<td>F: TTCTCAGTGAAAGCGGAGTG R: GAATCGGGAGTTGGGCTTG</td>
<td>88 bp</td>
<td>98.5</td>
<td>NM_009741.5</td>
</tr>
<tr>
<td>Bclxl</td>
<td>F: AGGCTGGCGATGAGT TTGAA R: TGAAACGCTCCTTG GCCCTTC</td>
<td>92 bp</td>
<td>93.6</td>
<td>NM_00128971 7.1</td>
</tr>
<tr>
<td>Bim</td>
<td>F: GGAGATACCGATTGCACAGGAG R: CCTTCTCCTACCAGACGGAAG</td>
<td>159 bp</td>
<td>89.4</td>
<td>NM_009754.3</td>
</tr>
<tr>
<td>Gpx1</td>
<td>F: GTTCGGACACCAGGAGAA R: GCCTTCTCACCATTCTCCTTCC</td>
<td>126 bp</td>
<td>95.6</td>
<td>NM_00132952 8.1</td>
</tr>
<tr>
<td>Ogg1</td>
<td>F: ATTGCCCATCGTGACTAC R: AAAGTTTCCCCAGTTCTTTT</td>
<td>87 bp</td>
<td>98.3</td>
<td>NM_010957.4</td>
</tr>
<tr>
<td>Top1</td>
<td>F: AACGATTCCAGATCGAAG R: TCCCCGTCCTTATCCTTATC</td>
<td>119 bp</td>
<td>88.4</td>
<td>NM_009408.2</td>
</tr>
<tr>
<td>Top1-mt</td>
<td>F: CTTCCTCTACGATGGCAAAC R: GACTTCCTTGCTCGTACACTC</td>
<td>103 bp</td>
<td>91.5</td>
<td>NM_028404.2</td>
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<tr>
<td>Top-2a</td>
<td>F: CGCTGCAGCTGTAATG R: TCTTCATCGAAACCACATT</td>
<td>184 bp</td>
<td>86.5</td>
<td>NM_011623.2</td>
</tr>
<tr>
<td>Top-2b</td>
<td>F: CCTGGGTGAACATGTCTAC R: GTATCAGGACGAAGAGAATG</td>
<td>133 bp</td>
<td>93.6</td>
<td>NM_009409.2</td>
</tr>
</tbody>
</table>
4.3.1.2 Immunofluorescence

Immunofluorescence was performed on 5µm thick left ventricle sections, fixed in formalin. Sections were then embedded and sectioned with 2 sections per slide. Sections were then de-paraffinized in xylene followed by incubations in 100%, 90%, 80% and 70% ethanol. Sections were then rinsed and incubated in PBS for 5 minutes. They were then pretreated with 1mg/ml trypsin for 20 min at 37°C. To reduce nonspecific binding, sections were treated with 5% BSA for 20 min. For determination of total double and stranded DNA breaks the DeadEnd™ Flurometric TUNEL System was used (Promega, Madison, WI, USA). For single strand DNA breaks greater than 25 bp in length the anti-ssDNA, clone F7-26 (Millipore, Billerica, MA, USA) combined with the M.O.M (mouse-on-mouse) kit (Vector Laboratories, Burlingame, CA, USA) was used. Sections were incubated with M.O.M. biotinylated anti-mouse IgG and FITC-streptavidin. They were then counterstained with 4’6-diamidino-2-phenylindole (DAPI) to visualize nuclei, mounted and viewed on an Olympus IX81 fluorescent microscope. The number of stained nuclei was counted by a blinded observer and values were expressed as number positive per 100 nuclei.

4.3.1.3 ELISA assays

20-hydroxyeicosatetraenoic acid (20-HETE) and 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), were evaluated using commercially available ELISA kits (Detroit R&D, Detroit, MI, USA). Briefly for both ELISAs cells were homogenized in dH2O and acidified to a pH of 3.5 using acetic acid. Following which the samples were extracted using ethyl
acetate. An equal volume of ethyl acetate was added and the homogenates were vortexed and the organic phase was collected. This extraction procedure was performed 3 times for each sample. Following which the organic phases were combined and dried under nitrogen gas. The dried sample was then resuspended in N,N-dimethyl-formamide. Samples were then split in half with one half being diluted in 1x sample dilution buffer from the 20-HETE kit and the other half being diluted in 1x sample buffer from the 14,15-DHET kit. Samples were then centrifuged for 5 min at 10,000 rpm and the supernatant was then used in the ELISA assays. For both assays 100 µl sample or standard was added to each well. Then 100 µl of the appropriate diluted conjugate (20-HETE or 14,15-DHET) was added to the wells. Plates were then incubated for two hours and washed three times using 400 µl wash buffer. Plates were then dried by patting with paper towel and 200 µl of the substrate was added to wells. Plates were then incubated for 30 minutes at room temperature, followed by addition of 50 µl 2 N sulfuric acid. Absorbance at 450 nm was then measured using a GloMax Multi+ detection system (Promega, Madison, WI, USA). Concentration of either 20-HETE or 14,15-DHET was determined by comparing to standards. The total protein content of the samples was determined using a Bradford assay (Bio-Rad Laboratories Inc, Hercules, CA, USA). Total concentration of 20-HETE or 14,15-DHET per mg of protein was determined as follows:

\[
\text{Total 20-HETE or 14,15-DHET per mg protein} = \frac{\text{Total (pg/ml)}}{\text{Protein (mg/ml)}}
\]
4.3.1.4 Echocardiography

A Vevo® 2100 system (Fujifilm Visualsonics, ON, CA) was used to examine LV function in mice by noninvasive transthoracic echocardiography. Mice were anesthetized with 2% isoflurane and maintained with 1% isoflurane on a warming platform to maintain body temperature. Two dimensional M-mode and parasternal short and long- axis scans were made to assess changes in left ventricle (LV) fractional shortening and output²²⁴. After acquisition of the images calculations were performed using the left ventricle analysis package. Within the cardiac package the left ventricle trace tool was selected. Using this tool multiple cardiac cycles were traced so that the anterior (epicardium and endocardium) and the posterior (endocardium and epicardium) for each of these cycles was traced. A sample trace, including sample data for calculations is shown below in Figure 4.1. While heart rate was measured, for cardiac output heart rate, determined from the trace was used in this calculation. The formula used for cardiac output was as follows:

\[
\text{Stroke Volume} = \text{Systolic Volume- Diastolic Volume}
\]

\[
\text{Cardiac output} = \frac{\text{Heart Rate} \times \text{Stroke Volume}}{1000}
\]

\[
\text{Fractional Shortening} = \frac{\text{LV Diastolic (mm)- LV Systolic (mm)} \times 100\%}{\text{LV Diastolic (mm)}}
\]
Figure 4.1 Sample echocardiography left ventricle trace.

Green lines represent traces of the anterior and posterior epicardium and endocardium. Blue dotted lines represent systolic diameter and red dotted lines represent diastolic diameter. Abbreviations: HR, Heart Rate; D;s, Systolic Diameter; D;d, Diastolic Diameter; V;s, Systolic Volume; V;d, Diastolic Volume; SV, Stroke Volume; FS, Fractional Shortening; CO, Cardiac Output.
4.3.1.5 Statistical analysis

Statistical Analyses were preformed as previously described in section 2.3.2.4.

4.4 Results

4.4.1 A diet high in n-6 PUFA alters cardiac function after cardiac insult

To determine the effects of high fat feeding on the function of the heart after mild cardiac injury, animals fed OO or CO were injected with a single dose of 100 mg/kg ISO. 24 hours post ISO injection, heart function was evaluated using echocardiography in these animals. No changes in the heart function between the control groups OO and CO were observed (Table 4.2). However, ISO led to an increase in peak systolic diameter and a decrease in fractional shortening in OO fed animals within 24 hours. In contrast, CO fed animals had a significant decrease in cardiac output following ISO treatment but no difference in fractional shortening or peak systolic diameter was observed.
Table 4.2 ISO treatment alters cardiac function in corn oil fed animals.

*P<0.05 vs corresponding control group; n=4. Abbreviations: OO, Olive Oil; CO, Corn Oil; ISO, Isoproterenol.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OO Control</th>
<th>OO + ISO</th>
<th>CO Control</th>
<th>CO + ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac Output (mL/min)</td>
<td>26.78 ± 1.32</td>
<td>27.12 ± 1.65</td>
<td>26.25 ± 2.75</td>
<td>22.80 ± 1.12*</td>
</tr>
<tr>
<td>Stroke Volume (µL)</td>
<td>51.21 ± 1.83</td>
<td>52.14 ± 2.58</td>
<td>50.39 ± 6.75</td>
<td>44.25 ± 2.62</td>
</tr>
<tr>
<td>Diastolic Volume (µL)</td>
<td>58.74 ± 2.52</td>
<td>64.84 ± 4.71</td>
<td>58.24 ± 8.12</td>
<td>49.58 ± 3.26</td>
</tr>
<tr>
<td>Systolic Volume (µL)</td>
<td>7.53 ± 1.57</td>
<td>12.70 ± 2.16</td>
<td>7.85 ± 1.69</td>
<td>5.32 ± 0.75</td>
</tr>
<tr>
<td>Fractional Shortening (%)</td>
<td>57.51 ± 2.74</td>
<td>49.95 ± 2.68*</td>
<td>56.59 ± 2.76</td>
<td>59.58 ± 1.23</td>
</tr>
<tr>
<td>Systolic Diameter (mm)</td>
<td>1.58 ± 0.12</td>
<td>1.94 ± 0.16*</td>
<td>1.60 ± 0.15</td>
<td>1.40 ± 0.08</td>
</tr>
<tr>
<td>Diastolic Diameter (mm)</td>
<td>3.70 ± 0.06</td>
<td>3.86 ± 0.12</td>
<td>3.66 ± 0.21</td>
<td>3.45 ± 0.10</td>
</tr>
</tbody>
</table>

4.4.1.1 n-6 PUFA increases oxidative stress and inflammatory genes expression

To elucidate the cellular mechanisms involved with the observed changes in cardiac function following ISO treatment, qPCR gene arrays were conducted ISO treated OO and CO fed animals (Qiagen, Hilden Germany). As this was designed to be an overview in order to identify key pathways, no statistical analysis could be conducted due to low sample number (Fig. 4.2; n=2). However it appeared that there was a trend of decreased expression in the antioxidant genes Gpx2 and Sod2, but no trend was observed in Gpx1. There was also a trend of increased expression in heat shock proteins Hspd1 (also known as mitochondrial Hsp60), and Hspa1l (also known as Hsp70-1L), which have been shown to play a protective role against oxidative stress induced damage in Saccharomyces cerevisiae and in HeLa cells. However, there was a trend of decreased expression in heat shock protein (Hsp) 70
family members (Hspa4, Hspa5, Hspa8). As well, there was a trend of upregulation in Hmox1, which has also been shown to be involved in the cellular oxidative stress response (Fig 4.2)\textsuperscript{307}. Finally, there was trend of increased expression in cytochrome p450 genes (Cyp7a1, Cyp4a10, Cyp2c29, Cyp1b1, Cyp1a1), which are involved in the metabolism of arachidonic acid (Fig. 4.2)\textsuperscript{296}. As well, Cyps have also been shown to be involved in DNA damage repair responses \textsuperscript{308,309}. This trend of increased expression of Cyps will be further explored later in this chapter.
Figure 4.2 Corn oil alters expression of stress and toxicity genes after ISO treatment.

Analysis of qPCR results using Qiagen software (Hilden, Germany) for OO and CO + ISO treated animals; n=2. Abbreviations: Sod2, Superoxide dismutase 2; Sod1, Superoxide dismutase 1; Hspe1, Heat Shock Protein Family E (Hsp10) Member 1; Hspd1, heat shock protein family D (Hsp60) member 1; Hspb1, heat shock protein family B (small) member 1; Hspa8, heat shock protein family A (Hsp70) member 8; Hspa5, heat shock protein family A (Hsp70) member 5; Hspa4, heat shock protein family A (Hsp70) member 4; Hspa1l, heat shock protein family A (Hsp70) member 1 like; Hmox2, heme oxygenase 2; Hmox1, heme oxygenase 1; Gstm1, glutathione S-transferase mu 1; Gsr, glutathione reductase; Gpx2, glutathione peroxidase 2; Gpx1, glutathione peroxidase 1; Fmo5, flavin containing monooxygenase 5; Fmo4, flavin containing monooxygenase 4; Fmo1, flavin containing monooxygenase 1; Ephx2, epoxide hydrolase 2; Dnaj, DnaJ heat shock protein family (Hsp40) member A1; Cyp7a1, cytochrome P450 family 7 subfamily A member 1; Cyp4a10, cytochrome P450, family 4, subfamily a, polypeptide 10 ; Cyp1b1, cytochrome P450 family 1 subfamily B member 1; Cyp1a1, cytochrome P450 family 1 subfamily A member 1 ; Olive oil; CO, Corn oil; ISO, Isoproterenol.
4.4.1.2 Treatment with ISO alters mechanisms of cell death in CO fed hearts

In order to determine if the observed trends from the gene arrays resulted in varying mechanisms of cell death, hearts were collected and sectioned for immunohistochemistry (Fig. 4.3). It was found that in the absence of ISO, CO fed animals had higher levels of ssDNA breaks (Fig. 4.3). This implies that CO fed animals have higher levels of apoptosis. However, in the CO group after treatment with ISO, there was a significant drop in the number of ssDNA breaks observed, but a significant increase in the number of dsDNA + ssDNA breaks using TUNEL was observed. Therefore as the number of ssDNA breaks was reduced this indicates that there is an increase in the number of dsDNA breaks (Fig. 4.3). As dsDNA breaks arise as a result of increased oxidative or mechanical stress, increase in the occurrence of necrosis over apoptosis is suspected. In contrast to CO, OO fed animals ISO treatment did not significantly alter the levels of either ssDNA or dsDNA breaks.
Figure 4.3 Corn oil increases single strand DNA breaks at baseline and double stranded DNA breaks following ISO.

Measurement of single stranded (SS) and double stranded (DS) DNA Breaks (DB) in olive oil (OO), and corn oil (CO) fed animals with and without ISO treatment. a) ssDNA Break >25bp per 100 nuclei. b) TUNEL staining per 100 nuclei. Data was analyzed using one-way ANOVA with Tukey's post hoc analysis (n=4 control, n=6 ISO) with different letters indicating significant difference (p<0.05). Abbreviations: ssDNA, Single-Stranded DNA Breaks; dsDNA, Double-Stranded DNA Breaks; OO, Olive oil; CO, Corn oil; ISO, Isoproterenol.

4.4.1.3 High fat feeding alters topoisomerase expression

As shown above in Fig 4.3, following treatment with ISO, CO fed animals had differing amounts of single stranded versus double stranded DNA breaks. In order to determine if the difference in breaks were a result of differential enzymes responsible for unwinding super-coiled DNA, we investigated the level of expression of topoisomerase genes. In particular, we investigated the differences between the two classes of
topoisomerases, topoisomerase 1 and topoisomerase 2. Topoisomerase 1 creates ssDNA breaks during replication, and although it is generally found in the nucleus, there is also a mitochondrial specific form known as mitochondrial topoisomerase \((\text{top1}-\text{mt})\)\(^{314,315}\). Unlike topoisomerase 1, topoisomerase 2 creates dsDNA breaks to allow for the separation of chromosomes during cell division\(^{316}\). After treatment with ISO, OO fed animals had a significant decrease in expression in \(\text{top1}-\text{mt}\). In contrast, there was an increased expression of topoisomerase 2a (\(\text{top2a}\)), in OO fed animals. Feeding with CO did not significantly alter the expression of topoisomerase genes in either the control or in ISO treated animals (Fig. 4.4).

**Figure 4.4** *Corn oil rich diets prevents high fat diet-induced alterations in topoisomerase expression*

qPCR of topoisomerase genes relative in OO, and CO fed animals with and without ISO treatment normalized to OO control. Data was analyzed using one-way ANOVA with Tukey's post hoc analysis \((n=4\) control, \(n=6\) ISO) with different letters indicating significant difference \((p<0.05)\). Abbreviations: \(\text{top1}\), Topoisomerase 1; \(\text{top1-mt}\), Topoisomerase 1-mitochondrial;; \(\text{top2a}\), Topoisomerase 2a; \(\text{top2b}\); Topoisomerase 2b; OO, Olive oil; CO, Corn oil; ISO, Isoproterenol.
4.4.1.4 Isoproterenol decreases antioxidant genes and DNA repair pathway in CO fed animals

In order to investigate how DNA repair and antioxidant genes were affected by high fat feeding, various antioxidant and DNA repair genes were analyzed by qPCR (Fig. 4.5). After treatment with ISO, CO fed mice had reduced oxidative stress repair genes as well as double stranded DNA repair and anti-apoptotic genes (Fig. 4.5). In contrast to CO, OO fed animals had an increase in the anti-apoptotic gene Bclxl after ISO treatment (Fig. 4.5).
Figure 4.5 *ISO* attenuates DNA repair and anti-apoptotic genes in corn oil fed hearts.

qPCR of DNA repair and apoptosis genes in OO, and CO fed animals with and without ISO treatment normalized to OO control. 1: Oxidative DNA damage repair genes 2: Single stranded DNA damage repair gene 3: Double stranded DNA damage repair genes 4: Pro-apoptotic genes 5: Anti-apoptotic genes. Data was analyzed using one-way ANOVA with Tukey's post hoc analysis (n=4 control, n=6 ISO) with different letters indicating significant difference (p<0.05). Abbreviations: *Ogg1*, 8-Oxoguanine Glycosylase 1; *Xrcc1*, X-Ray Repair Cross Complementing 1; *Xrcc2*, X-Ray Repair Cross Complementing 1; *Rad50*, Rad50 double strand break repair protein; *Bim*, Bcl-2 Like Protein; *Bax*, Bcl 2 Associated X Protein; *Bcl2*, B Cell Lymphoma 2; *Bclxl*, B Cell lymphoma Extra Large; OO, Olive oil; CO, Corn oil; ISO, Isoproterenol.
4.4.1.5  n-6 PUFA alters levels of ARA metabolites

As mentioned earlier, cytochrome p450 genes are involved in ARA metabolism and DNA repair. As ARA is derived from LA, n-6 PUFA feeding can result in increased levels of ARA. As previously mentioned, ARA can be metabolized into two major classes of molecules EETs/DHETs and HETES, with EETs being cardioprotective and HETES being cardiotoxic \(^{297}\). Therefore, to verify the results of the gene array, a subset of the cytochrome p450 genes were analyzed using qPCR. Unlike the array results, it was found that after treatment with isoproterenol, animals fed a CO diet had an increase in cytochrome p450 genes Cyp2j6 and Cyp4a12b (Fig 4.6). Since cytochrome p450 genes are involved in the metabolism of arachidonic acid \(^{296}\), the increase in these genes in the CO diet could be due to increased arachidonic acid metabolism. In order to determine if there was an increase in arachidonic acid metabolism, two separate metabolites of this pathway, 20-hydroxyeicosatetraenoic acid (20-HETE) and 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), were evaluated using commercially available ELISA kits (Detroit R&D, Detroit MI). In all CO fed animals (control and ISO), there was a significant increase in 20-HETE levels over the corresponding OO group (Fig 4.6). However, no increase in 14,15-DHET was observed. Interestingly, after ISO treatment, OO fed animals had increased levels of both 20-HETE and 14,15-DHET, which was not seen in CO fed animals (Fig. 4.6).
**Figure 4.6** *n*-6 PUFA increase levels of cardiotoxic 20-HETE.

a) qPCR of cytochrome p450 genes in olive oil (OO), and corn oil (CO) fed animals with and without ISO treatment normalized to OO control. b) ELISA of 20-HETE and 14,15-DHET in OO and CO fed hearts with and without ISO treatment. Data was analyzed using one-way ANOVA with Tukey's post hoc analysis (n=4 control, n=6 ISO) with different letters indicating significant difference (p<0.05). Abbreviations: CYP2J6, cytochrome P450 family 2 subfamily J member 6; CYP4A10, cytochrome P450, family 4, subfamily a, polypeptide 10; CYP4A12B, cytochrome P450 family 1 subfamily A polypeptide 12B; EPHX2, epoxide hydrolase 2; Olive oil; CO, Corn oil; ISO, Isoproterenol.
4.5 Discussion

The effects of dietary fatty acids on the modes of cell death (apoptosis, necrosis and necroptosis) remain unclear. Furthermore, due to its perceived cardiotoxicity, research on cardiac cell death tended to focus on the effects of SFAs and not n-6 PUFA. When the heart has undergone damage, collagen is required in order to repair the damaged tissue. This adaptation often leads to fibrosis. During fibrosis, collagen I fibers are less flexible than that of collagen III, which can lead to a stiffening of the heart muscle. We showed in earlier studies that n-6 PUFA led to an increase in collagen I, thus altering the collagen I/III ratio resulting in stiffening of the heart muscle. Thus, it was not surprising that ISO treated CO fed animals had a decrease in cardiac output that was not observed in any other diet group (Table 4.1).

ISO is a non-selective beta-adrenergic agonist drug, which in rats leads to left ventricular dysfunction in a dose-dependent manner after 1-3 doses. However, ISO treatment in mice requires 5-7 days in order to see changes in cardiac function. As an example, in adult Swiss-Webster mice, 5 days of 100mg/kg ISO for 10 to 14 days resulted in endocardial injury, increased fibrosis, and hypertrophy of myocytes. The purpose of using a single low dose of ISO (100mg/kg) in this study was to subject mice hearts to a mild insult in order to evaluate cellular responses, which are activated within 24-48 hours. Following ISO treatment, the mice did suffer from tachycardia and recovered within 12 hours after simulating a mild cardiac event.

To identify clues for specific damage pathways following diets and ISO, toxicity gene arrays were conducted (Fig. 4.2). As mentioned due to low sample number statistics could
not be conducted. But, in our study, $Gpx1$ did not change and $Sod2$ tended towards decrease after ISO in CO-fed hearts (Fig. 4.2). Decreases in $Sod2$ can propagate CVD risk. This was accompanied by a trend of increased expression in heat shock protein ($Hsp60$, and $Hsp70$-IL) and a trend of decreased expression in heat shock Hsp70 family members ($Hspa4$, $Hspa5$, $Hspa8$) genes (Fig. 4.2). Like antioxidants, heat shock protein 70 prevents oxidative stress induced damage by ensuring proper protein folding.

Under conditions of oxidative stress, loss of Hsp70 increases in necrotic cell death due to increased oxidation of LA and ARA, leading to the formation of 4-hydroxy-2-nonenal (4-HNE). 4-HNE is a highly toxic lipid peroxidation product of linoleic acid metabolism. 4-HNE increases the generation of ROS and results in calcium overload, ultimately leading to failure of the myocardium. 4-HNE is removed from the cell by conjugation to GSH and subsequent removal via GST. Dysfunction in GSH homeostasis can lead to increased levels of 4-HNE, which could explain why diets rich in LA are cardiotoxic.

After feeding of CO, as shown in chapter 2, there is decreased levels of GSH, which could result in increased levels of 4-HNE. As well, we show that CO leads to increased levels of apoptosis not seen in OO fed animals (Fig. 4.3). Increased levels of 4-HNE have been shown to increase apoptosis. Therefore, increased 4-HNE, coupled with decreased GSH, could explain why higher levels of apoptosis are observed after CO feeding. ISO is known to cause oxidative stress. Thus, in the CO diet group after ISO treatment, with a loss of antioxidants like $Sod2$, unchanged $Gpx1$ coupled with a loss of $Hsp70$ and increased of 4-HNE could induce higher oxidative insult, promoting necrosis pathways. This is supported by the observation that ISO challenge promoted more dsDNA breaks and not ssDNA breaks.
in CO-fed mice compared to CO controls (Fig. 4.3). However, while there was an increase in the type of DNA damage, no necrotic damage was observed in the tissues. This is likely due to the short time frame (24 hours) between the last injection and collection of samples. Further experimentation is required to determine if the alterations in the DNA damage pathways lead to the formation of necrotic zones over time.

In CO-fed hearts, increased levels of dsDNA indicate that there is a lack of dsDNA repair enzymes, which leads to increased levels of necrosis \(^{312,313}\). Topoisomerases are enzymes involved in the overwinding or underwinding of DNA. Topoisomerase 1 creates ssDNA breaks during replication \(^{314,315}\). Topoisomerase 2 creates dsDNA breaks to allow for the separation of chromosomes during cell division \(^{316}\). As mentioned, hearts fed CO had increased ssDNA breaks over OO fed hearts (Fig. 4.3). This corresponded to a decrease in topoisomerase 1 genes \((\text{top}1, \text{top}1-mt)\) in CO fed hearts (Fig. 4.4). Interestingly, after ISO treatment, topoisomerase 2a increased in OO but not in CO fed hearts (Fig. 4.4). Another clue to such repair pathways was provided by oxoguanine glycosylase \((\text{Ogg}1)\), the gene involved in oxidative DNA damage repair. Increased levels of \(\text{Ogg}1\) can prevent \(^{335}\) oxidative stress damage \(^{336}\). It has been previously shown that n-6 PUFA reduces \(\text{Ogg}1\) in rat hearts \(^{200}\). In this study, a decreased expression of \(\text{Ogg}1\) was noted in ISO-treated CO-fed mice hearts. Other DNA repair enzymes encoded by \(\text{Xrcc}1\) and \(\text{Rad}50\) were also reduced in this group (Fig. 4.5) \(^{337,338}\). Interestingly, the ssDNA repair gene, \(\text{Xrcc}1\), remained unchanged across groups.

When the classical BCL-2 regulated pathway of cardiac cell death was analyzed, a significant decrease in the anti-apoptotic genes \(\text{Bcl}2\) and \(\text{Bclxl}\) were noted in ISO treated CO-fed mice (Fig 4.5). Inhibition of \(\text{Bcl}2\) and \(\text{Bclxl}\) is known to cause a pro-necrotic effect \(^{339,340}\).
Therefore, the decrease in Bcl2 and Bclxl, combined with decreases in oxidative DNA
damage repair and dsDNA damage repair genes could provide clues as to why necrosis is
observed in CO fed hearts after ISO treatment.

From our array results, we also observed an increase in specific cytochrome p450
genes (Cyp7a1, Cyp4a10, Cyp2c29, Cyp1b1, Cyp1a1) in CO-fed hearts following ISO
treatment. One function of the cytochrome p450 enzyme, besides DNA repair, is the
production of EETs and HETEs, as mentioned earlier. Feeding of CO, but not OO, led
to increases in 20-HETE. Interestingly, after ISO treatment, there was an increase in 20-
HETE in OO, but not CO fed hearts. However, this increase was still lower than the levels of
20-HETE, observed during CO feeding alone (Fig. 4.6). In contrast to 20-HETE, after
injection with ISO, OO hearts displayed a significant increase in 14,15-DHET as well, which
was not observed in CO hearts (Fig. 4.6). Because the effects of EETs can be long lasting,
a higher level of 14,15-DHET could indicate that OO diets may be cardioprotective in
response to insult. These novel data suggests that CO diets may in fact be cardiotoxic, at least
partially due to increased levels of 20-HETE and reduced 14,15 DHET. However, more
research needs to be done to determine exact molecular mechanisms of such toxicity.

### 4.6 Chapter Conclusion

In summary, as mentioned in section 1.7 the specific hypothesis for this chapter was
*in the heart linoleic acid increases cell death and decreases repair pathway responses*. To
answer this hypothesis we demonstrate that after ISO treatment n-6 PUFA feeding decreases
cardiac output (objective 1) and increases cytochrome p450 genes Cyp2j6 and Cyp4a12b
(objective 2). Furthermore n-6 PUFA feeding plus ISO alters type of DNA damage with animals had higher levels of SSBs. However, following ISO treatment, n-6 PUFA fed animals had higher levels of DSBs, indicating a switch from apoptosis to necrosis. This is directly related to an attenuation of DSB repair genes and oxidative stress repair genes (objective 3). Finally, we demonstrated that CO fed animals have higher levels of 20-HETE than OO fed animals. However after ISO treatment, while there is an increase in 14,15-DHET in OO fed animals, this was not observed in CO animals (objective 4). Taken together, these data indicate the n-6 PUFA alters the type of DNA damage in the cell, and is cardiotoxic due to increased production of toxic metabolites from ARA metabolism.
Chapter 5: Conclusion

5.1 Limitations of the work

The work within this thesis was primarily carried out in cell culture and mouse models. Therefore one limitation of the research is its applicability to humans. However, as shown in chapter 2, when human PBMCs were tested (Fig. 3.7), they exhibited a response similar to that observed in mouse macrophages.

5.2 Significance

Despite recent recognition that n-6 PUFA is cardiotoxic, current dietary recommendations encourage consumption of n-6 PUFA with limited amounts of SFA. Besides direct sources of SFA, in a recently published study, I showed that n-6 PUFA is also present in significantly higher amounts in commercial, but not grass-fed butter. As such individuals that wish to limit their intake of n-6 PUFA may be obtaining significant amounts of it through other dietary sources such as meat and dairy. As an example, farmed salmon which should be rich in n-3 PUFA, has recently been shown to be higher in n-6 PUFA due to the use of n-6 PUFA containing oils as a source of food. Currently, previous studies have focused on interventions using n-6 PUFA after cardiac events. The results of this thesis highlight the necessity for further research in humans to determine the threshold at which intake of n-6 PUFA becomes cardiotoxic.

5.3 Conclusion
Despite efforts to reduce the occurrence of CVD, the rates of heart disease continue to climb\textsuperscript{343,344}. Originally it was hypothesized that SFAs were responsible for the development of CVD. But recent research has shown that n-6 PUFA may be a more likely candidate\textsuperscript{67,69,345}. Despite evidence that n-6 PUFA may be cardiotoxic, the mechanisms through which this cardiotoxicity occurs are not well understood. This thesis provides the following evidence which sheds new light on the mechanisms of n-6 PUFA toxicity \textit{in vivo}:

1. A n-6 PUFA-rich diet increases pro-inflammatory chemokines, oxidative stress and impairs GSH synthesis genes. Such observations \textit{in vivo} were validated \textit{in vitro} using pure LA, the primary dietary PUFA. Further investigations revealed that such impaired GSH synthesis might be modulated by MCP-1, a master pro-inflammatory regulator.

2. Despite increases in basal inflammation as mentioned above, n-6 PUFA actually impairs macrophage functionality, as evident when challenged with either live bacteria of bacterial LPS. Interestingly, such impairment under high n-6 PUFA is restored by increasing cellular GSH through either pharmacological or genetic means. These data suggests that impairment of macrophage function seen in n-6 PUFA treated cells, is a direct result of depletion in GSH.

3. While trying to evaluate the final fate of cells treated with n-6 PUFA, we identified that n-6 PUFA lead to increased cardiac apoptosis over MUFA - fed mice. However, when these hearts were challenged with isoproteronol, a cardiac stressor, the cell death switched from apoptosis to necrosis, with a reduced response from DNA damage repair genes, signifying a more widespread, unregulated damage pattern. Novel pathways like cytochrome p450 genes
involved in arachidonic acid metabolism, not previously reported in cardiolipotoxicity, were also upregulated with n-6 PUFA diet and isoproterenol challenge.

My results of reduced GSH following high fat feeding of a n-6 PUFA rich diet are consistent with previously published studies that n-6 PUFA rich diets leading to the creation of a pro-inflammatory environment\(^{188,202}\). As such, this thesis raises major concerns about studies in which n-6 PUFA is used as a secondary prevention for heart disease. As mentioned earlier, literature has shown that n-6 PUFA can lead to increased all cause mortality\(^69,75\).

Finally another concern raised by this thesis is that of the alteration of mechanisms of cell death. By itself n-6 PUFA led to increased levels of apoptosis. However, following cardiac insult there was increased levels of necrosis which were not observed in MUFA controls. Interestingly, our n-6 PUFA + ISO results are consistent with current literature in which necrosis is the predominant mode of cell death during heart failure\(^{346,347}\). Therefore this change in mechanisms of cell death could also explain why use of n-6 PUFA as a secondary prevention may leads to increased mortality\(^75\). Taken together this work cautions against continued high levels of n-6 PUFA in the western diet.
# References

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Appendices

Appendix A: Fatty acid analysis of OO, and CO fed mice

Full fatty acid analysis of hearts using GC-FID for c57/bl6 animals fed OO, or CO. A selected portion of this data is shown in table 3.1. Data was analyzed using two-way ANOVA with Bonferroni tests (n=4), p<0.05. *p<0.05 vs OO. Abbreviations: OO, Olive Oil, CO, Corn Oil.

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</tr>
</thead>
<tbody>
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<td>C15:0</td>
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<td>20.743 ± 0.705</td>
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<td>C22:6 n-3</td>
<td>3.805 ± 0.373</td>
<td>5.560 ± 0.150 *</td>
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<td>n-6 Polyunsaturated fatty acids</td>
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Appendix B: Holm-Bonferroni Multiple comparison correction of p values

See section 2.3.2.4. Abbreviations: co, corn oil; coi, co + iso; cof, corn + fish; cofi, co + iso; oo, olive oil; ooi, oo + iso; b, bso; buthionine sulfoximine; n, N-acetylcysteine.

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