



Whole plasma lipoprotein oxidation in women : influence of season, infection, age, physical activity, and dietary intake
by Shelly Patricia Hogan

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Health and Human Development
Montana State University
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Abstract:

Atherosclerosis may be viewed as an inflammatory process that takes place more readily during instances of acute infection. In response to an illness the body appears to release free radicals in an attempt to eliminate infectious antigens and inadvertently other cellular particles may be damaged. Injury to the walls of arteries associated with atherosclerotic development may be due to the existence of oxidized lipoproteins. Thus, individuals may be more susceptible to lipoprotein oxidation during periods of infection. Other factors that may influence the susceptibility of lipoproteins to oxidation may include seasonal variation and age. Purpose: The aim of this study was to determine whether the response to an infectious illness, seasonal variation, or age influenced the susceptibility of whole plasma lipid peroxidation in younger (18-40 y, n = 23) and older (>50 y, n = 19) healthy women. Method: Subjects logged nutrient intake and physical activity for three days, then fasting blood samples were collected and anthropometric measures were taken on two consecutive mornings. Formation of lipid conjugated dienes (CD) in diluted whole plasma was measured spectrophotometrically at 234 nm during eight hours of incubation with CuCl₂. Plasma fibrinogen concentration and complete blood counts (CBC) were performed using standard clinical techniques. Comparisons of the measured variables were made between the younger and older groups, between winter and summer seasons, and between periods of illness. Results: Susceptibility of whole plasma lipoproteins to oxidation was not detected to be significantly different ($P > 0.05$) in winter compared to summer or in the presence of an infectious illness. During periods of infectious illness subjects (n = 13) tended to have approximately 10 minute shorter ($P = 0.17$) periods of antioxidant protection (lag time) as compared to summer or winter assessments. Plasma cholesterol and LDL concentrations were found to be lower ($P = 0.03$, $P = 0.02$, respectively) during periods of illness as compared to healthy summer assessments. Plasma fibrinogen concentrations were higher for older women as compared to younger women ($P < 0.05$) during both winter and summer assessments. Subjects reporting an illness had higher ($P < 0.05$) concentrations of fibrinogen during periods of illness as compared to healthy summer or winter assessments. Conclusion: The effect of age, seasonal variation or response to an infectious illness were not found to be significant influencing factors on the susceptibility of whole plasma lipoproteins to oxidation related to atherosclerosis in the group of healthy women living in Montana. However, the statistically non-significant influence of infection on lipoprotein oxidation may have been confounded by the decrease in cholesterol and LDL concentrations measured during illness assessments. What might have been a significant decrease in lag time (period of antioxidant protection) during infectious periods may have been offset by the concomitant decrease in lipid substrates (decrease in plasma cholesterol and LDL concentrations).

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A thesis submitted in partial fulfillment
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in

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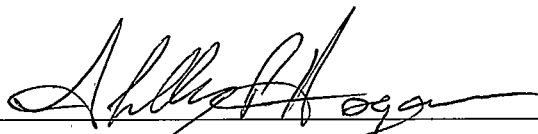
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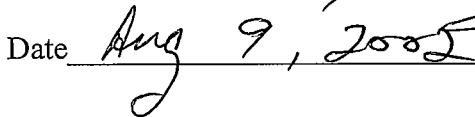
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TABLE OF CONTENTS

1. INTRODUCTION	1
Statement of the Problem.....	7
Research Hypotheses	8
Delimitations.....	9
Limitations	9
Definitions of Terms.....	10
2. LITERATURE REVIEW	12
Oxidation of Lipoproteins.....	13
Measurement of Lipoprotein Oxidation.....	16
Whole Plasma Lipoprotein Oxidation	19
Lipoprotein Oxidation related to Atherosclerosis.....	21
Seasonal Variation Related to Atherosclerosis	23
Infection and Lipoprotein Oxidation	26
Aging and Lipoprotein Oxidation.....	29
Other Factors Related to Lipoprotein Oxidation	32
Diet.....	32
Physical Activity.....	38
Summary of Literature Review.....	44
3. METHODS	47
Subjects.....	47
Experimental Design.....	48
Blood Collection and Plasma Separation.....	49
Blood Lipids.....	51
Whole Plasma Oxidation	52
Fibrinogen.....	54
Anthropometric and Body Composition Measurements.....	54
Physical Activity Assessment.....	55
Dietary Assessment.....	56
Infection Surveillance	57
Statistical Analysis and Sample Size Estimation.....	58

4. RESULTS	60
Subject Descriptive and Anthropometric Characteristics	60
Dietary Intake for Summer and Winter Groups.....	61
Estimated Physical Activity for Summer and Winter Groups	63
Plasma Lipid Profiles	64
Fibrinogen and CBC	69
Day to Day Reliability of Lipid Oxidation	70
Whole Plasma Oxidation Kinetics for Summer and Winter Groups	70
Plasma Lipid Profiles for Summer, Winter and Ill Groups	73
Fibrinogen and CBC for Summer, Winter, and Ill Conditions	76
Whole Plasma Oxidation Kinetics for Summer, Winter and Ill Groups.....	77
5. DISCUSSION.....	79
Whole Plasma Oxidation and Influence of Season.....	80
Whole Plasma Oxidation and Influence of Infectious Illness.....	83
Whole Plasma Oxidation and Influence of Age	86
Recommendations for Future Research	90
Summary	90
REFERENCES CITED.....	94
APPENDICES	106
APPENDIX A: INFORMED CONSENT	107
APPENDIX B: THREE-DAY PHYSICAL ACTIVITY RECORD	113
APPENDIX C: THREE-DAY DIETARY RECORD	117
APPENDIX D: INFECTIOUS ILLNESS LOG SHEET.....	120
APPENDIX E: BIS DATA SHEET	123
APPENDIX F: SOLUTIONS AND BUFFER RECIPES	125

LIST OF TABLES

Table	Page
1. Descriptive and Anthropometric Characteristics of Women.....	61
2. Selected Dietary Intake of Women.....	63
3. Time Spent in Physical Activity.....	64
4. Fibrinogen and CBC Measurements of Women.....	69
5. Fibrinogen and CBC Measurements of Women Reporting an Illness.....	77

TABLE OF FIGURES

Figure	Page
1. Proposed Oxidation Mechanism.....	4
2. Formation and Molecular Structure of Lipid Peroxidation.....	15
3. Kinetics of Whole Plasma Lipid Oxidation.....	19
4. Illustration of Research Time Line.....	49
5. Plasma Cholesterol Concentrations.....	66
6. Plasma Triglyceride Concentrations.....	67
7. Plasma HDL Concentrations.....	67
8. Plasma LDL Concentrations.....	68
9. Plasma VLDL Concentrations.....	68
10. Lag Times (min) of Women.....	72
11. Propagation Rates of Oxidation for Women.....	72
12. Plasma Cholesterol Concentrations of Subjects Reporting Illness.....	74
13. Plasma LDL Concentrations of Subjects Reporting Illness.....	75
14. Plasma TGs, HDL, and VLDL Concentrations of Subjects Reporting Illness.....	75
15. Lag Times (min) of Subjects Reporting Illness.....	78
16. Propagation Rates of Subjects Reporting Illness.....	78

ABSTRACT

Atherosclerosis may be viewed as an inflammatory process that takes place more readily during instances of acute infection. In response to an illness the body appears to release free radicals in an attempt to eliminate infectious antigens and inadvertently other cellular particles may be damaged. Injury to the walls of arteries associated with atherosclerotic development may be due to the existence of oxidized lipoproteins. Thus, individuals may be more susceptible to lipoprotein oxidation during periods of infection. Other factors that may influence the susceptibility of lipoproteins to oxidation may include seasonal variation and age. Purpose: The aim of this study was to determine whether the response to an infectious illness, seasonal variation, or age influenced the susceptibility of whole plasma lipid peroxidation in younger (18-40 y, n = 23) and older (≥ 50 y, n = 19) healthy women. Method: Subjects logged nutrient intake and physical activity for three days, then fasting blood samples were collected and anthropometric measures were taken on two consecutive mornings. Formation of lipid conjugated dienes (CD) in diluted whole plasma was measured spectrophotometrically at 234 nm during eight hours of incubation with CuCl_2 . Plasma fibrinogen concentration and complete blood counts (CBC) were performed using standard clinical techniques. Comparisons of the measured variables were made between the younger and older groups, between winter and summer seasons, and between periods of illness. Results: Susceptibility of whole plasma lipoproteins to oxidation was not detected to be significantly different ($P > 0.05$) in winter compared to summer or in the presence of an infectious illness. During periods of infectious illness subjects (n = 13) tended to have approximately 10 minute shorter ($P = 0.17$) periods of antioxidant protection (lag time) as compared to summer or winter assessments. Plasma cholesterol and LDL concentrations were found to be lower ($P = 0.03$, $P = 0.02$, respectively) during periods of illness as compared to healthy summer assessments. Plasma fibrinogen concentrations were higher for older women as compared to younger women ($P < 0.05$) during both winter and summer assessments. Subjects reporting an illness had higher ($P < 0.05$) concentrations of fibrinogen during periods of illness as compared to healthy summer or winter assessments. Conclusion: The effect of age, seasonal variation or response to an infectious illness were not found to be significant influencing factors on the susceptibility of whole plasma lipoproteins to oxidation related to atherosclerosis in the group of healthy women living in Montana. However, the statistically non-significant influence of infection on lipoprotein oxidation may have been confounded by the decrease in cholesterol and LDL concentrations measured during illness assessments. What might have been a significant decrease in lag time (period of antioxidant protection) during infectious periods may have been offset by the concomitant decrease in lipid substrates (decrease in plasma cholesterol and LDL concentrations).

CHAPTER 1

INTRODUCTION

Cardiovascular disease (CD) is the leading cause of death in the United States (American Heart Association, 2000). Intense research continues to be conducted in efforts to combat this complex and multifactoral health problem. One particular area of cardiovascular research is addressing specific oxidative mechanisms associated with atherosclerosis (Wattananpitayakul & Bauer, 2001). For instance, several studies have indicated that oxidative modification of low-density lipoprotein cholesterol (LDL) in the arterial wall plays a central role in atherosclerotic plaque development (Esterbauer, Striegl, Puhl, & Rotheneder, 1989; Hamilton, 1997; Steinberg, 1997). Consequently, numerous investigations pertaining to cardiovascular research have focused on factors influencing the oxidative mechanisms involving lipoproteins in efforts to address the "oxidation hypothesis" of atherosclerosis. This hypothesis states that oxidative modification to lipoproteins, such as LDL is important to the progression of atherosclerosis (Witztum & Steinberg, 2001).

Atherosclerosis is frequently described as an inflammatory process occurring in the walls of arteries as a response to injury in the vascular endothelium (Noll, 1998; Ross, 1999; Van der Wal & Becker, 1999). Similarly, the immune response to an infection is closely associated with inflammation and may be a risk factor for atherosclerosis (Noll, 1998). For instance, the increased incidence of infectious illnesses during the winter months has been proposed to initiate a chain of physiological events that contribute to

destabilization of atherosclerotic plaques and subsequent thrombosis (Crawford, Sweeney, Coyle, Halliday, & Stout, 2000; Woodhouse, Khaw, Plummer, Foley, & Meade, 1994).

The rationale behind this infection-based postulation is that the presence of infection will attract and activate immune cells. For instance, one population of the immune cells, neutrophils, responds to an infection by producing an "oxidative burst" consisting of free radical production (Krause, Mastro, Handte, Smiciklas-Wright, Miles, & Ahluwalia, 1999). Those free radicals help to eliminate infection. However, they also initiate an uncontrolled and potentially damaging chain reaction leading to the oxidation of unintended targets, such as lipoproteins (Esterbauer et al., 1989). If oxidized, these lipoproteins (i.e. LDL) may be more atherogenic and promote uptake and activation of macrophages (Steinberg, 1997).

The theoretical framework relating infection to atherosclerosis depends on the presence of macrophages. Esterbauer, Schmidt, & Hayn (1997) described macrophages as scavenger white blood cells, which consume the oxidized lipoproteins and eventually become lipid-laden foam cells. Once formed, the foam cells populate in the inner surface of the arterial wall forming atherosclerotic plaque (Esterbauer et al., 1997). The progression of atherosclerotic plaques eventually restricts blood flow (Jialal & Devaraj, 1996). In addition to the development of plaque, evidence suggests that oxidized LDL can decrease the stability of atherosclerotic plaques by inducing apoptosis within the smooth muscle cells of the arteries, thus increasing the likelihood of a thrombotic event, such as a myocardial infarction (MI) (Hsieh, Yen, Yen, & Lau, 2001; Okura,

Brink, Itabe, Scheidegger, Kalangos, & Delafontaine, 2000; Penn, Cui, Winokur, Bethea, Hamilton, DiCorelto, & Chisolm, 2000; Zaman, Helft, Worthley, & Badimon, 2000).

A proposed model of the mechanism linking infection to LDL oxidation and to MI is illustrated in Figure 1. This proposed mechanism is based on past studies associating atherosclerotic conditions (plaque formation and destabilization) with LDL oxidation. Additionally, the illustration offers a tentative explanation for the chain of events that may occur *in vivo* in response to the immune defense system in human populations. For instance, the oxidation of whole plasma lipids, such as LDL, is likely to increase when individuals experience an episode of an infectious illness contributing to the manifestation of atherosclerosis. Ultimately, increasing the understanding of the mechanistic steps in the atherogenic process provides targets to inhibit this potentially damaging oxidative pathway.

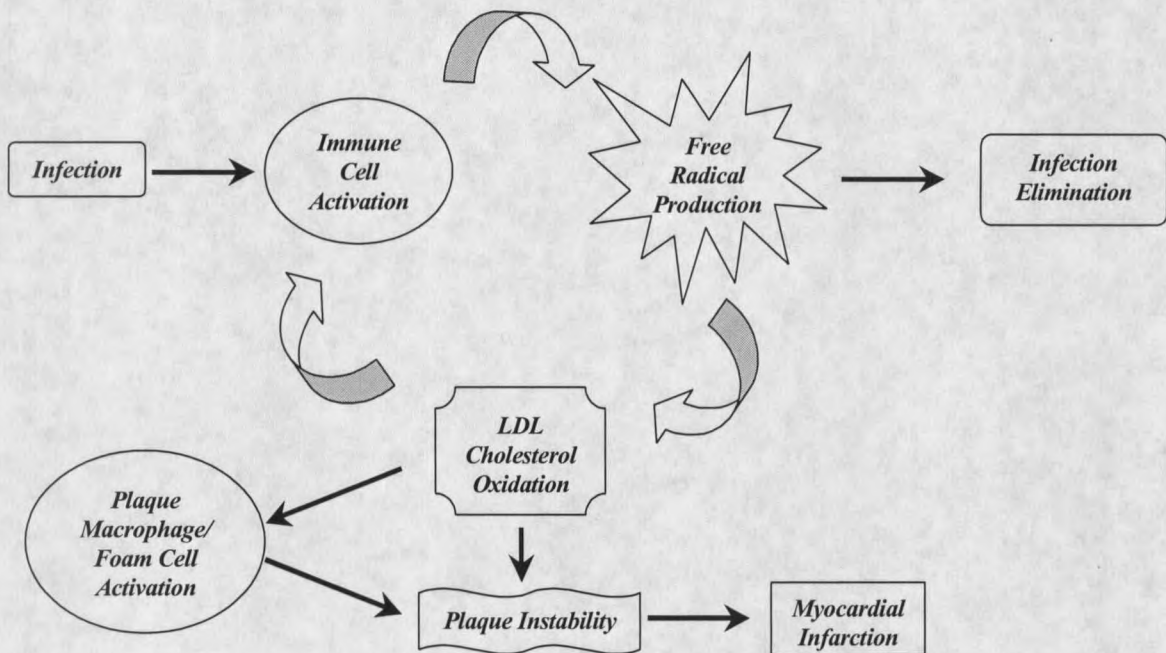


Figure 1. Proposed Oxidation Mechanism

Proposed mechanism of the chain of events that may link infection to atherosclerotic plaque formation, and/or instability. The presence of infection attracts and activates immune cells. One population of the immune cells, neutrophils, responds by producing an oxidative burst consisting of free radical production. Those free radicals are helpful in eliminating infection. However, they also may initiate a self-perpetuating cycle of oxidation of unintended targets, such as LDL. When oxidized, LDL is more atherogenic and promotes uptake and activation by macrophages/foam cells residing in arterial plaques. Oxidized LDL may decrease the stability of atherosclerotic plaques, thus increasing the likelihood of a thrombotic event, such as a myocardial infarction.

Various factors influence the susceptibility of lipoproteins to oxidative modification, such as, aging, response to infections, and seasonal variation. For instance, a 53% increase in acute MI incidence from summer to winter was reported to the National Registry of Myocardial Infarction (Spencer, Goldberg, Becker, & Gore, 1998). These researchers suggested that seasonal changes in behavior or environment could induce instability of atherosclerotic plaques, concomitantly increasing the risk for an individual to have a MI. Furthermore, researchers have indicated that physiological adaptations to the cold, particularly an increase in plasma fibrinogen levels, may contribute to the increase in MI occurrence in the winter (de Lorenzo, Sharma, Scully, & Kakkar, 1999). However, the increase in MI during the winter occurs even in warm climates (Spencer et al., 1998), suggesting that cold temperature is not the exclusive factor that increases MI incidence.

One important area of inquiry is to determine if the susceptibility of whole plasma lipoproteins to oxidative modification is influenced seasonally and /or in response to infectious illness. Although there is evidence to suggest that infection can increase oxidized LDL in both *in vitro* and *in vivo* animal models (Kalayoglu, Hoerneman, LaVerda, Morrison, Morrison, & Byrne, 1999; Memon, Staprans, Noor, Holleran, Uchida, Moser, et al., 2000), an increase in oxidized lipoproteins has not been demonstrated with respect to an acute infectious illness, such as a common cold, in humans.

Age appears to be a factor that increases both the susceptibility of lipoproteins to oxidation and risk of MI. Mosinger (1997) reported that post-menopausal women had a

significantly higher amount of LDL oxidation when compared to pre-menopausal women ($P < 0.001$). In elderly populations, infections have been observed to be more common in the winter and may contribute to higher incidences of MI (Sheth, Nair, Muller, & Yusuf, 1999). Additional data on the potential role of seasonal variation and/or age in the oxidative modification of lipoproteins related to cardiovascular disease has been acquired by other studies (Cristol, Abderrazick, Favier, Michel, Castel, Leger et al., 1999; Ji 1993; Meydani, Evans, Handelman, Biddle, Fieldings, Meydani, et al., 1993; Sheth et al., 1999). For instance, Sheth et al. (1999) reported a greater prevalence of mortality during the winter months from acute MI and stroke among elderly than younger individuals in a 300,000 person sample of North Americans. According to these investigators, the seasonal mortality variation in MI deaths (winter vs. summer) increased with increasing age. Taken as a whole, these findings indicate that an individuals increased susceptibility to oxidation may be dependent on age, seasonality, and response to infection.

A variety of methods have been developed to monitor parameters related to oxidation; however, no universal procedure has been established to reflect *in vivo* states of lipid oxidation (Witztum, 1994). It has been suggested that the oxidation of all major plasma lipoproteins, not just LDL, be assessed when measuring oxidative parameters of plasma lipoproteins (Kontush, Spranger, Reich, Kjahansouzi, Karten, Braesen, et al., 1997). Researchers have observed that the arterial walls contain not only LDL but also other lipoproteins, such as high density lipoproteins (HDL), very low density lipoprotein (VLDL), and also chylomicrons (Nordestgaard, Wootton, & Lewis, 1995). All of these particles may be oxidatively modified and form structures characteristic of oxidative

damage (Spranger, Finckh, Fingerhut, Kohlschutter, Beisiegel, & Kontush, 1998). In light of these postulations, Kontush et al. (1997) and Spranger et al. (1998) examined the oxidizability of plasma lipoproteins as a measure of the susceptibility of whole plasma to oxidation. Kinetic parameters that were measured include: (1) a period of oxidative protection, referred to as lag time and (2) the maximum rate of oxidation, known as propagation rate.

Further research concerning the seasonal effects and the influence of acute infection on whole plasma lipoprotein oxidation warrants investigation in human studies. Based on the potential association of acute infection with aging, monitoring whole plasma lipoprotein oxidation at summer and winter months may provide additional insight to the underlying mechanisms influencing the higher incidence of MI during the winter. Consequently, the goals of this study were to evaluate the influence of seasonal variation, infectious illness, and age on the susceptibility of whole plasma lipoproteins to oxidation in a group of healthy women.

Statement of the Problem

The objectives of this study were to determine whether the susceptibility of whole plasma lipids to oxidation is (1) greater in the winter compared to the summer months, (2) greater during an acute incidence of an infectious illness, and (3) greater in older women compared to younger women.

Research Hypotheses

Three hypotheses were tested for this study.

Hypothesis 1: Whole plasma lipids are significantly more susceptible to oxidation (shorter period of oxidative resistance and faster rate of oxidation) during winter assessments as compared to summer assessments independent of age and infection.

$$H_0: WPO_w \leq WPO_s \quad H_a: WPO_w > WPO_s$$

Where: WPO_w = susceptibility of whole plasma lipid oxidation measured in the winter.

WPO_s = susceptibility of whole plasma lipid oxidation measured in the summer.

Hypothesis 2: Whole plasma lipids are significantly more susceptible to oxidation during a period of an acute infectious illness independent of age and season.

$$H_0: WPO_{inf} \leq WPO_{well} \quad H_a: WPO_{inf} > WPO_{well}$$

Where: WPO_{inf} = susceptibility of whole plasma lipid oxidation measured during an infectious illness.

WPO_{well} = susceptibility of whole plasma lipid oxidation measured when healthy.

Hypothesis 3: Whole plasma lipids are significantly more susceptible to oxidation with older women compared to younger women.

$$H_0: WPO_{old} \leq WPO_{young} \quad H_a: WPO_{old} > WPO_{young}$$

Where: WPO_{old} = susceptibility of whole plasma lipid oxidation in older women.

WPO_{young} = susceptibility of whole plasma lipid oxidation in younger women.

Delimitations

There were two primary delimitations to this study.

1. This study was restricted to female subjects living in Montana throughout the 9-month duration of this investigation.
2. The age range for the subjects was limited to women between 18-40 years of age and to women 50 years or older.

Limitations

1. The results of this study cannot be generalized to men. Additionally, the results only reflect the response of women living in one geographical location year-round (i.e. Montana).
2. The results of this study may not be generalized to women younger than 18 years of age or between the ages of 41 and 49 years of age.
3. Collecting samples from subjects during periods of illness is a limitation due to subject's availability and/or compliance for blood collection at the laboratory.
4. Because there are no universally recognized standard procedures for measuring the extent of lipoprotein oxidation, generalizability may be limited to the chosen conjugated diene protocol used in this study.

Definitions of Terms

Atherosclerosis is a disease which involves the accumulation of cholesterol, lipids, smooth muscle cells and other biological particles in the wall of arteries, forming plaque that inhibits the flow of blood until a clot eventually forms, obstructs an artery and causes a heart attack or stroke.

Conjugated dienes refer to a particular molecular structure of two carbon-carbon double bonds separated by a single carbon-carbon bond. This structure is unusual in polyunsaturated fatty acid (PUFA) and is a consequence of oxidation. Conjugated diene can be measured at 234 nm spectrophotometrically in order to determine the degree of polyunsaturated fatty acid oxidation.

Fibrinogen is an acute phase protein in the blood that increases during instances of inflammation.

Free radicals are highly reactive biological particles, containing unpaired electron(s), capable of existing independently and of attacking biological molecules (i.e. protein, DNA, and lipids).

Infectious illness occurs when symptoms of an infection (upper respiratory tract, common cold, flu, etc.) persist for two or more consecutive days, or if a physician diagnosis the presence of an acute infection. The severity of these symptoms was ranked subjectively according to the following scale: 1) mild, 2) moderate, and 3) severe. Additional, complete blood counts (CBC) quantitatively measure the severity of illness.

Lag time is the length of time (min) that can be determined which reflects the period that lipoproteins are protected from oxidative modification (antioxidant protective phase).

Low-density lipoproteins (LDL) are compounds containing lipids and proteins, which can be characterized by their densities. LDL is isolated from plasma based on its density range of 1.019-1.063 g/mL.

Lipoprotein oxidation occurs when a free radical attacks a lipid molecule (e.g. LDL) by abstracting hydrogen, leaving the lipoprotein with an unpaired electron. *In vitro*, copper ions can initiate oxidation of lipids as a model for what may happen in arterial walls.

Macrophages are scavenger white blood cells. With respect to atherosclerosis, they are responsible for the uptake of oxidized LDL-cholesterol until they become foam cells that invade the hard fatty plaque on inner surfaces of arteries.

Myocardial infarction (MI), also known as a heart attack, occurs when a narrowed coronary artery becomes completely blocked, usually by a blood clot causing muscle tissue death.

Neutrophils are white blood cells and function primarily to eliminate bacterial microorganisms through phagocytosis (killing and consuming bacterial microorganisms) and/or production of toxic oxygen radicals.

Oxidation is a chemical process occurring in various metabolic conditions where an atom loses one or more of its electrons. If uncontrolled, unintended targets can be oxidized (including lipids) causing damaging oxidative stress.

Plaque stability is an important characteristic associated with atherosclerosis. Oxidized LDL may decrease the stability of atherosclerotic plaques and increase the likelihood of a thrombotic event (such as acute MI).

Propagation phase is observed during continuous monitoring of the oxidation of lipoproteins. During this phase the conjugated dienes rapidly increase to a maximum value because the lipoprotein, e.g. LDL, is depleted of its antioxidants (antioxidant unprotective phase). The slope of the propagation phase can be determined, which reflects the maximum rate of conjugated diene oxidation (units $\times 10^{-3}/\text{min}$).

White blood cell (WBC) is a measurement consisting of two components. These components consist of the total number of WBCs (leukocytes) in 1 mm^3 of peripheral venous blood and the percentage of each type of leukocyte present in the same sample. An increased total WBC count usually is indicative of infection or inflammation. Three types of WBCs were identified in the present study including: neutrophils, lymphocytes, and monocytes.

Whole plasma is comprised of the lipoproteins, such as LDL, HDL, VLDL, and chylomicrons. These lipoproteins are able to carry lipid peroxidation products in human plasma and are considered to reflect the *in vivo* oxidizability of whole plasma.

CHAPTER 2

LITERATURE REVIEW

Cardiovascular disease (CD) encompasses a variety of conditions and is the major cause of morbidity and mortality in the United States. According to the American Heart Association Heart and Stroke 2000 Statistical Update, CD claimed 949,619 lives in the United States in 1998. The principle examples of CD are MI and stroke (Keaney, 2000). Major known CD risk factors include age, male gender, family history, hypertension, elevated LDL cholesterol (>160 mg/dl), decreased HDL cholesterol (<35 mg/dl), cigarette smoking, physical inactivity and diabetes (Schaefer, 2001; Bowman & Russel, 2001; Brooks, Fahey, White, & Balwin, 2000). Atherosclerosis is a particular cardiovascular condition in which cholesterol-carrying macrophages, smooth muscle cells, endothelial cells, and other biological proteins accumulate in the wall of arteries, forming plaque that inhibits the flow of blood and narrows the diameter of an artery (Ganz, Creager, Fang, McConnel, Lee, Libby, & Selwyn, 1996; Keaney, 2000; Steinberg, 1997).

Researchers have hypothesized that oxidative modifications to low density lipoprotein cholesterol (LDL) within arteries contribute to the development of atherosclerosis (Esterbauer et al., 1989; Parks, German, Davis, Frankel, Kappagoda, Rutledge et al., 1998; Steinberg, Parthasarathy Carew, Khoo, & Witztum, 1989; Witztum, 1994). However, the exact mechanisms involved in the pathogenic relationship between oxidized lipoproteins and atherogenesis remain unknown (Chisolm & Steinberg, 2000;

Lenz, Hughes, Mitchell, Via, Guyton, Taylor et al., 1990). Therefore, identifying the specific atherogenic factors that lead to the promotion of atherosclerosis may help in developing strategies to reduce the degree of oxidation occurring in the walls of arteries. Additionally, this information may provide potential targets for therapy in reducing the risk of developing atherosclerosis and other life threatening cardiovascular diseases. The aim of this review is to summarize research regarding known associations and potential effects of seasonal variation, infectious illness, aging, physical activity, and diet on lipid oxidation associated with the manifestation of atherosclerosis. The literature is presented under the following topics: (1) oxidation of lipoproteins (2) measurement of lipoprotein oxidation (3) whole plasma lipoprotein oxidation (4) lipoprotein oxidation and atherosclerosis (5) influence of seasonal variation on lipoprotein oxidation (6) infection and inflammatory response (7) aging (8) other factors related with lipoprotein oxidation, such as diet and physical activity.

Oxidation of Lipoproteins

One focus of CD research has been on the effect of oxidative stress on endothelial cells. Generation of oxygen free radicals is occurring in cells throughout the life span of an individual (Halliwell & Chirico, 1993; Ji, 1993). Continuous exposure to either endogenous or exogenous oxidants can lead to oxidative stress (Heinecke, 1998).

Oxidative stress is defined as a situation that occurs when oxidants exist in amounts much greater than protective antioxidants (Halliwell & Chirico, 1993; Ji, 1995; Niki, Noguchi, Tsuchihashi, & Gotoh, 1995). For instance, pro-oxidants, such as highly

reactive oxygen molecules, are generated continually as by-products of aerobic metabolism, contraction of muscles, ultraviolet light exposure, hypoxia, pollution and other stresses (Jenkins & Goldfarb, 1993). Reactive oxygen molecules, which have an unpaired electron, have an affinity to attack proteins, lipids, and DNA (Halliwell & Chirico, 1993). Overall, this disrupting process results in alterations in important biological functions of cells and causes cellular damage (Halliwell & Chirico, 1993). The balance of the production and removal of these reactive oxygen species determines if oxidative stress occurs (Halliwell & Chirico, 1993; Meydani, 2000; Cristol, et al., 1999)

Lipid peroxidation may be one of the results of oxidative stress. Lipoproteins are susceptible to oxidative modification due to the innate molecular structure of certain lipids (Jiali & Scaccini, 1994). Specifically, the fatty acids, which are susceptible to oxidation, are called polyunsaturated fatty acids (PUFA) (Frei & Gaziano, 1993; Hamilton, 1997; Lenz et al., 1990). The PUFAs are susceptible to oxidative stress due to their double bond structure, which allows for increased release of hydrogen atoms (Halliwell & Chirico, 1993). Figure 2 illustrates the consequence of hydrogen removal from a PUFA molecule due to the presence of free radicals. The alteration of PUFA structure can potentially result in the formation of lipid peroxidation (Halliwell & Chirico, 1993).

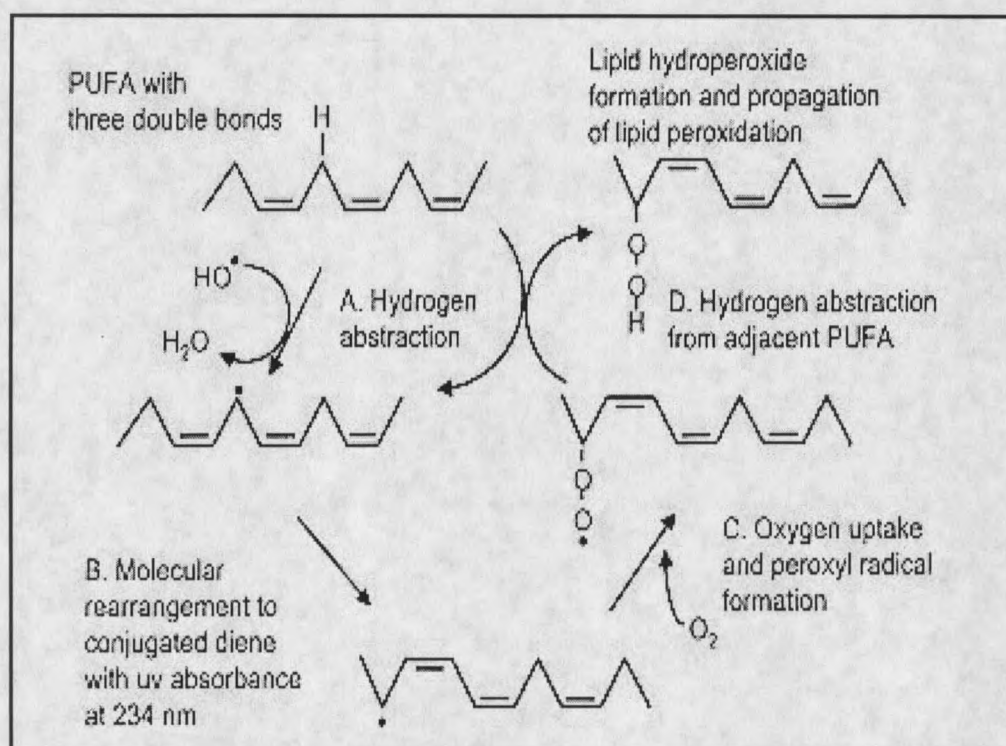


Figure 2. Formation and Molecular Structure of Lipid Peroxidation

Oxidative modification to lipids involves (A) the removal of a hydrogen atom from a PUFA molecule, (B) the formation of conjugated diene, (C) the reaction with oxygen to form peroxide radicals, (D) the hydrogen abstraction from another PUFA to form lipid peroxides.

When unsaturated lipid molecules are oxidized, a self-perpetuating chain reaction of more free radicals is produced (Kanter, 1997). Once lipid peroxides are formed, it is more feasible to generate additional radicals especially when metal ions, such as copper or iron, are present (Heinecke, 1998). Consequently, oxidation of lipoproteins, such as LDL, has the potential for creating an uncontrolled chain reaction of oxidative stress and to alter the integrity of cellular membranes (Esterbauer et al., 1989; Steinberg, et al., 1989).

Measurement of Lipoprotein Oxidation

Because oxidation of LDL *in vitro* is accompanied by changes of chemical, physical, and biological properties, a variety of methods are used by scientists for determining the extent and/or rate of lipid oxidation (Esterbauer, Schmidt, & Hayn, 1997). Quantifying the amount of lipid oxidation can be acquired by measuring (1) losses of unsaturated fatty acids, (2) amounts of primary peroxidation products (lipid peroxides), and (3) amounts of secondary products, i.e. hydrocarbon gases (Halliwell & Chirico, 1993). Presently, there is no universal protocol employed to determine the degree of oxidative stress in biological systems.

Esterbauer and colleagues (1989) introduced a popular method for the measurement of lipid peroxidation *in vitro*. This protocol measures formation of conjugated dienes as a result of oxidation of lipoproteins. Conjugated dienes are particular molecular structures identified as double bonds in fatty acid (Hamilton, 1997). Various researchers have employed the conjugated diene method for monitoring LDL oxidation *in vitro* with success (Ahotupa, Marniemi, Lehtimäki, Talvinen, Raitakari, Vasankari et al., 1998; Ahotupa & Vasankari, 1999; Esterbauer et al., 1989; Kleinveld, Hak-Lemmers, Stalenhoef, & Demacker, 1992; Vasankari, Ahotupa, Toikka, Mikkola, Irjala, Pasanen et al., 2001). Monitoring conjugated dienes has been validated and reported in detail by Ahotupa and colleagues (1998). This protocol is widely used for measuring the susceptibility of LDL to oxidation and measuring the resistance of

lipoproteins to oxidation (Ahotupa & Vasankari, 1999) and was chosen as the method of analysis for the present study.

When using the conjugated diene method for measuring oxidative activity of lipids, two measurements are particularly relevant. First, the degree of lipoprotein oxidation can be measured spectrophotometrically. For instance, when the PUFAs within a LDL particle are oxidized, a molecular rearrangement of two carbon-to-carbon double bonds separated by a single bond is formed (general structure: $-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CHOOH}-$) (Corongiu & Banni, 1994; Esterbauer et al., 1989). This particular molecular rearrangement is called a conjugated diene, which can react with oxygen to form lipid peroxide radicals. Figure 2 illustrates the molecular structure of a conjugated diene as a result of a hydrogen atom removal from PUFA. Like other free radicals, lipid peroxide radicals can create an uncontrolled chain reaction of molecularly altered LDL particles (Corongiu & Banni, 1994; Hamilton, 1997).

The kinetic parameters of the conjugated diene formation (the change of absorbance vs. time) can be divided into three phases: lag phase, propagation phase, and decomposition phase (Esterbauer et al., 1989). In the present study only the lag phase and propagation phase were used as indices for determining the susceptibility of whole plasma lipoprotein oxidation (refer to Figure 3).

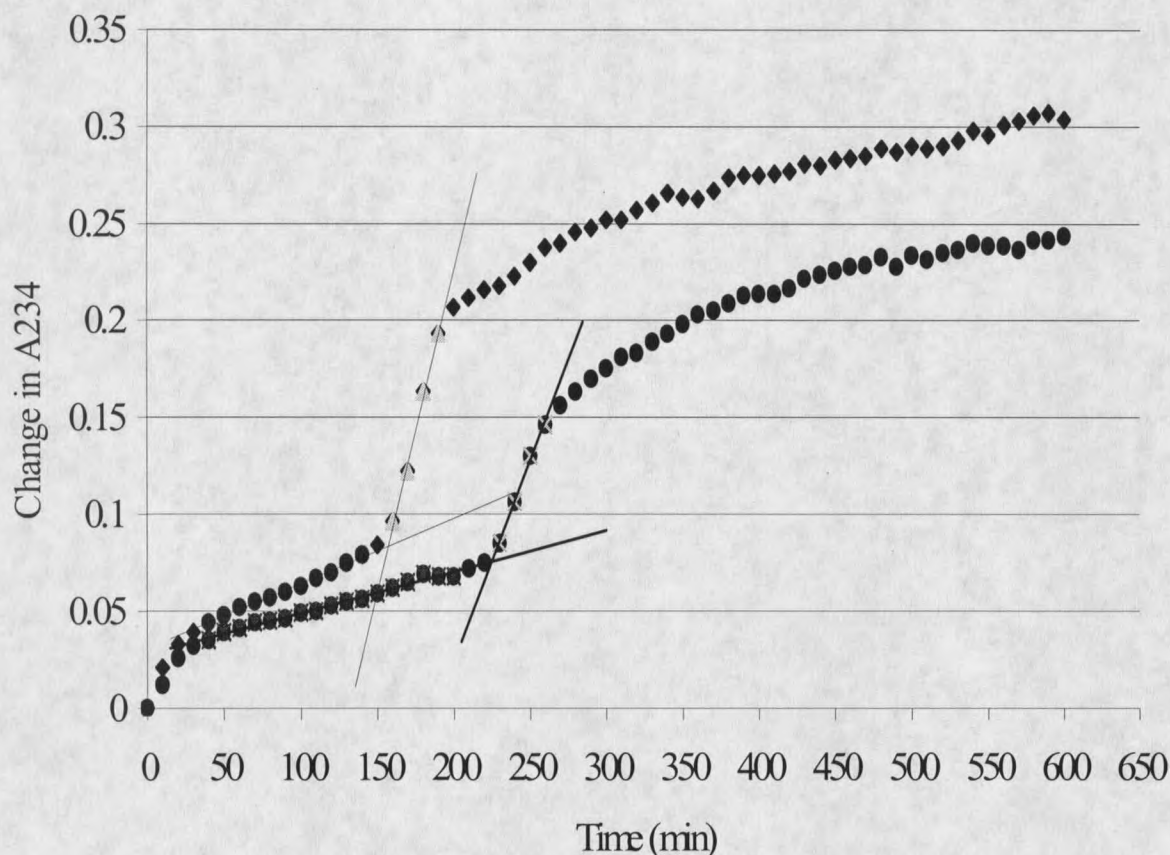


Figure 3. Kinetics of Whole Plasma Lipid Oxidation

The progress of whole plasma oxidation can be continuously monitored at 234 nm. Kinetics of whole plasma lipid oxidation can be characterized by lag time and propagation rate. The time of antioxidant protection, referred to as the lag phase was determined as the intercept of the best fit lines for the lag phase and propagation slope. The antioxidant molecules within the lipid particles are able to resist oxidation and this increase in oxidation is slow. However, when the antioxidant capacity of the lipids has been exceeded, the rate of conjugated diene formation will increase more rapidly (propagation rate). Thus, an ideal combination would be the bottom dotted sample demonstrating a longer lag time (longer resistance to oxidation) and a slower propagation rate (flatter slope) of oxidation.

The first phase or lag phase is characterized by the non-existence or slight increase in conjugated diene formation. The ability of the lipoproteins to resist oxidation is an important measurement. During the lag phase, the endogenous antioxidants protect the PUFAs against oxidation until the lipid particle is depleted of its antioxidants (Esterbauer et al., 1989; Frei & Gaziano, 1993). Thus, this length of time of oxidative resistance is referred to as the lag time. Lipoprotein samples that are more resistant to oxidation will have a longer lag time. Spectrophotometrically determined lag phase values are considered to be reliable. For instance, results of recent studies demonstrated reliable time values either spectroscopically or using high performance liquid chromatography (HPLC) technique (Raveh, Pinchuk, Fainaru, & Lichtenberg, 2001; Bagnati, Perugini, Cau, Bordone, Albano, & Bellomo, 1999; Ueda, Anzai, Miura, & Ozawa, 1999).

After the lag phase the conjugated dienes increase very rapidly to a maximum value referred to as the propagation rate (Esterbauer et al., 1989). In essence, the depletion of antioxidant protection leaves the PUFAs vulnerable to oxidative modification and subsequent formation of conjugated dienes. Thus, the rate of conjugated diene formation will increase more rapidly. This rapid increase in conjugated diene formation reflects the rate of lipid peroxidation.

Whole Plasma Lipoprotein Oxidation

The oxidation of all major plasma lipoproteins, not just LDL oxidation, may be reflective of the oxidizability *in vivo* when measuring oxidative parameters of plasma

lipoproteins *in vitro* (Kontush et al., 1997). The arterial walls contain not only LDL but also other lipoproteins, such as high density lipoproteins (HDL), very low density lipoprotein (VLDL), and also chylomicrons (Nordestgaard et al., 1995; Spranger et al., 1998). All of these particles may be oxidatively modified and form conjugated diene structures similar to oxidized LDL (Esterbauer et al., 1989; Spranger et al., 1998). In particular, Kontush et al. (1997) and Spranger et al. (1998) examined the oxidizability of plasma lipoproteins as a measure of the susceptibility of whole plasma to oxidation spectrophotometrically. While monitoring conjugated diene formation at 234 nm, they observed characteristics of the oxidation kinetics similar to the lag, propagation and decomposition phases of LDL oxidation described by Esterbauer and colleagues (1989).

Significant correlations between the oxidizabilities of whole plasma and isolated LDL were identified when the propagation rate and lag time of oxidized plasma was measured ($r = 0.57$, $P = 0.005$; $r = -0.44$, $P = 0.03$, respectively) (Kontush, 1997). Based on the results of these and other investigators, the spectrophotometric whole plasma oxidation method is recognized to be a useful assay. Furthermore, this particular protocol has been employed to aid in determining the susceptibility of plasma lipids to oxidation of many successful laboratory investigations (Karten, Beisiegel, Gercken, & Kontush 1997; Kontush et al., 1997; Kontush & Beisiegel, 1999; Schnitzer, Pinchuk, Bor, Fainaru, Samuni, & Lichtenberg, 1998; Spranger et al., 1998). Monitoring conjugated diene formation of whole plasma was the method chosen for calculating the susceptibility of lipoproteins to oxidation *in vitro* for this particular investigation.

Lipoprotein Oxidation related to Atherosclerosis

Fundamental factors believed to influence the development of atherosclerosis were explained by Brown and Goldstein in 1982. They proposed that damage to the thin layer of endothelial cells that line an artery initiate plaque formation. This damaged layer begins to accumulate lipid particles along with blood platelets. LDL has received increased attention because it is thought to be a key risk factor linked to cardiovascular disease (Chisolm & Steinberg, 2000; Esterbauer et al., 1997; Keaney, 2000). However, LDL itself may not be the primary factor contributing to the development of atherosclerosis. Researchers generally agree that, LDL needs to be oxidatively modified before being associated with plaque formation (Hamilton, 1997; Steinberg, 1997; Witztum & Steinberg, 2001).

Injury to the walls of arteries is often a resulting consequence when LDL is oxidized (Chisolm & Steinberg, 2000). As a result, smooth muscle cells in the layer of cells below the endothelium increase and migrate to the damaged area of the artery (Brown & Goldstein, 1982). Next, monocytes are activated and invade the area of injury (Steinberg et al., 1989, Steinbrecher, Parthasarathy, Leake, Witztum, & Steinberg, 1984). Eventually, the monocytes differentiate to become macrophages, which consume both the oxidized lipoprotein and smooth muscle cells. Specific, scavenger receptors on macrophages are believed to be responsible for most of the uptake of oxidized lipids within the walls of arteries (Dhaliwal & Steinbrecher, 1999).

Plaque formation and possible plaque destabilization are characteristic of alteration to the integrity of arteries (Brown & Goldstein, 1982, Penn et al., 2000). The primary function of smooth muscle cells and macrophages is to consume oxidized LDL particles. The accumulation of macrophages eventually becomes the cholesterol rich foam cells (refer to Figure 1). The foam cells begin to form plaque on the walls of arteries (Brown & Goldstein, 1982). In essence, the combined accumulation of cholesterol, smooth muscle cells and macrophages will eventually lead to plaque formation within the artery. Additionally, the formation of plaque increases the risk of a thrombotic event, such as MI (Hsieh et al., 2001; Okura et al., 2000; Penn et al., 2000; Zaman et al., 2000). Thus, this type of damage to arteries can ultimately impair the function of an artery and potentially promote MIs or strokes (Brown & Goldstein, 1982).

Research has shown oxidative susceptibility of LDL to be associated with atherosclerosis. For instance, oxidized LDL was demonstrated to be significantly elevated ($P < 0.01$) in subjects with diagnosed CD compared to a healthy control group (Wang, Xiao-Zhuan, Yi-Yi, & Lu-Yan, 2000). In accordance with earlier studies, Wang and colleagues interpreted the data as strong evidence demonstrating that oxidized LDL plays an important role in the development of CD (2000). Recently, Vasankari et al., (2001) investigated the relation between lipids including oxidized LDL, and the severity of atherosclerosis in middle-aged men. They determined the amount of LDL oxidation by measuring the baseline levels of conjugated dienes in LDL. Subjects with CD who did not take lipid lowering therapy had a 41% higher oxidized LDL: LDL ratio than healthy subjects ($P = 0.033$). This study provided additional information relating LDL

oxidation to atherosclerotic conditions. Particularly, if individuals have elevated LDL lipid profiles then they may have increased potential for oxidative stress.

Seasonal Variation Related to Atherosclerosis

The National Registry of Myocardial Infarction (MI) reported that there is a 53% increase in the incidence of MI during the winter months as compared to summer months (Spencer et al., 1998). Spencer et al. (1998) suggested that seasonal changes in behavior or environment could induce instability of atherosclerotic plaques, a condition predisposing an individual to MI. Studies have provided results indicating that physiological adaptations to the cold, particularly an increase in plasma fibrinogen levels, may contribute to the increase in MI occurrence in the winter (de Lorenzo et al., 1999). However, the winter increase in plasma fibrinogen levels occurs even in warm climates (Spencer et al., 1998), suggesting that cold temperature is not the primary factor that increases MI incidence.

Researchers have attempted to acquire a better understanding of the seasonal variation phenomenon. Specifically, seasonal variations of serum lipids in human population have been investigated with respect to possible influence on cardiovascular conditions. For example, seasonal variation of plasma cholesterol levels was observed in 1446 men in the Lipid Research Clinics Coronary Primary Prevention Trial (Gordon, Trost, Hyde, Whaley, Hannah, Jacobs, & Ekelund, 1987). A significant seasonal effect was found where plasma cholesterol levels were 7.4 mg/dl higher in December or January months than in June or July months. However, not all studies have demonstrated

seasonal trends of plasma cholesterol or lipoproteins. The fasting serum concentrations of total cholesterol, LDL and HDL cholesterol, and triglycerides (TG) were measured for 60 weeks in 28 healthy young adults (Mjos, Rao, Bjor, Henden, Thelle, Forde, & Miller, 1979). No statistically significant ($P < 0.05$) seasonal changes were detected in any variable. Thus, there are conflicting results on lipid parameters in response to seasonal variation.

Infections have been found to be more common in the winter and may contribute to higher winter cases of MI, specifically in the elderly population (Sheth et al., 1999). Consequently, data on the potential role of seasonal variation and/or age and infection on the oxidative modification of LDL and cardiovascular disease has been the focus of recent studies (Cristol et al., 1999; Ji 1993; Meydani, Evans, Handelman, Biddle, Fieldings, Meydani et al., 1993; Sheth et al., 1999). Sheth et al. (1999) demonstrated a greater prevalence of mortality during the winter months from acute MI and stroke among elderly than younger individuals in a 300,000 person sample of North Americans. These investigators reported that, the seasonal mortality variation in MI deaths (winter vs. summer) increased with increasing age: 5.8% for < 65 years vs. 15.8% for > 85 years ($P < 0.005$).

Crawford et al. (2000) proposed that increases in cardiovascular occurrences during the winter are the result of a seasonal effect on cardiovascular risk factors. In contrast to past studies that measured a number of possible risk factors of cardiovascular disease, Crawford and colleagues (2000) focused their investigation specifically on seasonal variation and the acute phase protein, fibrinogen. These scientists hypothesized

that fibrinogen proteins are involved in cardiovascular disease due to an increase during the inflammation process. Although fibrinogen had a significant seasonal variation with 23% ($P < 0.003$) increase during winter, this study provided no evidence that winter infections are responsible for the seasonal variation in fibrinogen (Crawford et al., 2000).

Additional seasonal variations were identified by Shahar and colleagues, who observed changes in specific dietary intake in summer and winter (Shahar, Froom, Harari, Yerushalmi, & Lubin, 1999). These investigators screened 94 males (42.4 ± 11.9 y) once in January and once in July. From summer to winter the mean dietary intake of fat increased by 7% (from 99.1 to 106.0 g, $P = 0.0016$) and dietary cholesterol intake increase by 7.7%. Various vitamins were measured in the study. Vitamin E intakes were 13.4 % higher ($P = 0.0073$) in winter time. While vitamin A was reported to be 2.8% lower during the winter. Vitamin C was not accounted for in this particular investigation. The investigators reported that total serum cholesterol increased from 200.8 to 208.6 mg/dl ($P = 0.001$) and LDL concentration increased 8% (125.2 mg/dl in the summer to 134.9 mg/dl in winter). Shahar's group concluded that seasonal changes occur in dietary intakes. The seasonal increase in dietary fat and cholesterol intake at winter is related to the increase in serum cholesterol.

Evidence of a potential seasonality factor linked to atherosclerosis in terms of LDL oxidizability has been reported. When 96 volunteers aged 65-74 years studied at 2-month intervals for one year, seasonal variation influenced lipid profiles (Woodhouse, Khaw & Plummer, 1993). Specifically, calculated LDL was highest in winter in men ($P < 0.0001$), and TG were higher in winter for women ($P = 0.002$). These findings may

help explain the relation between dietary patterns and the increased prevalence of certain CD occurring during winter seasons.

Van de Vijver, Van Duyvenvoorde, Buytenhek, Van der Laarse, Kardinaal, Van Den Berg, & Princen (1997) detected no seasonal variability in the susceptibility of LDL oxidation. Van de Vijver's group (1997) reported that this was the first study to examine the influence of seasonality specifically on LDL oxidation. No differences were found in the rates of formation of oxidized LDL *in vitro* for 10 volunteers (6 men, 4 women) at 4 different periods over a year. They monitored the kinetic parameters (lag time and propagation rate of copper ion-induced oxidation) of 10 healthy volunteers. No significant ($P < 0.05$) differences of these parameters over the seasons were detected. However, it was recognized that this study was comprised of a small number of subjects, who had a mean age 39.6 ± 7.4 years.

Overall, these studies demonstrate the complexity and inconsistencies related to seasonal variation associated with atherosclerotic development. It is evident that future studies may provide further insight into the potential influence of the time of the year on the susceptibility of oxidation of specific lipoproteins in human populations.

Infection and Lipoprotein Oxidation

A growing body of evidence suggests that inflammation plays a major role in atherogenesis (Ross, 1999). This evidence suggests that atherosclerosis may be a result of a chronic inflammatory process within arteries as a response to injury in the walls of arteries (Noll, 1998; Ross, 1999; Van der Wal & Becker, 1999). The increased incidence

of infectious illness in the winter may trigger a chain of physiological events that increase the occurrence of thrombotic events resulting from plaque ruptures (Crawford et al., 2000; Woodhouse et al., 1994). Additionally, recent evidence suggests that oxidized LDL can decrease the stability of atherosclerotic plaques, increasing the likelihood of a thrombotic event, such as a MI (Penn et al., 2000).

The potential link between an immune response and oxidation of lipoproteins is due, in part, to the activation of the immune cells (Ross, 1999). Specifically, leukocytes are a type of immune cells that may promote a cyclic chain reaction of LDL oxidation (Ross, 1999). Once formed, the oxidized LDL is highly atherogenic in that it promotes plaque formation (Steinberg, 1997). Thus, the development of atherosclerosis is often characterized as an inflammatory response (Ross, 1999).

Several infectious agents have been proposed to stimulate this inflammatory process associated with atherosclerosis. For instance, the presence of infections like *Chlamydia pneumonia* was described as being a contributing factor to an inflammatory response linked atherosclerosis (Mahony & Coombes, 2001). *Chlamydia pneumonia* has been described as an infectious respiratory bacteria in bronchitis and is thought to be a potential contributor to the process of atherosclerosis (Noll, 1998; Leinonen & Saikku, 2002). Some studies suggested that *Chlamydia pneumonia*, which can reside in macrophages, may be one of the stimulating agents for the oxidation of LDL (Kalayoglu et al., 1999). Specifically, infection was linked to LDL oxidation during the incubation of *Chlamydia pneumonia* with hamster monocytes *in vitro* and initiated LDL cholesterol

oxidation (Kalayoglu et al., 1999). This study suggests that periods of acute infection influence oxidative status of LDL.

In addition to *Chlamydia pneumonia* other infectious bacteria have been investigated in terms of oxidative stress. *In vivo* injection of bacterial lipopolysaccharide (LPS) resulted in a 2.2 fold increase ($P < 0.005$) in LDL oxidation in the blood of hamsters (Memon et al., 2000). Memon et al. (2000) demonstrated that a decrease occurred in the lag time of oxidation when monitoring conjugated diene production (control 92.5 minutes versus LPS-treated hamsters 31.2 minutes; $P < 0.001$). This study provided additional evidence to suggest that periods of infections can increase the susceptibility of lipoproteins to oxidation in both *in vitro* and *in vivo* animal models. Hence, human models will need to be developed and further researched to better understand the possible link between infection and the manifestation of atherosclerosis.

Recently, investigators have looked at the possible association between lipid oxidation and fibrinogen concentrations. An investigation of 144 middle-aged Finnish men provided data on the association between plasma fibrinogen level and lipid peroxidation (Rankinen, Hietanen, Vaisanen, Lehtio, Penttila, Bouchard, & Rauramaa, 2000). Results from this investigation suggested that increased lipid peroxidation was associated with elevated plasma fibrinogen levels on lipid oxidation. These studies support the rationale that the influence of seasonal variation and affect of fibrinogen levels in humans needs to be further investigated to better understand the potential contribution of this factor the progression of atherosclerosis.

During periods of inflammation the concentration of fibrinogen increases. Fibrinogen has been considered to be a risk factor for cardiovascular disease (Crawford et al., 2000; Stout & Crawford, 1991). In particular, a number of studies have noted fibrinogen as one of the acute phase proteins that might be elevated post-infection or in the winter and associated with an increased occurrence of MI (Woodhouse et al., 1994; Crawford et al., 2000; Stout & Crawford, 1991). The data of these researchers suggest that the influence of both seasonal variation and incidence of infection may have synergistic influence on atherosclerotic markers.

Aging and Lipoprotein Oxidation

The aging process in humans is generally associated with changes in physical characteristics and the decline in various physiological functions (Meydani, 2000; Brooks et al., 2000; Ji, 1993). Meydani (2000) explained that aging may be the consequence of increased accumulation of free radicals over time, which can lead to oxidative stress. This stress may be responsible for the oxidant-antioxidant balance associated with aging and age-related diseases (Jenkins & Goldfarb, 1993; Ji, 1993; Meydani, 2000). Some of these age-related diseases include: atherosclerosis, cancer, MI, retinal degeneration (Meydani, 2000).

Various researchers have attempted to investigate whether differences in susceptibility of lipoproteins to oxidation differ between younger and older individuals (Cristol, et al., 1999; Ji 1993; Meydani et al., 1993; Mosinger, 1997; Reaven, Napoli, Merat, & Witztum, 1999). In general, Ji (1993) explained that the aging process is

associated with a decline in protein synthesis and cell proliferation. Consequently, this decrease in cellular synthesis may be a factor affecting an older individual's natural antioxidant defense capacity because proteins are needed for the generation of endogenous antioxidants (Ji, 1993). Additionally, age-related oxidative stress may cause adaptational changes in the antioxidant enzyme status within the cell, affecting the balance of reactive oxygen species (Ji, 1993).

Cellular antioxidant defense systems may decline with aging (Leeuwenburgh, Fiebig, Chandwaney, & Ji, 1994). Leeuwenburgh and colleagues (1994) investigated the relationship between age, exercise, and oxidative stress. In particular, glutathione content and antioxidant enzyme activities in skeletal muscles of rats were measured. Glutathione is a major nonenzymatic antioxidant, which has several important functions related to free radical metabolism. For example, glutathione is a substrate for one of the main antioxidant enzymes found in the body (Leeuwenburgh et al., 1994). According to Leeuwenburgh and colleagues, there is strong evidence that age may be associated with significant alterations in nonenzymatic antioxidants (i.e. glutathione) status in rat skeletal muscle. Muscle lipid peroxidation was increased 2.5 fold ($P < 0.08$) in old rats compared to young rats. Although the precise mechanism for these age-related changes is unknown, Leeuwenburgh et al. hypothesized that changes were most likely due, in part, to increased oxidative stress associated with the inherent aging processes.

Other studies also have measured antioxidant enzymes in aging populations in order to gain a better understanding of the mechanisms of LDL oxidation (Cristol et al., 1999; Mosinger, 1997). For instance, Cristol et al. (1999) suggested that there may be an

impairment of antioxidant defense mechanisms in elderly women. According to Cristol's group, there were significant decreases in the enzymatic antioxidant, superoxide dismutase (SOD), and in the nonenzymatic antioxidant, glutathione (GSH) ($P < 0.01$, $P < 0.05$) in a group of elderly women. In terms of oxidative modification to LDL and the influence of age among women, Mosinger (1997) found that post-menopausal women had significantly higher amounts of LDL oxidation when compared to pre-menopausal women ($P < 0.001$). Overall, the antioxidant system in elderly individuals may be more vulnerable to free radical tissue damage due to an insufficient balance between oxidants and antioxidants.

In agreement with other aging investigations, a group of researchers monitored the variation between serum lipid peroxides in a group of 100 healthy men and women ranging in age from 20 to 70 years (Miquel, Ramirez, Soler, Diez, Carrion, Diaz et al., 1998). These investigators found an age related increase in the concentration of lipid peroxides. Specifically, results from simple linear regression analysis showed a strong positive correlation between age and lipid peroxides in 50 women in age from 22 to 70 years ($P < 0.01$). This data suggests that in certain individuals, aging may be linked to a decline of the pro-oxidant/antioxidant balance. Overall, the aging process may leave an elderly individual more vulnerable to oxidative stress and concomitantly increased lipid peroxidation.

On the contrary, inherent differences in susceptibility of lipoproteins to oxidation have not been documented between young and old individuals in all studies. For instance, in a small study (Schmuck, Fuller, Devaraj, & Jialal, 1995) conducted on 13

older individuals and 11 younger subjects, no statistical difference in the LDL oxidation after exposure to a copper oxidizing agent was observed between age groups. The “elderly” study group contained mainly women ranging in ages from 59-80 years. Schmuck et al. (1995) speculated that the relatively young age of some of the “elderly” subjects made interpretation of the results difficult. Thus, more studies are warranted for further understanding of the contributing role that aging has on lipid peroxidation related to atherosclerosis.

Other Factors Related to Lipoprotein Oxidation

Diet

Substantial evidence suggests that modifications of dietary habits can reduce the risk of developing CD. In fact, the scientific evidence of modifying dietary habits in regards to CD has been so convincing that the United States Food and Drug Administration (FDA) approved two health claims identifying a relationship between certain nutrient/foods and CD (FDA, 1993). For instance, one of the FDA claims states that, while many factors affect CD, diets low in saturated fat, cholesterol and total fat may reduce the risk of CD. Since this claim was proposed, there has been accumulating scientific evidence establishing that diets high in saturated fats and cholesterol are associated with increased levels of total cholesterol and LDL, and, thus, with increased risk of CD (Yu-Poth, Zhao, & Etherton, 1999; Parks et al., 1998).

Because diets that are high in cholesterol and saturated fats are known to contribute to unhealthy plasma lipid levels, the goal of dietary intervention is mainly to

affect the lipid profile positively, i.e., reduce TG, cholesterol, and LDL and increase HDL (Yu-Poth et al., 1999). Studies have shown that a reduction of dietary saturated fats, cholesterol, fried foods, and animal proteins positively affects CD risk (Parks et al., 1998).

In response to the proposed oxidation hypothesis, various studies altering diets have been conducted with the intent to better understand the role that lipid peroxidation plays in CD. Parks et al. (1998) investigated the oxidative susceptibility of LDL from patients with coronary artery disease. A lifestyle altering treatment program was developed to increase exercise, reduce stress, and decrease the consumption of fat in the diet. After 3 months of participation in this program intended to decrease susceptibility of LDL to oxidation, the authors concluded that the LDL particles became resistant to oxidation (Parks et al., 1998). For instance, the antioxidant protective phase of lipid peroxidation, lag time, was increased by 24% and the propagation rate slowed 29%. At the time, this was one of the first investigations to document reduction in oxidative stress in patients with coronary artery disease in response to alterations in diet and physical activity patterns.

The consumption of a diet high in fiber and fruits and vegetables is recommended (Yu-Poth, Etherton, & Reddy, 2000). In light of substantial scientific evidence, the FDA approved the health claim stating that, diets low in saturated fat and cholesterol and rich in fruits, vegetables, and grain products that contain some types of dietary fiber, particularly soluble fiber are associated with reducing the risk of CD (FDA, 1993).

The benefit of a high fruit and vegetable diet is thought to be due, in part, to the increase of plant antioxidants (Shils, Olson, Shike, & Ross, 1999). Antioxidants such as vitamin C (ascorbic acid), vitamin E (alpha-tocopherol), and beta-carotene (provitamin A) are exogenous nutrients that have the potential to provide oxidative protection (Clarkson & Thompson, 2000; Wattanapitayakul & Bauer, 2001). Most antioxidant vitamins A, E, and beta-carotene are found concentrated in certain fruits and vegetables and some organ meats (Bowman & Russel 2001; Shils et al., 1999).

As a result of these FDA claims research in regards to the oxidation hypothesis of atherosclerosis has focused on the therapeutic potential of antioxidants. Cardioprotective nutrients (i.e. foods containing antioxidants) may help to prevent damage to lipids that contribute to the development of CD. Antioxidants help to protect the unsaturated fatty acids in cell membranes from lipid peroxidation caused by free radical oxygen species (Lenz et al., 1990). Therefore, when antioxidants have been depleted, free radicals attack vulnerable lipid molecules. In particular, PUFA are potential targets for attack and consequently oxidative damage (Halliwell & Chirico, 1993; Kanter, 1997) (Figure. 2).

Human studies have shown that increased amounts of dietary PUFA increase LDL oxidation. For instance, Schnell, Anderson, Stegner, Schnidler, & Weinberg (2001) observed that subjects on a high PUFA diet had shorter LDL oxidation lag times as compared to a high monounsaturated fatty acid (MUFA) diet. Other oxidative markers have been measured in addition to lag time, such as measuring the propagation rate of oxidation. For example, some studies have detected that a high PUFA intake increases LDL oxidation susceptibility measured as propagation rate of conjugated diene formation

(Abbey, Belling, Noakes, Hirata, & Nestel, 1993). Contradictory to Abbey et al.'s findings, Schnell group observed no effect on PUFA lipoprotein in terms of oxidation rate.

Other investigations have been conducted with the intent to better understand oxidative status and diet. Yu-Poth et al. (2000) demonstrated that lowering dietary saturated fat and total fat reduces the oxidative susceptibility of LDL in healthy men and women by measuring the kinetic parameters of conjugated diene formation assessed *in vitro*. These researchers found that when plasma LDL levels are reduced, beneficial values associated with oxidative susceptibility and cardiovascular disease are reflected. Specifically, conjugated diene production and oxidation rates were significantly lower ($P < 0.05$) in response to lower levels of LDL as a result of a modified diet (Yu-Poth et al., 2000). Likewise, Parks et al. (1998) showed that diets rich in fruits and vegetables increased the resistance of LDL to oxidation *in vitro*. In essence, fruits and vegetables may increase antioxidant capacity in blood to potentially protect against *in vivo* lipid peroxidation in humans.

Scientists have conducted studies, particularly on vitamins C, E, and beta-carotene to find out what role they play in protecting the body from free radical production. Vitamin C, or ascorbic acid, is a water-soluble vitamin (Wattananpitayakul & Bauer, 2001). This vitamin is a free radical scavenger and interacts with free-radicals in the water compartment of cells as well as in the fluids between cells (Bowman & Russell, 2001). Vitamin C is considered to be the first line of antioxidant defenses against endothelial cell damage (Heller, Paulig, Grabner, & Till, 1999; Huang, Vita,

Venema, & Keaney, 2000). Additionally, vitamin C has a sparing effect on vitamin E as it regenerates vitamin E from the tocopheroxyl radical after it has neutralized free radicals (Niki et al., 1995; Shils et al., 1999). Thus, vitamin C may prevent LDL oxidation and therefore act as a cardiovascular protective agent (Bowman & Russell, 2001).

Another potentially protective antioxidant is the carotenoids, a group of red, orange, and yellow pigments found in plant foods, particularly fruits and vegetables (Bowman & Russell, 2001). Some carotenoids like beta-carotene act as precursors of vitamin A (Shils et al., 1999). Beta-carotene is an effective antioxidant as it is one of the antioxidant vitamins able to neutralize free radicals (Niki et al., 1995). However, the current evidence linking carotenoids with CD prevention is limited and remains inconsistent. For example, Kushi, Folsom, & Prineas (1996) and Rimm, Stampfer, Ascherio, Giovannucci, Colditz, & Willet (1993) found no evidence for a protective association with beta-carotene and CD.

Vitamin E is a fat-soluble compound found in all cellular membranes and is mainly stored in adipose tissue, the liver and muscle (Bowman & Russell, 2001). Vitamin E is a principal antioxidant in the body and protects PUFA in cell membranes from peroxidation (Shils et al., 1999). Over the past few decades, research has continuously shown that vitamin E offers protection against CD. The Cambridge Heart Antioxidant Study tested the hypothesis that treatment with vitamin E would reduce the risk of MI (Stephens, Parsons, Schofield, Kelly, Cheeseman, Mitchinson, & Brown, 1996). In this randomized, placebo-controlled, double-blind study of 2,002 patients with

diagnosed CD, vitamin E reduced the rates of non-fatal MI by 77%, with beneficial effects apparent after 1 year of treatment.

Adding supplements of vitamin E to the diet have been shown to increase the antioxidant content of the LDL and thus protect it from oxidative modification (Dieber-Rotheneder, Puhl, Waeg, Striegl, & Esterbauer, 1991; Jialal & Grundy, 1992; Jialal, Fuller, & Huet, 1995). Results from a clinical intervention trial showed that 1600 mg/day (800 IU) vitamin E taken for 5 months reduced LDL susceptibility to oxidation by 50% (Reaven et al., 1993). Additionally, the greatest risk reduction of coronary heart disease occurred in men and women who took 100 mg of vitamin E per day (the recommended dietary allowance [RDA] is 15 mg for women and men) (Stampfer, Hennekens, Manson, Colditz, Rosner, & Willett, 1993; Rimm et al., 1993). These human studies established that supplementation with antioxidant vitamins protect LDL from oxidation.

Jialal et al. (1995) found that the minimum dose of vitamin E needed to significantly decrease CD risk is 400 mg/dl day. In this placebo-controlled, randomized trial the investigators examined the effect of vitamin E dose by supplementing the diets of subjects (8 per group) for 8 weeks with a placebo or one of the following doses of vitamin E: 60, 200, 400, 800, or 1200 IU/day. Copper-mediated oxidation of LDL *in vitro* was assessed by monitoring the formation of CDs and thiobarbituric acid-relating substances (TBARS). Vitamin E concentrations in plasma and LDL increased with all levels of vitamin E supplementation and were not associated with changes in lipoprotein profile or in plasma concentrations of vitamin C or beta-carotene (Jialal et al., 1995). Jialal's group concluded that the minimum dose of vitamin E necessary to significantly

decrease the susceptibility of LDL to oxidation (i.e., prolong the lag phase and decrease the oxidation rate) was 400 IU/day. However, a study on women who had gone through menopause refuted these vitamin E supplemental findings. Women who ate moderate amounts of foods rich in vitamin E had about half the chance of dying from heart disease than women who avoided vitamin E-rich foods. But there was no decrease in risk for women who took vitamin E supplements (Kushi et al., 1996).

Overall, McCall and Frei (1999) explained in a recent review article, that if oxidative stress is critical factor in the development of atherosclerosis, then antioxidant vitamins may play an essential role in the prevention of this epidemic disease. However, these experts in the field of oxidative research concluded that the current evidence is insufficient to conclude that antioxidant vitamin supplementation reduces oxidative damage in humans. The evidence of oxidative protection is strongest for vitamin E, although studies may be confounded by other nutrient intakes and antioxidant nutrients (e.g. beta-carotene and vitamin C). According to Miller, Appel, & Risby (1998), the extent of lipid peroxidation depends on at least 3 factors: generation of oxygen free radicals, the presence of lipid substrates, and the activity of antioxidants. Hence, further research regarding lipoprotein oxidation should also assess antioxidant intake.

Physical Activity

The benefits of engaging in regular exercise are well documented. A recent review of the epidemiological evidence regarding physical activity and CD provided strong confirmation that physical activity is associated with reduced risk of CD

(Wannamethee & Shaper, 2001). For instance, major beneficial atherogenic adaptations of the lipoprotein profile to physical activity include: decrease plasma TG and LDL concentrations, and increased concentrations of HDL (Brooks et al., 2000; Wannamethee & Shaper, 2001).

Numerous studies have investigated the influence of regular physical activity on plasma lipoprotein profiles. For instance, 12 older and 12 younger male amateur cyclists had their plasma lipids examined during their training season and then following a detraining period (Giada, Vigna, Vitale, Baldo, Bertaglia, Crecca, & Fellin, 1995). The data acquired from the group of athletes was compared with a control group of 12 sedentary men. The researchers detected beneficial changes in lipoprotein profiles in response to cycling training independent of age. Specifically, HDL concentrations increased, while TG levels decreased significantly ($P < 0.05$) during training as compared to a period of detraining. In accordance with previous studies, the researchers concluded that a lifestyle of habitual physical activity is a major contributing factor in maintaining an antiatherogenic lipoprotein profile.

Despite the many known health benefits of exercise, accumulating research is speculating that there may be a negative side to exercise. Researchers have discovered that exercise with all its many benefits also increased the formation of dangerous free radicals (Ji, 2000). Under normal conditions the body has a natural antioxidant defense system that protects it from cellular damage. However, the healthy habit of exercising may not be considered normal due to the induced oxidative stress and concomitantly an increased potential for tissue damage (Polidori, Mecocci, Cherubini, & Senin, 2000,

Brooks et al, 2000; Moller, Wallin, & Knudsen, 1996). During exercise the body naturally produces more free radicals as a result of an increase flux of metabolic oxygen (Alessio, Hagerman, Fulkerson, & Ambrose, 2000; Clarkson & Thompson, 2000; Brooks et al., 2000; Jenkins & Goldfarb, 1993). This increase in radical production from exercise may increase the susceptibility of lipids to peroxidation (Alessio, 1993). Thus, current research is being conducted to further investigate oxidative stress of lipoproteins associated with exercise.

Recently, a group of researchers from Finland studied physical activity and lipid profiles and oxidizability in young athletes. Vasankari, Veromaa, Mottonen, Ahotupa, Irjala, Heinonen, Leino, & Viikari (2000) investigated the effect of physical activity on LDL oxidation and on lipid risk factors associated with CD in 183 teenage girls (9 – 15 years): 64 gymnasts, 61 runners, and 58 controls. The gymnasts had a 15% lower ratio of LDL conjugated dienes to LDL cholesterol (ox-LDL:LDL ratio, $P = 0.0052$) compared to controls. The gymnasts had a 12% higher ratio of HDL to total cholesterol than the controls. This study provided additional evidence that physical activity can favorably influence the risk of CD for young ladies by altering lipid profiles. Engaging in healthy lifestyle habits early in life may reduce the risk of developing CD.

Work in the field of physical activity and monitoring lipoprotein oxidation continues to accumulate. For example, Liu, Bergholm, Makimattila, Lahdenpera, Valkonen, Hilden, Jarvinen, & Taskinen (1999) detected that the LDL oxidizability (measured as lag time) of trained runners to be significantly shorter after the Helsinki marathon (180 ± 7 vs. 152 ± 4 min, $P < 0.001$). Liu et al. (1998) concluded that

strenuous aerobic exercise increases the susceptibility of LDL to oxidation. This may be due to the inability of the endogenous antioxidant defense system of the body to overcome an environment of increased oxidative stress.

In accordance with these findings, Benitez, Sanchez, Lucer, Arcelus, Ribas, Jorba, et al. (2002) investigated the effects of a 4-hour intense aerobic exercise on the oxidizability of both LDL and HDL in 11 male runners. LDL susceptibility to oxidation (measured as lag time) increased in all subjects after the race (64.4 ± 12.7 vs. 57.2 ± 9.1 min, $P < 0.05$). The lag time of HDL was slightly shorter after the race, but was not statistically significant. These researchers recognized that following intense aerobic exercise LDL is more susceptible to oxidation (shorter antioxidant protective period), which may be attributed to oxidative stress (Benitez et al., 2002).

Further evidence of an exercise-induced increase in antioxidant capacity associated with an increase in markers of oxidative stress has been documented. Alessio et al. (2000) found significant ($P < 0.01$) increased lipid peroxides in serum after nonaerobic isometric exercises. This was the first study to monitor the influence of isometric exercise on oxidative parameters as opposed to endurance exercise. In an earlier investigation, Alessio (1993) looked at the impact of exercise intensity on oxidative stress. They concluded that when performing a single bout of exercise, there may be an intensity threshold related to the degree of oxidative stress. For instance, moderate exercise did not induce oxidative stress, whereas higher intensity exercise associated with exhaustion did promote oxidative stress (Alessio, 1993).

On the contrary, various studies have revealed that exercise can be beneficial in terms of resistance to lipid oxidation. In particular, there is strong evidence that supports the idea that adaptations to regular exercise seem to have an antioxidant protective effect in addition to favorable lipid profiles (Ji, 2000; Liu et al., 1999; Moller et al., 1996; Powers, Ji, & Leeuwenburgh, 1999). For instance, regular exercise results in increased endogenous antioxidant capacity within cells, such as skeletal muscles (Brooks et al., 2001; Powers, et al., 1999). Additionally, regular exercise causes adaptation of antioxidant enzymes and enhances tissue repair systems all of which could decrease the degree of oxidative damage and increase resistance to oxidative stress (Radak, Taylor, Ohno, & Goto, 2001).

Liu and colleagues (1999) found the endogenous defense mechanism, total peroxyl-radical trapping capacity of plasma (TRAP), increased among men immediately after a marathon run and 4 days later. This finding provided evidence that trained athletes can adapt to strenuous oxidative conditions associated with intense and prolonged exercise in terms of enhancement of endogenous antioxidant capacity.

Similarly, several other studies have been published and examined the presence of oxidative stress after exercise (Ginsburg, O'Toole, Rimm, Douglas, & Rifai, 2001; Vasankari et al., 1997, Vasnakari, Kujala, Vasankari & Ahotupa, 1998). Evidence on physical activity and LDL oxidation was obtained in a study where sedentary subjects participated in an exercise program. After investigation of the effect of a 10-month exercise program on LDL oxidation in 34 sedentary middle-aged men and 70 women, the investigators demonstrated that LDL oxidation declined by 23% ($P < 0.001$) and 26% (P

< 0.0001) in men and women, respectively (Vasankari et al., 1998). These authors suggested that atherogenic risk may be reduced by habitual exercise due to the decrease of circulating oxidized LDL. These observations were in accordance with results for a study that reported oxidized LDL is reduced in young female gymnasts (Vasankari et al., 2000).

Furthermore, Ginsburg et al. (2001) found that the susceptibility of plasma lipids to peroxidation was reduced by 61% ($P < 0.001$) in men and 14% (NS) in women after participating in the Hawaii Ironman triathlon. Data collected from these well conditioned athletes suggested that endurance exercise favorably influences oxidative factors associated with CD. LDL oxidation was not affected by acute physical exercise in studies with sedentary adults or healthy endurance athletes (Vasankari et al., 1997).

Clearly, more studies are needed to better understand the potential factors related to atherosclerosis, such as seasonal variation, illness, aging, exercise and dietary antioxidants. For example, by far the most promising studies about antioxidants and exercise have centered on vitamin E. These studies have shown that vitamin E reduces free radical production and oxidation related to exercise (Evans, 1996; Ji, 1995; Kanter, Nolte & Hollosky, 1993; Meydani et al., 1993).

Dietary supplementation of vitamin E may increase tissue resistance to exercise-induced lipid peroxidation. For instance, Kanter et al. (1993) showed that daily supplementation of a vitamin mixture containing 600 mg vitamin E for 6 weeks significantly decreased levels of lipid peroxidation both at rest and after 30 minutes of treadmill exercise at 60% and 90% maximum oxygen consumption (VO_{2max}).

Similarly, Evans found that subjects over age 55 who ran and walked downhill benefited from a daily 800 mg vitamin E supplement. Subjects under age 30, however, did show decreased lipid peroxidation benefits. Evans concluded that as people age, their vitamin E levels decrease, but their need for them increases, and that supplements may help. Furthermore, Meydani et al. (1993) found that vitamin E (800 IU/d for 48 days) significantly increased the concentration of vitamin E in skeletal muscle after eccentric exercise. They also found that vitamin E supplementation reduced oxidative injury after eccentric exercise. On the whole, these investigators provided additional evidence of a relationship existing between CD, physical activity and dietary antioxidants that warrant further research.

Summary of Literature Review

The reviewed literature describes a variety of data demonstrating some of the potential factors that may directly affect the susceptibility of lipids to oxidation and concomitantly to atherosclerosis. For instance, due to seasonal variation, plasma LDL concentrations tended to be higher during the winter compared to summer months (Woodhouse et al., 1993). Furthermore, there is convincing evidence suggesting that there may be a link between infection and atherosclerosis as a result of *Chlamydia pneumonia* investigations (Kalayoglu et al., 1999; Memon et al., 2000). Additionally, aging was related to oxidative stress in that it may be a contributing factor related to atherosclerosis as a result in a decline of the aging cell's antioxidant defense capacity (Cristol et al., 1999; Ji, 1993; Leeuwenburgh et al. 1994; Meydani et al., 2001; Miquel et

al., 1998; Mosinger, 1997). Both dietary and physical activity patterns have been documented to have an influence on the risk of developing CD (Brooks et al., 2001; Bowman & Russel, 2001). For instance, the research is overwhelming that exercise does promote health in terms of cardiovascular health and enhanced adaptations to oxidative stress (Giada et al. 1995; Ginsburg et al., 2001; Ji, 2000; Liu et al., 1999; Parks et al. 1998; Vasankari et al., 1998; Vasankari et al., 2000). However, new research also addresses the fact that exercise increases the production of damaging oxidants, which concomitantly diminish oxidative protection of lipids (Alessio, 1993; Alessio et al., 2000; Benitez et al., 2002; Liu et al., 1999).

In the future it will be important to establish whether oxidized lipoprotein molecules and infection are involved in the mechanism(s) that decrease the stability of atherosclerotic plaques and trigger MI in humans. Ultimately this piece of information will be important for developing strategies to prevent infection or infection-induced lipid oxidation and provide additional insight into the pathogenesis of atherosclerotic. Although several factors influencing lipid oxidation have been identified, many unknown influences and exact mechanisms need to be further researched to fill in the gaps between the theoretical and practical aspects of CD. For instance, determining if whole plasma lipoprotein oxidation is reflective of *in vivo* oxidation would have practical importance in the clinical field for identifying a person's susceptibility to damaging uncontrolled oxidation. According to Kritchevsky (1996), there are many underlying variables and factors that need to be further investigated before intervention and prevention of cardiovascular diseases can be made. The reviewed literature suggests that the role of

oxidative stress(es) in addition to lipoprotein oxidation, warrant investigation as cause(s) of atherosclerosis, such as seasonality, infection, aging, physical activity and dietary influences.

CHAPTER 3

METHODS

Subjects

Study participants consisted of 44 women living in Montana throughout the duration of the study. Women were recruited for volunteer participation and classified into one of two groups according to their age: a group of 24 younger women (18-40 years of age) and a group of 20 older women (50 years or older). Major exclusion criteria for eligibility were that the women must be non-smokers, free of known cardiovascular disease and diabetes, and not taking medications for hyperlipidemia. Because antioxidant vitamins may affect oxidation of LDL cholesterol (Niki & Noguchi, 1997; Meydani et al., 1993; Vasankari et al., 1997), women who had been taking regular supplements of the anti-oxidant vitamins (e.g. beta-carotene, vitamin E, vitamin C, or coenzyme Q₁₀) were excluded in the younger group. However, the older volunteers were asked to continue with their regular vitamin supplemental habits and provide frequency and dosage of supplement intakes if applicable. Data regarding the cardioprotective effect of estrogen on oxidation of LDL cholesterol are mixed (Hwang, Peterson, Hodis, Choi, & Sevanian, 2000; Abbey, Owen, Suzakawa, Roach, & Nestel, 1999). Women who were taking estrogen in the form of birth control or estrogen replacement therapy were not excluded from participation. However, women taking exogenous estrogen provided frequency and

dosage of the particular type of estrogen. Subjects were informed of the risks of participation and gave written informed consent prior to study enrollment, which was approved by the Human Subjects Committee at Montana State University-Bozeman (Appendix A).

Experimental Design

After screening for participation eligibility, fasting blood lipids were acquired four times: twice during the summer (end of July to the first week in September) and twice during the winter (January and February). Summer and winter assessments included fasting blood lipids total cholesterol (TC), TG, HDL, VLDL, and LDL and whole plasma oxidation induced with copper chloride. Assessment of reliability of blood lipids and whole plasma oxidation, the primary dependent variables, were obtained using the two measurements made for these variables during each season. Plasma fibrinogen concentrations were assessed from the second blood collections during each season. To aid in the interpretation of differences in blood lipid variables from season to season, anthropometric measures, body composition, physical activity, and diet were also evaluated as part of the summer and winter assessments. The experimental design is illustrated in Figure 4.

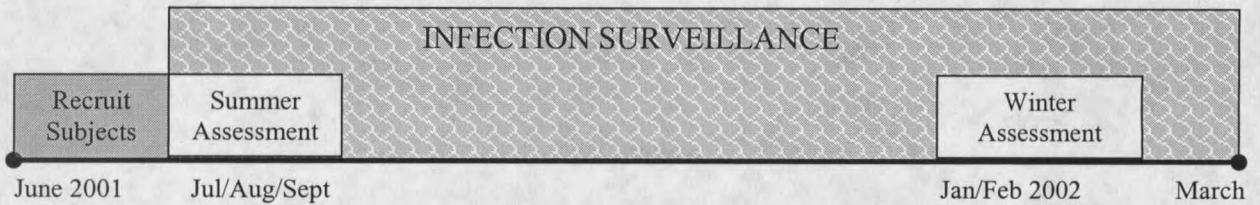


Figure 4. Illustration of Research Time Line

Summer and winter assessments included fasting blood lipids, whole plasma oxidation and fibrinogen, body composition, physical activity, and dietary recall. These measures were not made during an active infection or within 12 days of onset of that infection. If subjects experience an infectious illness during the infection surveillance period, assessment of fasting blood lipids, whole plasma oxidation and fibrinogen were made within a window of 3 to 8 days following the onset of the symptoms.

To determine whether acute infection, e.g. common colds or flu, influences the oxidation of whole plasma, each woman kept a log of illness symptoms from the time of enrollment through the end of March. If self-reported moderate or severe symptoms of an infection persist for two or more consecutive days, or if the physician of the subject diagnosed the presence of an acute infection, then the subjects contacted the investigator and scheduled a fasting blood collection within the next five days. Menstrual cycle phase was noted and recorded for younger subjects if they were ill and scheduled for the infection blood draw.

Blood Collection and Plasma Separation

All blood samples were collected after an overnight, 12-hour fast. Subjects were asked to drink water in order to remain hydrated and instructed to refrain from vigorous

physical activity in the 24 hours prior to blood collection. Subjects reported to the MSU Nutrition Research Laboratory for collection of all blood samples. The premenopausal women had blood drawn in the first seven days of their menstrual cycle to alleviate potential effects of hormonal variations. Subjects were seated for 10 minutes, and then samples were collected from an antecubital vein into evacuated tubes using standard venipuncture technique. Subjects were instructed to return to the lab the following morning if feasible or within 48 hours of the first blood draw.

Blood was collected using a 21 gauge Vacutainer ® needle (Becton Dickinson and Company, Franklin Lakes, NJ) into previously ice water-chilled centrifuge tubes: 6 mL vacuum collecting Vacuette® tubes without additive for determination of blood lipids; 6 mL lithium heparin Vacuette® tubes for whole plasma oxidation; and 3 mL sodium citrate and EDTA Vacuette ® tubes for fibrinogen and complete blood count (CBC) determination, respectively. Shortly after collection, plasma and serum was separated from cells using a refrigerated 21000 Marathon centrifuge (Fisher Scientific, Pittsburgh, PA). The centrifuge was programmed at 16 °C for 10 minutes at 2500 rpm. As a result of this procedure clear plasma was obtained. Aliquots of each plasma sample were pipetted into labeled and coded micro-centrifuge tubes. Next, the plasma aliquots were stored at -80°C in a REVCO Ultima II freezer (Legaci Refrigeration System, Asheville, NC) until further assay. After centrifugation the EDTA plasma samples were delivered to Bozeman Deaconess Laboratory Services for determination of CBC. In regards to plasma fibrinogen concentrations the blood samples of each subject was collected in sodium citrate Vacuette ® centrifuge tubes and stored at -80°C in a REVCO

Ultima II freeze (Legaci Refrigeration System, Asheville, NC) until delivery to off-site laboratory (approximately 2 weeks). For a given analysis, all samples for an individual (e.g. summer, winter, and infection) were analyzed in the same run of the assay. Thus, inter-assay variability amongst conditions did not systematically influence assay results.

Blood Lipids

Plasma TC, HDL, and TG concentrations were measured in duplicate using a KODAK Ektachem analyzer (VitrosDT60, Rochester, NY) (Lie et al., 1976; Fossati et al., 1982) and the appropriate slides and kits. The analyzer was calibrated with DT Calibrator Kits (195-7927 Vitros, Ortho-Clinical Diagnostics, Inc., Rochester, NY). Total cholesterol was measured at 555 nm using DT Cholesterol slides (153-2175 Vitros, Ortho-Clinical Diagnostics, Inc., Rochester, NY). A 10 μ L drop of plasma was deposited on a slide. Each sample was incubated for a period of 5 minutes at 37 °C inside the analyzer. Next, the analyzer calculated the amount of cholesterol present in the samples and printed off results. HDL cholesterol was measured at 660 nm using DT HDL Cholesterol Kits (148-0664 Vitros, Ortho-Clinical Diagnostics, Inc., Rochester, NY). Triglyceride concentrations were determined at 555 nm using DT Triglyceride slides (153-2159 Vitros, Ortho-Clinical Diagnostics, Inc., Rochester, NY). LDL cholesterol was determined by the method of Friedewald, Levy, & Fredrickson (1972). Specifically, LDL was calculated from the following Friedewald equation: $[\text{LDL}] = [\text{TC}] - [\text{HDL}] - ([\text{TG}]/5)$ where $[\text{TG}]/5$ is an estimate of VLDL and all concentrations are expressed in mg/dL (Friedewald et al., 1972). The Friedewald equation was used

because all fasted subjects' TG concentrations were less than 400 mg/dL (4.66 mmol/L) and chylomicrons were not visibly present, to avoid falsely overestimating VLDL and underestimating LDL.

Whole Plasma Oxidation

A method was used for evaluating oxidation of whole plasma with the capacity to handle small multiple samples involved using a 96-well microtiter plate (Wallin, Rosengren, Shertzer, & Camejo, 1992). Specifically, whole plasma was oxidized to measure the oxidizability of whole plasma lipoproteins (LDL, HDL, and VLDL), which has been shown to provide results similar to that obtained using a common LDL oxidation assay (Kontush et al., 1997). Heparin plasma samples were used to measure the oxidation of whole plasma following the procedures described by Kontush et al. (1997) modified for micro-method analysis.

The thawed heparin plasma aliquots were immediately diluted 1:125 with phosphate buffer saline (PBS), 250 μ L of this dilution was added in triplicate into a 96 well flat bottom UV-microplate (Costar-3635, Corning Incorporate, Acton, MA). A fresh solution of CuCl_2 was mixed to a concentration of 300 μ M in PBS solution (Appendix F). To initiate the oxidation in the samples, 50 μ L of PBS plus the CuCl_2 solution was added to all wells using a multi-channel pipette. This yielded a final dilution of plasma at 1:150. Three blank wells consisted of PBS and CuCl_2 without plasma. The plates were sealed with an UV-readable plate sealer (T329-1FS, EXCEL Scientific, Wrightwood, CA) to prevent evaporation. Each plate was read every 10 minutes for 8 hours at 234 nm

in the μ Quant Universal microplate spectrophotometer (Bio-Tek Instruments, Inc. Winooski, VT). The μ Quant Universal microplate spectrophotometer was controlled for temperature using a Precision gravity convection incubator (GCA Corporation, Chicago, IL) set at 37°C. All of the sequential absorbance readings were saved on KC Junior software (Bio-TEK® Instruments, Inc., Winooski, VT) and plotted to determine the dependent variables: lag time and propagation rate of conjugated diene formation of the lipoproteins in whole plasma.

The susceptibility of whole plasma to oxidation was characterized by the two phases similar to the lag and propagation phases of LDL oxidation (Esterbauer et al. 1989). The lag phase was determined by calculating the length of time (minutes) it takes for the lipoproteins in whole plasma to resist oxidation. The change of absorbance of the conjugated diene versus time profile is illustrated in Figure 3. The absorbance reading for each time point is corrected for the absorbance of the blanks and then plotted against time. A previously written National Instruments LabVIEW™ 5.1 program determined the lag time and the maximum rate of conjugated diene formation (propagation rate). For instance, the time of the lag phases were determined as the intercept of the best-fit line for the lag phase and the line through the steepest portion of the propagation phase. The maximum propagation rate of conjugated diene formation was calculated by determining the slope of the propagation phase using the first derivative of diene versus time curve (Esterbauer et al., 1989). Thus, conjugated diene formation was determined as the change of the rates of oxidation as a function of time. The average of the lag time and maximum propagation rate of the duplicate samples was used for statistical analysis.

Fibrinogen

This investigation measured the acute phase protein, fibrinogen, so that comparisons could be made to the responses of oxidized whole plasma parameters to a measure representative of the acute phase process during an infection. The appropriate equipment was not available in the Nutrition Research Laboratory. Frozen samples were sent to the Clinical Laboratory at Bozeman Deaconess Hospital for analysis. Plasma fibrinogen concentrations were determined by the Clauss fibrinogen method using bovine thrombin as the reagent (bioMerieux, France). The Clauss method for fibrinogen measurement is a standard assay using the rate of fibrinogen conversion to fibrin in the presence of excess thrombin. The concentration is determined by using a reference curve prepared from the clotting times of reference plasma dilutions with known fibrinogen concentrations.

Anthropometric and Body Composition Measurements

Summer and winter assessments involved collecting standard anthropometric and body composition measurements. Specifically, during the summer and winter laboratory visits, body mass (kg), body heights (cm), waist-to-hip circumference ratios (cm:cm), and BMI (kg/m^2) measurements were recorded. Total body water content was estimated using a Hydra ECF/ICF Bio-Impedance Spectrum Analyzer (Model 4200, Xitron Technologies, Inc., San Diego, CA) (Appendix E). This measurement was used to determine fat free mass according to the appropriate equation developed and validated by

Segal et al. (1988). This equation was validated for women of mixed racial backgrounds. All measurements were made in the morning, when no vigorous exercise had been performed for 24 hours, and for pre-menopausal women in the first 7 days of the menstrual cycle. These conditions minimize potential sources of error with bioelectrical impedance analysis (Heyward, 1998).

Physical Activity Assessment

Because physical activity in the form of an aerobic exercise training program was shown to alter the level of LDL oxidation in men and women (Vasankari et al., 1998), estimations of the level of physical activity for subjects during each of the summer and winter seasons were made. The subjects were instructed to maintain their usual level of physical activity during the study, but to avoid any strenuous exercise 24 hours prior to blood collection. All subjects completed a Bouchard Three-Day Physical Activity Record (Bouchard, Tremblay, LeBlanc, Lortie, Savard, & Theriault, 1983, Appendix B) during the course of both the summer and winter assessments. Subjects recorded a physical activity category for each 15-minute block of the day. There were 9 activity categories with descriptions of activity levels, e.g. 1=lying down, 5=light manual work or activity such as housework or golfing, and 9=intense manual work such as running, cross-country skiing, or sawing with a handsaw. Each category was assigned an energy expenditure value per kg/15 minutes and daily energy expenditure was calculated. Additionally, the amount of time spent specifically in moderate and intense activity was calculated. The three days of activity monitored included two weekdays and one

weekend day that the subjects felt were typical with respect to physical activity. The test-retest reliability of this measure was high for adults, intraclass correlation, $r = 0.97$ (Bouchard et al., 1983).

Dietary Assessment

A three-day dietary survey (McArdle, Katch, & Katch, 1999) was used in which subjects recorded all food and beverages consumed (Appendix C). The use of antioxidants (vitamins C, E, and beta-carotene) that may influence the oxidation of whole plasma lipoproteins were recorded to estimate the consumption of these compounds by subjects in both summer and winter (Dieber-Rotheneder et al., 1991; Jialal & Grundy, 1992; Jialal et al., 1995). Similarly, dietary intake of several variables that may influence blood lipid levels and oxidation state was assessed, including total and saturated fat consumption (Parks et al., 1998; Yu-Poth et al., 2000). Overall, the dietary variables of interest from the three-day dietary records included: protein, carbohydrate, fat, cholesterol, saturated fatty acids, monounsaturated fatty acid, PUFA, vitamins C, E, beta-carotene, and alcohol. Subjects were asked to maintain their usual diet during the study. Prior to completing the survey, each subject was given instruction as to serving sizes and the types of details needed to accurately record intake. Additionally, subjects performed a one-day practice diet record in the presence of the investigator so that feedback could be provided to improve documentation before the actual survey was performed. Data from this survey was reduced to daily nutrient intakes using Nutritionist Pro, version 2.2

(San Bruno, CA). This method was chosen because the acute nature of the assessment will help to detect changes from season to season.

Infection Surveillance

Logbooks to cover the period of time from subject enrollment in the summer to the end of the following March were given to subjects (Appendix D). This logbook is based on the method used by Nieman, Henson, Gusewitch, Warren, Dotson, Butterworth, & Nehlsen-Cannarella, (1993) to document the occurrence of illness based on the following categories: (1) no health problems today, (2) cold symptoms (i.e. runny stuffy nose, sore throat, coughing, sneezing, colored discharge), (3) allergy (i.e. itchy eyes, stuffy nose, clear discharge), (4) flu symptoms (i.e. fever, headache, general aches and pains, fatigue and weakness, chest discomfort, cough), (5) nausea, vomiting, and/or diarrhea, (6) other health problems by description. Additionally, the volunteers ranked subjectively the severity of these symptoms according to the following scale: (1) mild, (2) moderate, and (3) severe. Subjects were asked to see their physician if moderate or severe symptoms persisted for more than 2 days, so that they received a professional diagnosis to improve the accuracy of the log, but this was not required. If self-reported moderate or severe symptoms of an infection persist for two or more consecutive days, or if the physician of the subject diagnosed the presence of an acute infection, then the subjects contacted the investigator and scheduled a fasting blood collection within the next five days. Menstrual cycle phase was noted and recorded for younger subjects if they were ill and scheduled for the infection blood draw.

Statistical Analysis and Sample Size Estimation

Based on reported mean and standard deviation of cross-sectional data, post-menopausal women had about 25% greater oxidized LDL (Mosinger, 1997).

Consequently, it was estimated that the sample size of 20 subjects per group (younger and older) is sufficient to detect an 18% change in LDL oxidation at a power level of $\beta = 0.80$ and $\alpha = 0.05$ (Park & Schutz, 1999). Thus, the proposed sample size was sufficiently powered to detect the magnitude of change that is likely to be measured within this investigation in order to reject the null hypotheses.

The dependent variables to be analyzed included lipid oxidation kinetics for whole plasma (lag time and propagation rate). Assessment of reliability of blood lipids and whole plasma oxidation was done using the two measurements made for these variables during each season. Within each season, reliability was assessed using a Pearson product-moment correlation, which computed the correlation for whole plasma oxidation and other blood variables (TC, HDL, LDL, VLDL, and TG). Additionally, stability was assessed using a paired t-test.

Two-way analysis of variance (ANOVA) with repeated measurements was used to compare whole plasma oxidation variables (lag time and propagation rate) and blood lipid profiles (TC, HDL, LDL, VLDL, and TG) at summer, winter, and infection assessments for both younger and older women. In particular, a two-way ANOVA (2x2) with repeated measures was used to detect main effect differences between groups (older and younger women) and seasons (summer and winter). For the subjects who

experienced an acute infectious illness, a two-way ANOVA (2x3) with repeated measures was used to detect differences between groups (older and younger) and across summer, winter, and infectious conditions. If significant interactions were detected, then the Tukey-HSD Post-hoc test was used to identify the location of the differences. All results are expressed as means $\pm$ standard deviations (SD). Statistical significance was set at an alpha-level equal 0.05. The quality of the assays was controlled by measuring the intra-assay coefficient for variability, which was not higher than 10.9 % for all parameters measured.

For the two-way ANOVA (2x2) all subjects model, statistical analysis was used for assessing the physical activity (total energy expenditure) and selected nutrients from diet analysis (cholesterol, total fat, saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids, vitamins C, E, beta-carotene) between summer and winter seasons. The statistical analyses were performed using Statistica software package for Windows, 1999 Edition (StatSoft[®], Tulsa, OK).

CHAPTER 4

RESULTS

Subject Descriptive and Anthropometric Characteristics

Forty-three subjects completed the study. The mean age for the younger group was 29 ± 6.6 years as compared to the older group, which had a mean age of 61 ± 8.2 years. One younger subject could not be re-contacted for winter assessments and therefore her acquired summer data was not included in the interpretation of the results. Additionally, one older subject was dropped from analysis of the investigation as a result of a winter back operation and subsequent blood transfusion. Overall, statistical analyses was performed on 23 younger women and 19 older women. The group descriptive and anthropometric characteristics are reported in Table 1. There was no significant difference detected in height, weight, BMI, or waist to hip ratios between the younger and older women or between summer and winter assessments. However, the older women had a significantly higher ($P = 0.002$) percent body fat compared to younger women.

Ten out of the 19 older subjects used some form of vitamin supplementation before entering the study. For these subjects, vitamin usage remained constant throughout the 9-month study period. Additionally, of the 19 older women participating in this study five were taking some form of estrogen. In the younger group, no subject reported regular vitamin supplementation at least one month prior to the first summer

assessment. Seven out of the 23 women in the younger group reported taking some form of estrogen during the study period.

Table 1. Descriptive and anthropometric characteristics of women.

	<u>Summer</u>		<u>Winter</u>	
	Younger (n=23)	Older (n=19)	Younger (n=23)	Older (n=19)
Height (cm)	167.7 ± 7.3	165.4 ± 7.5	167.3 ± 7.2	165.4 ± 7.4
Weight (kg)	68.6 ± 16.5	66.9 ± 8.6	69.4 ± 17.2	67.2 ± 9.0
BMI (kg/m ²)	24.4 ± 4.3	24.5 ± 3.2	24.7 ± 5.4	24.7 ± 3.7
Body fat (%) [†]	35.0 ± 7.2	41.4 ± 5.3	35.0 ± 6.4	41.1 ± 6.0
Waist:Hip ratio	0.74 ± 0.06	0.75 ± 0.05	0.73 ± 0.7	0.74 ± 0.05

Values are means ± SD. [†] P < 0.01 main effect for age.

Dietary Intake for Summer and Winter Groups

Selected measurements of nutrient intake potentially important for lipoprotein oxidation *in vitro* were considered in both younger and older women in summer and winter assessments (Table 2). Energy intake was significantly higher (P = 0.02) for the younger compared to older women. Additionally, there was a significant season by age interaction for energy intake (P = 0.02), with most of this variability occurring in the summer between the young and old group (P = 0.04). Similarly, significant (P = 0.02) season by age group interaction was found for protein intake. Pos hoc testing detected that the majority of the difference occurred in the younger group between summer and winter seasons (P = 0.08). Carbohydrate intake was higher (P = 0.01) for younger

women compared to older women during both seasons (summer and winter). Significant season by age group interaction ($P = 0.01$) was found with fat (g) intake. Post-hoc testing detected that the majority of the difference occurring with the older group between summer and winter seasons ($P = 0.08$). The percent fat intake during the summer was significantly lower ($P = 0.05$) compared to winter for older women. Additionally, there was a significant season by age interaction for percent fat intake ($P = 0.05$), with higher fat intakes occurring in the winter for the old group ($P = 0.04$). Strong evidence of significant season by age group interaction ($P = 0.01$) was found with saturated FAs intake. Similarly, significant season by age group interaction ($P = 0.02$) was found with MUFA intake. Pos hoc testing detected that higher intakes of MUFA occurred during the summer compared to the winter for younger women ($P = 0.06$). No differences in cholesterol, PUFAs, or alcohol were detected.

Selected antioxidant vitamins (vitamins C, E, and beta-carotene) were analyzed in both younger and older groups of women during the summer and winter. Significant differences ($P < 0.05$) were found as a result of the main effect, season, for vitamin C intake. Specifically, vitamin C intake during the summer was significantly higher ($P = 0.02$) as compared to winter assessments for both younger and older women. Vitamin E intake was significantly higher ($P = 0.02$) for the older women compared to younger women during both summer and winter assessments. No differences in beta-carotene were detected.

Table 2. Selected dietary intake of women.

	<u>Summer</u>		<u>Winter</u>	
	Younger (n=23)	Older (n=19)	Younger (n=23)	Older (n=19)
Energy intake (kcal) ^{††}	2233.3 ± 489 ^a	1783.5 ± 355 ^a	2006.4 ± 381	1937.7 ± 425
Protein (g) [‡]	79.5 ± 20.1 ^a	68.7 ± 18.7	70.6 ± 20.3 ^a	73.1 ± 15.2
CHO (g) [†]	305.0 ± 76.7	229.8 ± 67.2	278.5 ± 72.7	243.5 ± 75.0
Fat (g) [‡]	75.5 ± 24.7	58.9 ± 20.5	66.6 ± 16.3	73.8 ± 28.4
FAT % of kcal ^{*†}	30.2 ± 6.7	29.7 ± 8.2 ^a	30.3 ± 6.2	33.8 ± 9.2 ^a
Cholesterol (mg)	216.6 ± 130.0	244.7 ± 124.7	191.1 ± 99.0	237.3 ± 152.0
Saturated FAs (g) [‡]	25.0 ± 7.9	18.9 ± 8.0	22.2 ± 5.6	22.0 ± 7.9
MUFAs (g) [‡]	22.2 ± 10.9	18.9 ± 7.0	16.9 ± 6.9	21.1 ± 8.5
PUFAs (g)	11.5 ± 6.0	12.0 ± 5.2	9.2 ± 3.4	13.9 ± 6.6
Alcohol (g)	8.66 ± 11.3	13.3 ± 17.6	7.2 ± 13.3	6.7 ± 12.0
Beta-carotene (µg)	1254.7 ± 949.9	1340.5 ± 916.5	841.1 ± 434.9	1387.5 ± 747.8
Vitamin C (mg) [*]	133.4 ± 104.2	239.0 ± 260.8	103.6 ± 122.2	126.9 ± 73.6
Vitamin E (mg) [†]	7.1 ± 4.9	11.6 ± 11.1	6.4 ± 5.7	9.7 ± 5.7

Values are means ± standard deviation. [†] P < 0.05 main effect for age. ^{*} P < 0.05 main effect for season. [‡] P < 0.05 season by age group interaction. ^a P < 0.05 between means with like superscript letters.

Estimated Physical Activity for Summer and Winter Groups

Data regarding estimated physical activity for younger and older women in summer and winter are reported in Table 3. No significant main effects (age or season) or interactions were detected for estimated time spent in physical activity (min) per day. However, the amount of time that subjects spent in moderate PA decreased significantly

($P = 0.0001$) as a result of the seasonal effect for both younger and older women. In contrast, the amount of time that subjects spent in vigorous PA increased significantly ($P = 0.02$) in response to the effect of season.

Table 3. Time spent in physical activity.

	<u>Summer</u>		<u>Winter</u>	
	Younger (n=23)	Older (n=19)	Younger (n=23)	Older (n=19)
Total PA (kcal/kg)/day	3183.4 $\pm$ 693.1	3028.3 $\pm$ 497.5	3141.8 $\pm$ 828.0	3025.6 $\pm$ 459.3
Moderate PA (min/day)*	139.6 $\pm$ 110.8 ^a	140.3 $\pm$ 129.2	29.1 $\pm$ 37.3 ^a	41.1 $\pm$ 56.7 ^a
Vigorous PA (min/day)*	13.3 $\pm$ 19.2	10.8 $\pm$ 30.4	33.9 $\pm$ 48.0	27.9 $\pm$ 44.1

Values are means $\pm$ standard deviation. * $P < 0.05$ main effect for season. ^a $P < 0.01$ between means with like superscript letters.

Plasma Lipid Profiles

All measured concentrations of plasma lipids for younger and older in the summer and winter, such as total cholesterol are illustrated in Figures 5-9. The intra-assay coefficient of variation of plasma total cholesterol in repeated measurements was 1.2 %. Total cholesterol concentrations in the plasma were higher ($P = 0.01$) for the older compared to younger women during both the summer and winter assessments (Figure 5). Specifically, during the summer, younger subjects had lower cholesterol levels (181.4 $\pm$ 32.8 mg/dl) as compared to the higher cholesterol levels of older subjects (202.7 $\pm$ 32.1

mg/dl). Similar concentrations of plasma cholesterol were measured during the winter resulting in significant differences between young and old groups (181.5 ± 25.8 mg/dl vs. 208.4 ± 31.6 mg/dl, respectively).

The intra-assay coefficient of variation of plasma TG in repeated measurements was 1.6 %. Figure 6 illustrates that no significant differences in TG concentrations were found between young and old groups ($P = 0.51$), or summer and winter assessments ($P = 0.45$). Likewise, no significant interaction was identified for TG concentrations ($P = 0.68$).

The intra-assay coefficient of variation of plasma HDL in repeated measurements was 1.9 %. Significant ($P = 0.03$) age effect was found on the response of the concentrations of HDL in the plasma (see Figure 7). In particular, during the summer, younger subjects had lower HDL levels (49.8 ± 14.1 mg/dl) as compared to the HDL levels of older subjects (57.8 ± 11.9 mg/dl). Similar concentrations of plasma HDL were measured during the winter resulting in significant differences between young and old groups (49.9 ± 11.4 mg/dl vs. 59.0 ± 14.7 mg/dl, respectively). No significant differences in HDL concentrations were detected for either a season by age interaction or seasonal main effect.

Figure 8 illustrates that no significant differences in LDL concentrations were identified for age ($P = 0.09$), season ($P = 0.22$), or for a season by age interaction ($P = 0.46$). Similarly, Figure 9 illustrates that no significant differences in VLDL concentrations was found between young or old groups ($P = 0.51$), summer or winter

assessments ($P = 0.45$). Additionally, no significant interaction was detected for VLDL concentrations ($P = 0.68$).

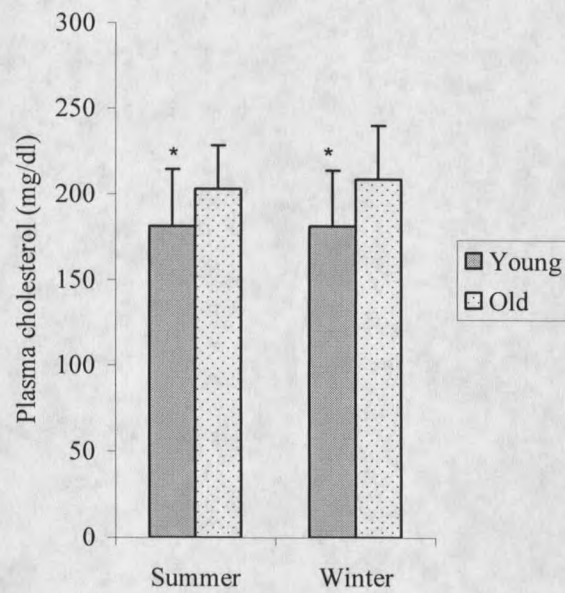


Figure 5. Plasma cholesterol concentrations of younger and older groups in the summer and winter. * $P < 0.05$ young versus old group during same season.

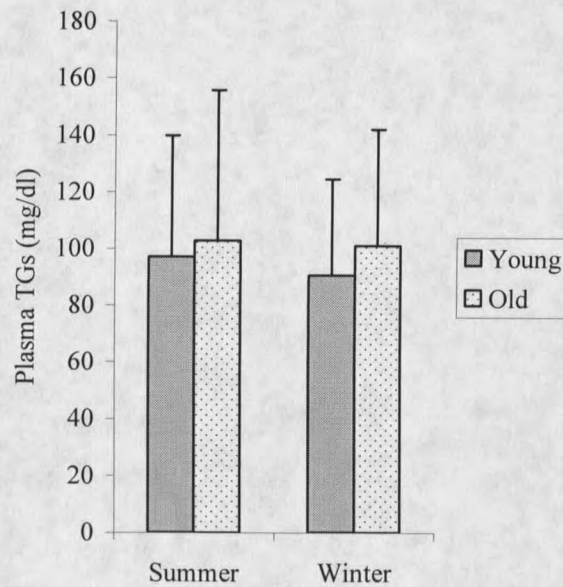


Figure 6. Plasma triglyceride concentrations of younger and older women during summer and winter assessments revealed no significant differences.

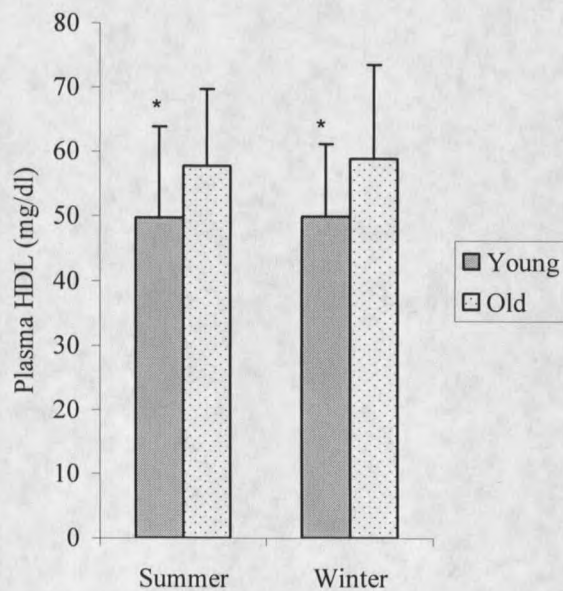


Figure 7. Plasma HDL concentrations of younger and older groups in summer and winter. * $P < 0.05$ young versus old group during same season.

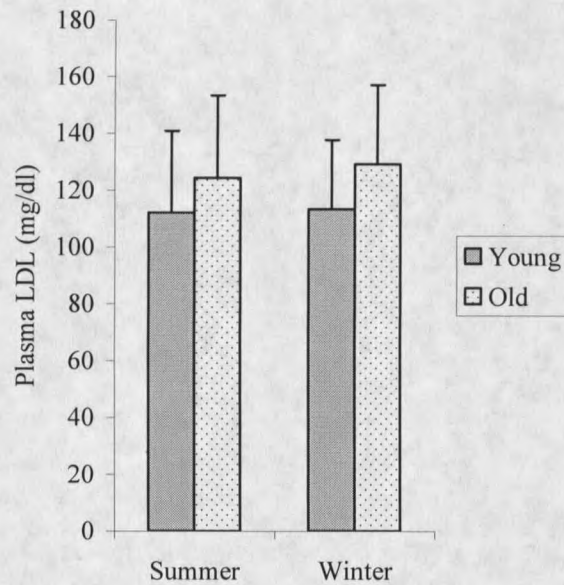


Figure 8. Plasma LDL concentrations of younger and older groups in summer and winter indicated no significant differences.

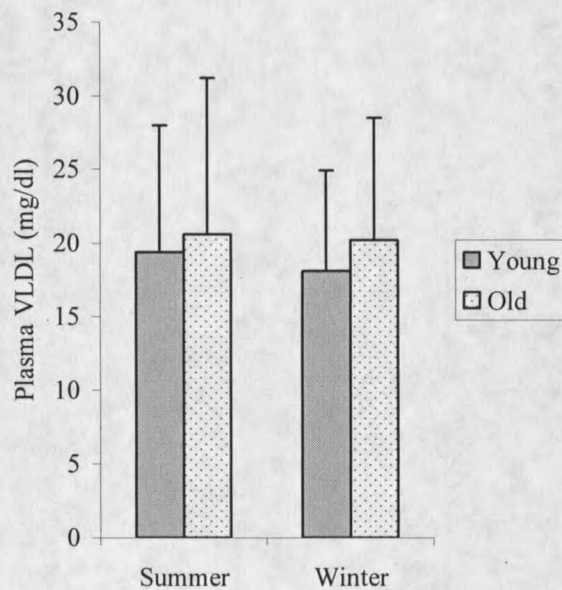


Figure 9. Plasma VLDL concentrations of younger and older groups in summer and winter showed no significant differences.

Fibrinogen and CBC

Quantitative measurements of selected inflammatory markers are presented in Table 4. No statistically significant ($P < 0.05$) interaction effect between season and age factors was found for any of the measured inflammatory markers. However, age was a factor that accounted for most of the total variability in fibrinogen and hemoglobin levels in the plasma. There were higher ($P = 0.02$) mean fibrinogen concentrations for older women compared to younger women independent of season. Additionally, there were lower ($P = 0.05$) mean hemoglobin concentrations for older women compared to younger women. There was no evidence of an interaction, age or seasonal main effect for the other inflammatory markers in the calculated data.

Table 4. Fibrinogen and CBC measurements of women.

	<u>Summer</u>		<u>Winter</u>	
	Younger (n=23)	Older (n=19)	Younger (n=23)	Older (n=19)
Fibrinogen (mg/dl) [†]	248.9 ± 38.3	280.0 ± 65.0	251.7 ± 42.6	287.0 ± 44.0
Hemoglobin (g/dl) [†]	14.5 ± 0.9	14.0 ± 0.7	14.2 ± 0.8	13.9 ± 0.8
WBC (x10 ³ /μl)	5.5 ± 1.4	5.1 ± 1.8	5.4 ± 1.6	5.0 ± 1.6
Neutrophils (x10 ³ /μl)	3.2 ± 1.1	2.9 ± 1.4	3.2 ± 1.2	2.8 ± 1.3
Lymphocytes (x10 ³ /μl)	1.7 ± 0.5	1.6 ± 0.4	1.7 ± 0.5	1.6 ± 0.5
Monocytes (x10 ³ /μl)	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.2
Platelet Count (x10 ³ /μl)	249.2 ± 46.2	252.1 ± 52.0	257.5 ± 45.1	256.6 ± 50.4

Values are means ± standard deviation. [†] $P < 0.05$ main effect for age during same season.

Day to Day Reliability of Lipid Oxidation

In accordance with data previously reported for human plasma (Kontush & Beisiegel, 1999), the absorbance increase of diluted plasma oxidized with 50 μM Cu(II) was characterized by two consecutive phases, the lag and propagation phases of oxidation (Figure 3). To quantitatively describe plasma oxidizability, the lag phase (min) and the maximum rate of oxidation in the propagation phase (units $\times 10^{-3}/\text{min}$) were calculated. Day to day reliability of whole plasma lipid oxidation kinetics within subjects was very high. Data collected from the 44 subjects during the summer season indicated that the lag time and propagation rate are stable (t-test $P > 0.05$) and reliable (correlation from day 1 to day 2, $r = 0.93$ for lag time, and $r = 0.81$ for propagation rate). The intra-assay coefficients of variation for same day same sample duplicates were: lag time = 6.6% and propagation rate = 10.9%.

Whole Plasma Oxidation Kinetics for Summer and Winter Groups

Plasma oxidation kinetics observed in the present study for the younger and older women in summer and winter are presented in Figure 10 and Figure 11. Results from the 2-way ANOVA with repeated measures for age by season interaction or main effects (age, season) did not indicate any significant differences among means for lag time or propagation rate of oxidation. Specifically, the effects of age or season and the interaction of age by season were not significant ($P = 0.60$, $P = 0.21$, $P = 0.14$, respectively) (Figure 10). The mean lag time (min) measurement for older women in the

summer was 101 ± 30 minutes as compared to the mean winter lag time determined to be 102 ± 27 minutes. Although not significant, the younger women had lag times approximately 12 minutes longer in the summer as compared to the winter (113 ± 44 min vs. 101 ± 43 min). There were no significant differences in propagation rate in either age ($P = 0.28$) or season ($P = 0.87$) effect, nor was an interaction identified to be significantly different ($P = 0.68$) (Figure 11). For propagation rate there was a trend indicating that older women in both summer and winter groups had faster rates of oxidation as compared to younger women. For instance, during the summer older women had propagation rates of 0.0033 (units $\times 10^{-3}/\text{min}$) as compared to the slower rates of younger women calculated as 0.0030 (units $\times 10^{-3}/\text{min}$). This statistically non-significant trend was observed during the winter as well.

Further statistical analyses were performed on the data to address the concern of a potential effect that estrogen intake may have on oxidative parameters. The whole plasma oxidative variables, propagation rate and slope, were analyzed using two-way ANOVA with repeated measures. There were no differences detected between women taking estrogen and women not taking estrogen for either variable (lag time: $P = 0.99$, propagation rate: $P = 0.92$). In particular, summer lag times of women taking estrogen were 102.5 ± 34.3 minutes versus women not taking estrogen 109.8 ± 39.5 minutes. During the winter the mean lag times for women taking estrogen and not taking estrogen were 105.3 ± 38.7 and 100.3 ± 35.7 minutes, respectively. Similar non-significant propagation rate values were found for women during summer and winter seasons.

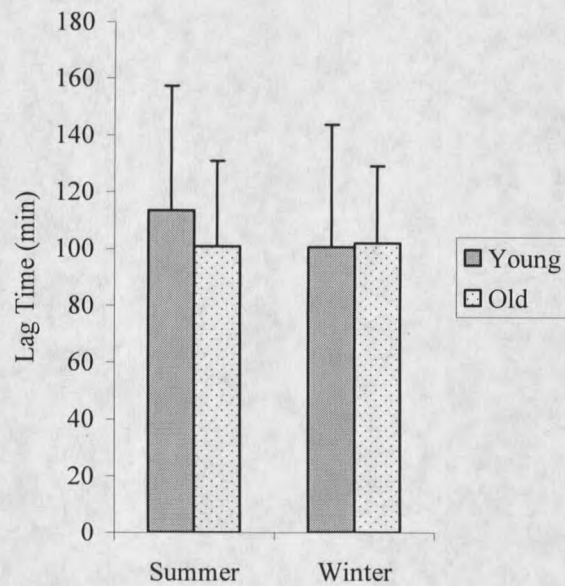


Figure 10. Lag times (min) of women younger and older groups in summer and winter were found to be non significantly different.

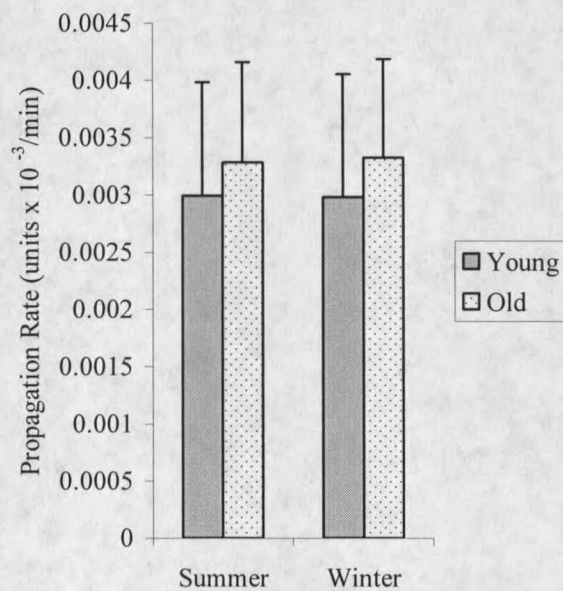


Figure 11. Propagation rates of oxidation for women. No significant differences were identified for propagation rates of oxidation for younger and older groups in summer and winter.

Plasma Lipid Profiles for Summer, Winter and Ill Groups

Thirteen subjects reported an episode of infectious illness for at least 2 days (subjectively rating illness as moderate or severe using the illness surveillance directions provided at enrollment in study). Subjects consisted of two older subjects and 11 younger subjects reporting and providing samples for analysis. Five of the 11 younger women reporting illness provided blood samples during their menstrual cycle. Of the 13 subjects assessed during or post illness 3 subjects were diagnosed by a physician with a staphylococcus aureus bacteria (staph infection), streptococcus pyogenes bacteria (strep throat), or the influenza virus. Four of the 13 ill subjects experienced 2 or more days of moderate to severe flu like symptoms (i.e. nausea, aches, chills and/or fever). The remaining 6 ill subjects reported at least 2 or more days of moderate to severe cold-like symptoms. Of the 13 ill subjects, 4 subjects reported illness between August and mid-October. The other 9 participants reported illness between November and February. All measured plasma lipids for younger and older summer, winter and ill groups are illustrated in Figures 12 - 14.

Plasma cholesterol concentrations were significantly higher ($P = 0.03$) for the subjects during the summer (195.5 ± 32.7 mg/dl) as compared to plasma cholesterol levels post-infectious illness (181.2 ± 30.8 mg/dl) (refer to Figure 12). Plasma LDL was lower ($P = 0.02$) during illness compared to summer assessments. For instance, higher levels of plasma LDL during the summer was measured (125.1 ± 29.2 mg/dl) compared to plasma LDL levels during a period of acute illness (110.0 ± 21.3 mg/dl) (see Figure

13). Figure 13 illustrates that plasma TGs, HDL, and VLDL cholesterol concentrations were not significantly ($P = 0.10$, $P = 0.31$, $P = 0.95$) influenced by summer, winter or illness assessments for the 13 subjects reporting a period of illness during the 9 month investigation.

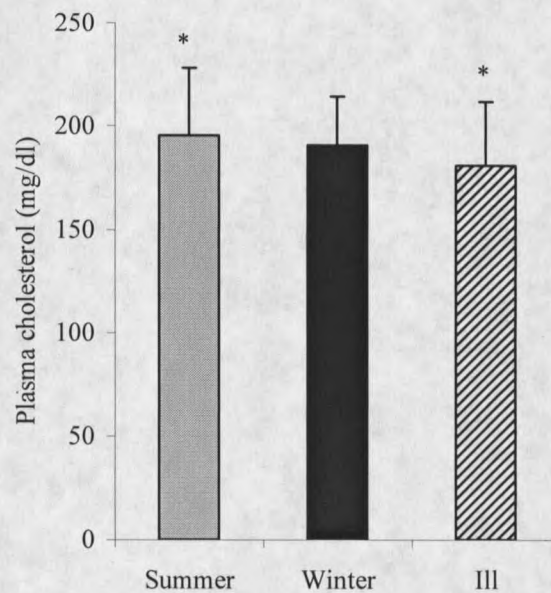


Figure 12. Plasma cholesterol concentrations of 13 subjects reporting illness. * $P < 0.05$ between means of summer and ill assessments.

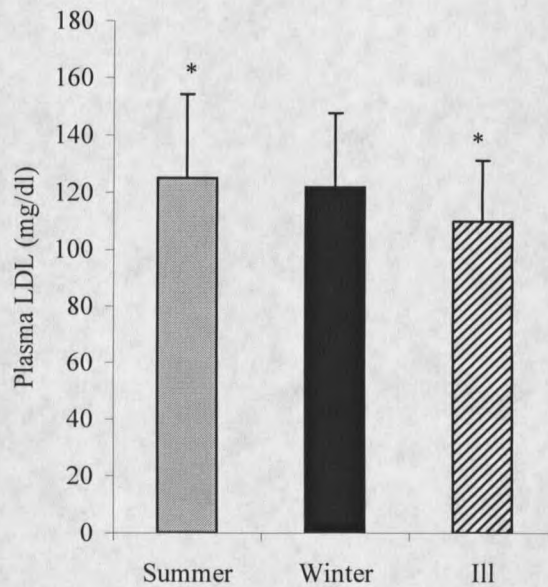


Figure 13. Plasma LDL concentrations of 13 subjects reporting illness. * $P < 0.05$ between means of summer and ill assessments.

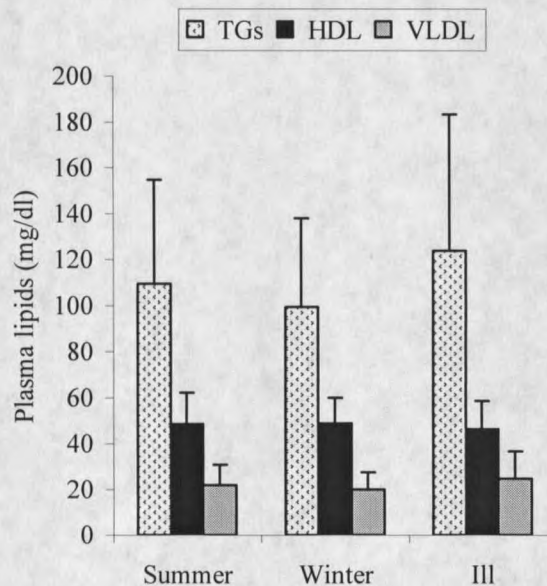


Figure 14. Plasma TGs, HDL, and VLDL concentrations of 13 subjects reporting illness. No significant differences were detected between summer, winter or ill conditions.

Fibrinogen and CBC for Summer, Winter, and Ill Conditions

Inflammatory markers were measured for the 13 subjects who experienced an acute illness (refer to Table 5). As expected, concentrations of plasma fibrinogen were significantly ($P = 0.0001$) influenced depending on illness. Specifically, the mean variability of fibrinogen level was higher ($P < 0.001$) during periods of infection as compared to either summer assessments or winter assessments. Additionally, plasma concentrations of hemoglobin were significantly ($P = 0.02$) different depending on illness, summer and winter assessments. Furthermore, post hoc testing indicated that the majority of this variability ($P = 0.018$) occurred between periods of summer and winter assessments. Subjects experiencing illness revealed significantly higher ($P < 0.001$) WBC counts as compared to summer or winter assessments. Women experiencing illness revealed higher ($P < 0.01$) neutrophil concentrations as compared to summer or winter assessments. Furthermore, subjects experiencing illness revealed higher ($P < 0.05$) monocyte levels as compared to summer or winter assessments. No significant ($P = 0.15$) mean differences of lymphocyte were detected between summer, winter or ill assessments. Likewise, no significant ($P = 0.69$) mean differences were identified for platelets in response to summer, winter, or ill conditions. This data reflects a period of illness from 13 subjects' subjective reporting of an acute illness or infection.

Table 5. Fibrinogen and CBC measurements of women reporting an illness.

	<u>Summer</u> (n=13)	<u>Winter</u> (n=13)	<u>Ill</u> (n=13)
Fibrinogen (mg/dl) [†]	255.6 ± 35.5 ^a	260.5 ± 34.7 ^b	308.5 ± 43.6 ^{ab}
Hemoglobin (g/dl) [†]	14.7 ± 0.8 ^a	14.3 ± 0.7 ^a	14.6 ± 0.9
WBC (K/mm ³) [†]	5.0 ± 1.2 ^a	4.9 ± 1.2 ^b	6.2 ± 1.6 ^{ab}
Neutrophils (K/mm ³) [†]	2.8 ± 0.7 ^a	2.8 ± 1.0 ^b	4.0 ± 1.6 ^{ab}
Lymphocytes (K/mm ³)	1.7 ± 0.6	1.5 ± 0.5	1.6 ± 0.5
Monocytes (K/mm ³) [†]	0.4 ± 0.1 ^a	0.4 ± 0.1 ^b	0.5 ± 0.2 ^{ab}
Platelet Count (K/mm ³)	254.2 ± 49.3	249.8 ± 33.9	241.7 ± 53.5

Values are means ± standard deviation. [†] P < 0.05 main effect for illness. From Tukey HSD Post-hoc test: ^a P < 0.05 between means with like superscript letters. ^b P < 0.05 between means with like superscript letters.

Whole Plasma Oxidation Kinetics for Summer, Winter and Ill Groups

Plasma oxidation kinetics observed in the present study for the 13 women assessed at summer, winter, and ill conditions are presented in Figure 15 and Figure 16. No significant effects of summer, winter, or ill conditions resulted in mean differences in either lag time (P = 0.17) or propagation rate of oxidation (P = 0.65). In regards to lag time, during periods of illness subjects tended to have shorter lag times (108.2 ± 26.0 min) as compared to winter lag times (118.2 ± 26.3 min) or summer lag times (119.9 ± 27.9 min), although not statistically different.

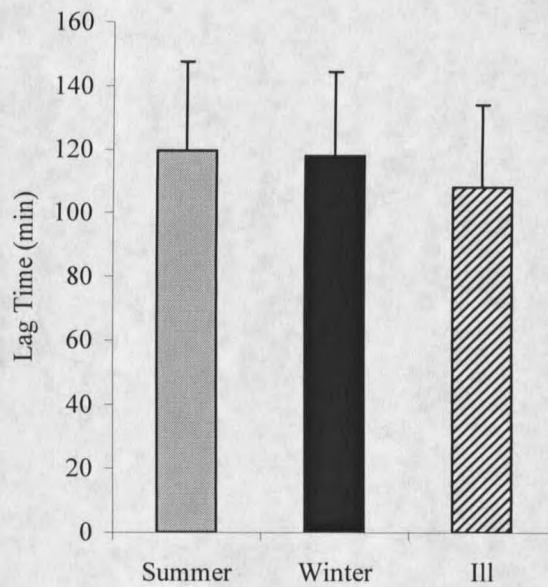


Figure 15. Lag times (min) of 13 subjects reporting illness. No statistically significant differences were detected in lag time.

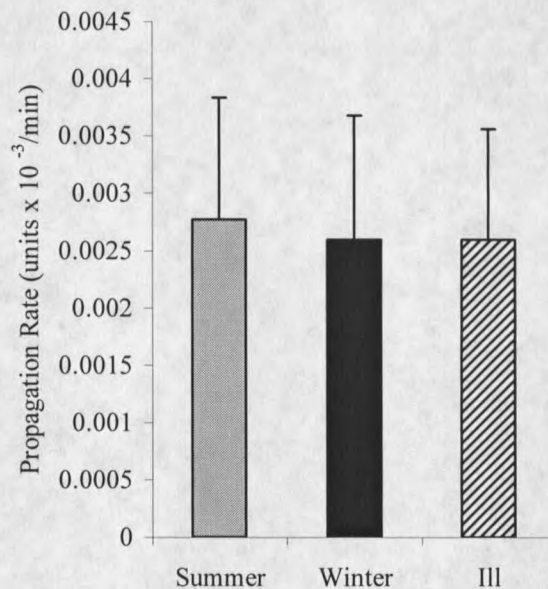


Figure16. Propagation rates of 13 subjects reporting illness. No statistically significant differences were detected in propagation rate.

CHAPTER 5

DISCUSSION

Previous investigations provide strong evidence in support of the hypothesis that oxidation of lipoproteins leads to atherosclerosis. However, there is a lack of research concerning factors that influence the susceptibility of lipoproteins to oxidation in human models. The goals of this longitudinal 9-month investigation were to determine whether age, seasonal variation, or response to illnesses had a significant influence on oxidative susceptibility of whole plasma lipoproteins in a group of healthy women living in Montana. Specific hypotheses of this study were to determine whether oxidation of whole plasma lipoproteins (1) increased in the winter compared to the summer months, (2) increased during incidences of an infectious illness, and (3) was greater in older (≥ 50 yr, $n = 19$) compared to younger (18 - 40 yr, $n = 23$) women. The lag time for the initiation of conjugated diene formation and the propagation rate of conjugated diene formation in diluted whole plasma exposed to CuCl_2 *in vitro* were used as indices to measure the susceptibility of whole plasma lipoproteins to oxidation. Additionally, inflammatory markers, such as the acute phase protein fibrinogen and white blood cell counts, estimates of dietary intake, and physical activity were measured to help explain oxidative results.

Whole Plasma Oxidation and Influence of Season

The first purpose of this study was to assess the influence of the season (summer vs. winter) on whole plasma lipid oxidation independent of age and infection. The data did not support the hypothesis that whole plasma lipoprotein oxidation may be influenced seasonally. In the sample of 43 healthy women, no interaction or main effects for whole plasma oxidative markers were observed. The period of antioxidant protection measured for older women in the summer was very comparable to the mean period of antioxidant protection in the winter. Although not statistically significant, the younger women had longer periods of antioxidant protection (approximately 12 minutes) in the summer as compared to the winter assessments. No significant differences existed between summer or winter assessments in the propagation rate of lipid peroxide formation.

The results of the present study are in agreement with the study conducted by Van de Vijver et al. (1997). Specifically, no seasonal change for lag time or propagation rate of copper induced lipoprotein oxidation was reported by these investigators. Van de Vijver's group measured parameters of oxidizability of LDL in contrast to the measurement of whole plasma lipoproteins in the present study. During a period of one year, these investigators collected blood from 10 participants at four different times (February, May, September, and December). The subjects consisted of 6 men and 4 women who had a mean age of 39.6 ± 7.4 years. Two of the subjects were current smokers and two of the subjects had only 3 blood collections. The present study supports the conclusions of Van de Vijver, that there appears to be no seasonal pattern in

lipoprotein oxidation in terms of lag time and propagation rate of conjugated diene formation.

One limitation of the present study and that of Van de Vijvers et al. was the small number of sampling time points. One recommendation for future research designs concerning the influence of seasonality on the susceptibility of lipids to oxidation includes increasing the number of blood collections during a year (e.g. monthly). During the period of one year, two to four periods of blood collections may not be reflective of an individual's response to a true seasonal rhythm/pattern. A blood collection in July may actually be a period preceding an August trough in lipoprotein oxidative status. Whereas, a February collection may be the month following the actual lipoprotein oxidative peak of an individual occurring in January. Therefore, additional blood collection periods should be included into future experimental designs concerning seasonal variability on interested oxidative variables.

A variety of parameters were measured in this investigation with the intent to acquire a better understanding of oxidative differences of lipoproteins that may have been revealed in response to seasonal variation. One reason that we hypothesized that susceptibility to oxidation may increase in the winter was because of a potential decrease in physical activity and/or consumption of fruits and vegetables rich in antioxidants. Only a few of these measurements were influenced seasonally. For instance, the water soluble antioxidant vitamin C was the only dietary nutrient that was affected in response to the season. This particular vitamin was estimated to be consumed in lower amounts during the winter as opposed to summer months. The potential antioxidant properties of

vitamin C apparently did not influence the oxidative status of whole plasma lipids between summer and winter assessments significantly.

Fibrinogen concentrations did not change seasonally, neither did plasma lipid levels. This may be one of the reasons why this particular group of women did not demonstrate a seasonal pattern of increased oxidation during the winter. Fibrinogen has been documented to increase during winter months and is typically reflective of increased inflammatory activity (Crawford et al., 2000; Stout & Crawford, 1991; Woodhouse et al, 1994). Other measurements such as anthropometric characteristics, estimations of other selected dietary intakes, or estimations of time spent in physical activity did not change between the summer assessment and winter assessment. Taken as a whole, the relatively consistent values of these potential factors affecting oxidation status of lipids did not change between summer and winter assessments. Thus, the consistent response of these variables helps explain the similar oxidative values measured at summer and winter.

The lack of change in fibrinogen concentrations from summer to winter is not consistent with the study conducted by Crawford et al. (2000). Crawford et al. (2000) concluded that fibrinogen does vary seasonally with the potential to increase CD risk in winter months. They detected a relationship between elevated fibrinogen levels and seasonal variation with a definite winter peak occurring in mid-February. In this investigation 68 subjects had at least seven monthly measurements for fibrinogen (24 of the subjects had 12 consecutive monthly readings for fibrinogen). An advantage to the experimental design of Crawford's study was the multiple fibrinogen measurements taken during the one year investigation.

The inability or failure to show a seasonal influence on fibrinogen levels in the present study may indicate the lack of adequate assessments during the 9-month investigation. Due to financial limitations the present study was limited to two fibrinogen measurements, and this number of assessments did not identify significant differences in fibrinogen levels between seasons. As a result of the documented seasonal rhythm in human population for fibrinogen, future investigations should incorporate more frequent assessments of fibrinogen and/or other inflammatory markers when attempting to identify seasonal factors associated with infection or MI.

Whole Plasma Oxidation and Influence of Infectious Illness

The second hypothesis, concerning a potential increase in whole plasma susceptibility to oxidation during an episode of acute infectious illness was not supported by the data. Thirteen women in the study were assessed during an infectious illness during the 9-month investigation. The increase susceptibility of whole plasma lipoproteins to oxidation in response to periods of illness was not found to be statistically significant in the calculated oxidative indices (lag time or propagation rate of oxidation). In regards to the lag time, whole plasma lipid samples of ill subjects tended to be approximately 10 minutes shorter ($P = 0.17$) as compared to assessments of whole plasma when illness was not present.

As would be expected during conditions of an infectious illness, fibrinogen and leukocyte concentrations were elevated and reflected states of active infection in subjects during illness assessments, providing quantitative evidence of the existence of an active

infection. This study found a strong influence on selected inflammatory markers in response to periods of acute illness. Specifically, fibrinogen, hemoglobin, WBC, neutrophils, and monocytes were all found to be significantly higher when compared to states of non-infection (summer or winter assessments).

The data demonstrating a trend for decreased resistance of whole plasma to oxidation in response to a period of acute illness suggest that an increased generation of free radicals may have occurred in the blood. Although no human studies have been conducted to assess lipid oxidation and infection, these results are in agreement with the findings of Mileva, Bakalova, Tancheva, Galabov, & Ribarov, (2002). These investigators infected mice with the influenza virus and observed at least two-fold higher ($P < 0.001$) amounts of conjugated diene formation in the infected mice compared to that of control mice, reflecting lipid peroxidation. Collectively the findings from the present human model and other animal models suggest that during conditions of infections, the increase production of radicals during infections may result in lipid oxidation (Kalayoglu et al., 1999; Memon et al., 2000; Mileva et al., 2002).

A variety of parameters were measured in the present study with the intent to acquire a better understanding of oxidative differences of lipoproteins that may have been identified in response to an infectious illness. Periods of reported illness revealed differences in plasma cholesterol and LDL profiles. Of the plasma lipids measured, plasma cholesterol and LDL concentrations of the 13 women experiencing an infectious illness were found to be significantly lower during periods of illness. Decreased plasma cholesterol and LDL maybe be reflective of a characteristic response to infections.

Fernandez-Miranda, Pulido, Carrillo, Larumbe, Gomez, Ortuno et al., (1998) determined that plasma cholesterol and LDL concentrations decrease in patients with HIV infection. This lipid response provides additional mechanistic information in the infection base oxidative pathway where lipids are needed as substrates to yield lipid peroxides (Miller et al., 1998).

What may have been a significant response in decreased lag time (antioxidant protective phase) may have been off set by the significant decrease in plasma cholesterol in the subjects reporting illness. The data provide confounding results in terms of the oxidative theoretical framework that has been published. It was hypothesized that the susceptibility of whole plasma lipoprotein oxidation would increase due to the increased production of free radicals oxidatively modifying lipoproteins in response to an infectious illness. However, subjects reporting illness had lipid profiles with lower concentrations of plasma cholesterol and LDL. These findings help explain for the non significant differences of oxidation between states of illness or non-illness. In particular, Miller et al. (1998) explained that lipoproteins (found in lipids such as LDL) are substrates necessary to yield lipid peroxides. Thus, a decrease in lipid substrates will decrease the susceptibility of lipids to oxidation. These results suggest that the response to infectious illness may be key factor that influences lipid profiles and oxidative parameters and warrants further investigation.

Whole Plasma Oxidation and Influence of Age

Results addressing the influence of age on oxidative stress did not support the third research hypothesis. In particular, the susceptibility of whole plasma lipid oxidation was not significantly greater for older women (61 ± 8 years) compared to younger women (29 ± 7 years). However, there was a trend indicating that older women in both summer and winter groups demonstrated faster rates of oxidation as compared to the slower rates of oxidation of younger women. This effect of age on the response to mean oxidation rate was not found to be statistically significant.

The lack of significant difference between younger and older women in the present whole plasma oxidation investigation is not consistent with previous data concerning age and lipid oxidation. In a sample of 100 healthy men and women (20 to 70 years of age) serum lipid peroxides were measured (Miquel et al. 1998). These investigators showed that there was an age related increase in lipid peroxide. However, lag time and propagation rate were not the variables measured to reflect lipoprotein oxidation as was employed in the present study.

Mosinger et al. (1997) observed the effect of age on indices of LDL oxidation in women (36 pre-menopausal, 12 post-menopausal). The post-menopausal women had increased ($P < 0.01$) amounts of conjugated diene formation as compared to pre-menopausal women. However, the findings of Mosinger et al. did not reveal differences in lag times or propagation rates between the young and old women. These findings were in agreement with the results of the whole plasma investigation.

In the present project, other variables were measured to help identify differences in oxidative parameters influenced in response to the subject's age. As a whole both the young and old subjects were very similar in terms of most anthropometric characteristics. However, older women had body fat percentages that were significantly higher than the group of younger women. This characteristic appears not have a significant impact on the oxidative variables measured in young and old women.

Selected dietary intakes were found to be different depending on the age of subjects. For instance, energy intake and carbohydrate consumption were found to be higher for younger women as compared to the group of older women. Similarly, the lipid soluble antioxidant vitamin E intake was consumed at higher amounts in the older group of women as compared to the younger women.

A high intake of vitamin E from foods or supplements may inhibit lipoprotein oxidation by affecting the theorized oxidative mechanism. For instance, Jialal et al. (1995) concluded that supplementation of at least 400 IU/day of vitamin E was sufficient to decrease the susceptibility of LDL to oxidation. In the current study, the older women reported vitamin E intakes to be 11.6 ± 11.1 mg/d at summer assessments and 9.7 ± 5.7 mg/d at winter assessments (1 mg = 1.5 IU). This information provides a possible rationale as to why older women were comparable to younger women in terms of oxidative susceptibility to whole plasma lipoproteins. Although the older women reported high intakes of vitamin E, the amounts of vitamin were not sufficient enough to affect the susceptibility of whole plasma lipoproteins to oxidation.

This study detected differences between young and old women for plasma lipid profiles regardless of season. Plasma cholesterol and plasma HDL concentrations were found to be lower in the younger group of women compared to older women. Mosinger et al. (1997) assessed various risk factors of CD like age, gender, and cholesterol concentrations. Consistent with the present study, Mosinger's group found that the post-menopausal as compared to pre-menopausal women had increased levels of plasma cholesterol.

The data indicated that younger women had lower concentration of the acute phase protein, fibrinogen, and higher levels of hemoglobin as compared to the older group of women. No other marked differences between young and old women were found with respect to descriptive and anthropometric, estimated dietary intakes or time spent in physical activity. Consequently, it was concluded that the subjects in the present study were comprised of both healthy older and younger women. For instance, both groups of women spent similar amounts of time performing physical activity. It appeared that the older women had lipid oxidative values similar to younger women. This finding may be a consequence of the benefit of the older women engaging in regular physical activity in comparable amounts as younger women.

Vasankari et al. (1998, 2000) and Ginsburg et al. (2001) provide strong evidence supporting the rationale that habitual physical activity provides cardiovascular benefits. These benefits are characterized with lipid profiles reflecting decrease in cholesterol and LDL concentrations and increase in HDL. Additional cardiovascular benefits from regular participation in physical activity include improved lipid oxidative status, in terms

of longer antioxidant protection periods and slower rates of oxidation. These studies demonstrated that physically active individuals have decreased lipoprotein oxidative values. For instance, Vasankari et al. (1998) reported that an exercise program of 3-5 hours per week for 10 months was sufficient to reduce oxidized LDL by 14 and 18% in men and women who have been sedentary. The results of the present study indicate that the volunteers were individuals who may have adapted to the oxidative benefits of performing physical activity. The comparable results of oxidative parameters between the younger women and older women may be attributed to the healthy lifestyles of the subjects.

In addition to the present study, data suggesting that physical activity may attenuate the age associated increase in lipid oxidation have been presented by other researchers (Succari, Meneguzzo, Ponteziere, Boreis, Devanlay, Desveaux, et al., 1997) assessed markers of oxidative stress in fit, health-conscious elderly people. Lipid peroxidation index and antioxidant indicators were measured in 193 (103 men and 90 women), ages 70 – 89 years. They determined that markers of oxidative stress were not influenced by old age when healthy lifestyles and nutritional status are habitual patterns, as in the case of their population. Furthermore, Kostka, Draai, Berthouze, Lacour, & Bonneoy (2000) made similar findings in 26 healthy active elderly men. These investigators compared the older men with 12 younger men and concluded that indicators of anti-oxidant defense were not lower. In particular, the amounts of lipid peroxides, and antioxidant enzymes were measured in both younger and older group of men and were reported to be similar. These studies provide additional evidence to the notion that

regular physical activity can be a factor that improves oxidative profiles (lag time and propagation rate) and concomitantly decrease the risk of CD.

Recommendations for Future Research

The literature suggests that the use of one method of analysis may have been a limitation to present investigation. For instance, a number of protocols have been employed to monitor and measure various substances associated with oxidation. Thus, an improvement to the current study would be to monitor oxidative characteristics with more than one method. Additionally, it would be interesting to first isolate LDL and determine the concentrations per sample. Then both whole plasma and LDL oxidation protocols could be conducted and provide data for comparative analysis of adjusted whole plasma samples and LDL samples. Future research concerned with oxidative stress in humans could utilize a combination of methods to assess this complex system of oxidative stress due to the fact that no universally protocol has been established.

Summary

Cardiovascular disease remains one of the deadliest diseases in the United States. Research is continually being directed towards understanding the mechanistic pathways involved in the progression of specific CD such as atherosclerosis. In particular, intense research has been conducted with the intent to identify specific triggers of plaque formation or destabilization. One area of CD research revolves around the oxidative

hypothesis associated with lipids. When oxidatively modified, lipoproteins are believed to be critical in the mechanism involved with plaque development.

We hypothesized that the time of the year, periods of illness, and age would be factors that change the susceptibility of whole plasma lipoproteins to oxidation. In particular, we hypothesized that infections would increase lipid oxidation based on the inflammatory immune response to infections. For instance, when generally healthy individuals acquire an acute illness, their protective immunological response to help eliminate the infection may inadvertently be producing free radicals. The production of free radicals has been shown to have deleterious effects on various cells within the body. In particular, free radicals may initiate the peroxidation of lipoproteins, which attributes to the injury to cellular membranes, specifically the inner surfaces of arteries. If one can prevent or suppress inflammation, one might be able to lower the risk of atherosclerosis. Therefore, it is important to identify markers involved in this pathway and potentially prevent oxidative damaging during times of inflammation.

The primary goals of this longitudinal investigation were to determine if: (1) the susceptibility of whole plasma oxidation was influenced seasonally, (2) the susceptibility of whole plasma oxidation was influenced during incidence of acute infectious illness, (3) the susceptibility of whole plasma oxidation was influenced by age.

The susceptibility of whole plasma lipoprotein to oxidation was not influenced seasonally, in response to infection, or by age. First, there was no significant difference in the susceptibility of lipoproteins to oxidation when measured during the summer as compared to winter. Second, there was no significant difference in the susceptibility of

lipoproteins to oxidation when measured during an incidence of acute infectious illness as compared to healthy summer or winter assessments. Finally, there was no significant difference in the susceptibility of lipoproteins to oxidation between young women compared to old women.

The reasons for not detecting the hypothesized differences may be due, in part, to the similar physical activity and dietary intake patterns employed through out the investigation independent of season. Similarly, the younger group of women reported time spent in physical activity and dietary logs that were comparable to the older group of women. In essence, the proposed hypotheses were based on the assumption that physical activity and dietary intake in foods rich in antioxidant vitamins would decrease during winter months. The results are reflective of a generally healthy group of participants with comparable periods of oxidative resistance and comparable rates of whole plasma lipoprotein formation.

In regards to infection, the subjects reporting an illness tended to have approximately 10 minute shorter periods of antioxidant protection as compared to summer or winter assessments. Plasma cholesterol and LDL concentrations were found to be lower during periods of illness as compared to healthy assessments. The statistically non-significant influence of infection on lipoprotein oxidation may have been confounded by the decrease in cholesterol and LDL concentrations measured during illness assessments. What might have been a significant decrease in lag time (period of antioxidant protection) during infectious periods may have been offset by the

concomitant decrease in lipoprotein substrates (decrease in plasma cholesterol and LDL concentrations).

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APPENDICES

APPENDIX A
INFORMED CONSENT

SUBJECT CONSENT FORM FOR PARTICIPATION IN HUMAN RESEARCH
MONTANA STATE UNIVERSITY

Study Title: Determinants of LDL-Cholesterol Oxidation

Funding: This study is funded by a grant from Montana's NSF EPSCoR Program

Investigators: Mary P. Miles, Ph.D., Assistant Professor
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Hosaeus 101, Montana State University
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Phone: (406) 994-6678; FAX (406) 994-6314

Shelly P. Hogan, B.S., Graduate Student
Dept. of Health and Human Development
MSU Nutrition Research Laboratory
Bozeman, MT 59717
Phone: (406) 994-5001

You are being asked to participate in a study investigating the influence of age, seasonal variation, and incidence of infection on changing the properties of lipids. Specifically, this study will identify the 'bad' low-density lipoprotein cholesterol (LDL-cholesterol) from analysis of your blood during a 9-month investigation. This study is of interest because changes in the 'bad' cholesterol have been implicated in the development of cardiovascular disease, however the exact mechanisms involved are not completely understood. Therefore, identifying these variables may help to explain how the atherogenic process occurs. This study will assess whether the changes in LDL-cholesterol are affected by age, seasonal variation, and incidence of infection (i.e. common cold) during 9 months. We will be collecting blood during July/Aug and Jan/Feb and when you experience an infectious illness. You will be tested in the Montana State University Nutrition Research Laboratory in Herrick Hall.

The study has the following purposes:

- 1) To determine whether oxidation of LDL-cholesterol increases in younger and older women in the winter compared to the summer months.
- 2) To determine whether there is an increased susceptibility of LDL-cholesterol to oxidize after an infectious episode.
- 3) To determine whether the magnitude of the changes from summer to winter and following an infectious episode differ between younger and older women.

If you agree to participate in this study you will do the following things:

- 1) You will read and sign this document.
- 2) Report to the Nutrition Research Laboratory on the MSU campus twice during both the summer (July/Aug) and winter (Jan/Feb) and when you experience an illness. You will have blood collected for assessments which will include: blood lipids, LDL oxidation and fibrinogen. You will need to fast overnight (12 hours) and refrain from vigorous physical activity for a period of 24 hours prior to testing.
- 3) Your resting blood pressure will be assessed by qualified laboratory assistants using standard blood pressure cuffs.
- 4) Your body composition will be assessed using bioelectrical impedance, a non-invasive procedure that involves application of four electrodes to your wrists and ankles. A small electrical current is used to measure the amount of water in your body, but this current is not dangerous and you will not feel it.
- 5) You will complete a three-day dietary log of food intake that represents your normal eating pattern (including at least one weekend day) during both summer and winter to aid in interpreting your differences if found during the study.
- 6) You will complete a three-day physical activity log in which you designate a categorical activity level, i.e. from categories listing examples of activities relating to intensity of activity, for each quarter hour of the day.
- 7) You will refrain from cigarette smoking, and taking any antioxidant vitamin supplements (i.e. Vitamin E, A, CoQ₁₀, C) during the 9 months of investigation.
- 8) If you experience an infectious illness during the 9-month study (i.e. upper respiratory tract infection, common cold) you will report to the Nutrition Research Laboratory within 5 to 12 days following the onset of the symptoms for blood collection. However, you will not be asked to report to the testing facility more than twice as a result of infection during the 9-month enrollment. We would like you to see your physician to receive a diagnosis when you experience an illness, but this is not required.

Sometimes there are side effects from having blood drawn. These side effects are often called risks, and for this project, the risks are:

Approximately 4 teaspoons (2 tubes) of blood will be removed by putting a needle in your vein on at least four, but not more than six, occasions. This is the standard medical method used to obtain blood for tests. There is momentary pain at the time the needle is inserted into the vein, but other discomfort should be minimal. In about 10% of the cases there is a small amount of bleeding under the skin, which will produce a bruise. The risk of infection is less than 1 in 1,000. The pain and bruising is usually mild and a person trained in blood drawing will obtain your blood.

There may be benefits from your participation in this study. These are:

- 1) Information about your personal blood lipid profile (i.e., LDL-cholesterol levels) and body composition. However, you should be aware that these are research values and that you should contact your personal physician if you would like interpretation or actual clinical analysis of your blood lipids.
- 2) Exposure to a protocol for studying the determinants of LDL-cholesterol oxidation, along with an increased awareness of the possible factors linked to cardiovascular disease.
- 3) You will learn how to assess energy and nutrient intake from recording a three-day dietary log.

No other benefits are promised to you.

Confidentiality: The data and personal information obtained from this study will be regarded as privileged and confidential. A code number will identify the data that we collect from you, and all data will be kept in locked offices in the Nutrition Research Laboratory or in Dr. Miles' locked office. The information obtained in this study may be published in scientific journals, but your identity will not be revealed. They will not be released except upon your written request/consent. If during the study you decide to cease your participation, your name will be removed from our study records, and we will not contact you again regarding this study. You will not be penalized in any way.

You will not incur any costs beyond those related to your transportation to and from the Nutrition Research Laboratory at MSU as a result of your participation. If you choose to see your physician for diagnosis of illness when you experience symptoms, you are entirely responsible for charges incurred.

Freedom of Consent: You may withdraw consent in writing, by telephone, or in person with the investigator (Mary Miles at (406)994-6678 or Shelly Hogan at 994-5001) and discontinue participation in the study at any time and without prejudice or loss of benefits (as described above). Participation is completely voluntary.

In the event your participation in this research supported by MONTS results in injury to you, medical treatment consisting of basic first aid and assistance in getting to Bozeman

Deaconess Hospital will be available, but there is no compensation for such injury available.

Further information about this treatment may be obtained by calling Mary Miles at 994-6678.

You are encouraged to express any questions, doubts or concerns regarding this study. The investigator will attempt to answer all questions to the best of her or his ability. The investigator fully intends to conduct the study with your best interest, safety and comfort in mind. *The Chairman of the Human Subjects Committee, Mark Quinn can answer additional questions about the rights of human subjects at 406-994-4706.*

STATEMENT OF AUTHORIZATION

Study Title: Determinants of LDL-Cholesterol Oxidation

AUTHORIZATION: I have read the above and understand the discomforts,
inconvenience and risk of this study. I, _____

(PRINT YOU NAME), agree to participate in this research. I understand that I may later
refuse to participate, and that I may withdraw from the study at any time. I have received
a copy of this consent form for my own records.

Signed: _____
Subject's Signature

Date: _____

Witness: _____
(if other than the investigator)

Date: _____

Investigators: _____
Mary Miles or Shelly Hogan

Date: _____

APPENDIX B

THREE-DAY PHYSICAL ACTIVITY RECORD

Activity Codes for the Bouchard Three Day Physical Activity Record

Category of activity	Example of activity for each category	Approximate energy expenditure (kcal/kg/15 min)
1	Lying down: - sleeping - resting in bed	0.26
2	Seated: - listening in class - eating - writing by hand or typing - reading - listening to the radio or T.V. - taking a bath	0.38
3	Standing; light activity: - washing oneself - shaving - combing hair - dusting - cooking	0.57
4	Getting dressed Taking a shower Driving a car Taking a walk (strolling)	0.70
5	Light manual work: - housework (washing windows, sweeping etc.) - tailor - baker - printer - brewer - cobbler - mechanic - electrician - painter - lab-work - carpentry - masonry - driving a farm tractor - cleaning trees - working in the chemical or electric industries - feeding animals on a farm - doing the bed Riding a moped Moderately quick walking (going to school, shopping)	0.83
6	Light sport or leisure activities: - light canoeing - volleyball - table tennis - baseball (except the pitcher) - golf - rowing - archery - ninepins - croquet - sailing - cycling (leisure)	1.20
7	Moderate manual work: - machine operating (building industry) - repairing a fence - loading bags or boxes - plantation work - forest work (machine sawing and log handling) - mine work - shoveling snow	1.40
8	Moderate sport or leisure activities: - baseball (pitcher) - badminton - canoeing - cycling (race bike) - dancing - tennis - jogging (slow running) - horseback riding - Alpine skiing - cross-country skiing (leisure) - swimming - gymnastics - brisk walking	1.0

Category of activity	Example of activity for each category	Approximate energy expenditure (kcal/kg/15 min)
9	Intense manual work: - felling a tree with an ax - sawing with a hand-saw - working with a pitchfork (on a farm) - cutting tree branches Intense sport or leisure activities: - running in a race - boxing - mountain-climbing - squash - cross-country skiing - ice hockey - basketball - football - racquetball	1.95

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Date: / /
 day month year

	Minute	0-15	16-30	31-45	46-60
	Hour				
Last name: _____	0				
First name: _____	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
	11				
	12				
	13				
	14				
	15				
	16				
	17				
	18				
	19				
	20				
	21				
	22				
	23				

In each box, write the number which corresponds to the activity which you have carried out during this 15 minute period. Please consult the activity card that follows to establish the proper coding. If an activity is carried out over a long period (e.g. sleeping) you can draw a continuous line in the rectangular boxes which follow until such a time when there is a change in activity.

To understand this better, we suggest that you take a look at the example that follows.

APPENDIX C

THREE-DAY DIETARY RECORD

Directions for using the 3 - Day Food Diary:

1. Select 3 days to record all foods and beverages consumed.
Select 2 weekdays and 1 weekend day to provide a more accurate picture of normal eating habits.
2. Keep your food record current. List foods immediately after they are eaten. Do not wait until the end of the day to record entries. Please print all entries.
3. Be as specific as possible when describing the food or beverage: the way it was cooked (if it was cooked) and the amount that was consumed -for example: Chicken breast, skinless, grilled
4. Include brand names or name of restaurant whenever Possible.
5. Estimate amount -for example: cups, ounces, tablespoons, 1 deck of playing cards, etc...
6. Report only the food portion that was actually eaten -for example: T -bone steak, grilled, size of 1 deck of playing cards
7. Include method that was used to prepare the item -for example: fresh, stewed, broiled, fried, baked, canned, boiled, braised, raw, etc...
8. For canned foods, include the liquid in which it was canned- for example: peaches in heavy syrup, tuna in oil, pears in light syrup.
9. Do not alter your normal diet during the period you keep this record.
10. Remember to record the amounts of visible fats (oils, butter, salad dressings, margarines, sauces & condiments, etc.) you eat or use in cooking.
11. Whenever possible staple or paper clip the food or beverage label with nutritional information on it.

[illegible]

APPENDIX D

INFECTIOUS ILLNESS LOG SHEET

Instructions for Rating Daily Health/Illness Symptoms:

- The Illness Surveillance Logbook is used to record your daily health/illness over the nine-month study. The logbook is divided into six categories of health/illness.
- Each day rate your health/illness on a scale of 1-3; #1 – Mild, #2 – Moderate, and #3 – Severe. Base the illness severity on previous illness experiences. To understand this better, we suggest that you take a look at the example that follows.
- If your illness symptoms are moderate to severe, please report to Shelly at 994-5001. During illness days, if symptoms persist for more than two days, we ask that you see your physician. However, this is not required.
- Monthly Health/Illness Surveillance will be mailed to you along with a self-addressed envelope. Please mail the completed month log sheet to Shelly and continue logging your health/illness symptoms for the current month.

		ILLNESS SURVEILLANCE LOG - JULY																														
Name:		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1) No Health Problems today																																
2) Cold Symptoms																																
(runny stuffy nose, sore throat,																																
coughing, sneezing, colored discharge)*																																
3) Allergy																																
(itchy eyes, stuffy nose, clear discharge)*																																
4) Flu Symptoms																																
(fever, headache, general aches and pains,																																
fatigue and weakness, chest discomfort,																																
cough)*																																
5) Nausea, Vomiting, and/or Diarrhea*																																
6) Other Health Problems																																
coughing,																																

** If symptoms are moderate to severe, please contact Shelly at 994-5001.

APPENDIX E
BIS DATA SHEET

Subject ID _____ Date _____
 Age _____ yrs Body Mass(TM) _____ kg Height _____ cm
 Distance between electrodes on the arm _____ cm, on the leg _____ cm
 Extracellular H₂O (E) _____ L Total H₂O (T) _____ L
 Intracellular H₂O (I) _____ L Fat-Free Mass (L) _____ kg
 Fat Mass (FM) = TM -FFM = _____ kg %BF = ($\sim M/TM$)x100 = _____ %

Patient Preparation:

- Patient should NOT have recently eaten a large meal (within 3-4 hours).
- Patient should NOT have exercised above a light intensity within the previous 2-3 hours.
- Patient should be fully hydrated prior to arriving at the lab.
- Ideally, patient should be lying in a supine position for at least 5 minutes prior to testing.
- Patient with known electrolyte imbalances should NOT be tested.

Electrode Placement:

Needed materials -1 sheet of 4 electrodes, 2-3 disposable alcohol pads, a disposable shaving razor (for removing skin hair), and rubber gloves.

- First, have the patient recline on a level surface in the supine position with the legs and arms slightly apart (no extreme abduction or adduction of limbs).
- Next, shave and then clean (with alcohol pad) the intended electrode sites with the alcohol pads. Use rubber gloves during this process...
- Repeatable placement of electrodes for repeat measurements is absolutely critical! Place electrodes as follow:

- #1: Anterior ankle, in line with widest malleolar breadth (red lead).
- #2: Anterior foot, parallel with #1, 3-8 cms distal from #1 (black lead).
- #3: Posterior wrist, in line with widest breadth at the ulnar and radial styloid processes (red lead).
- #4: Posterior hand, parallel with #3, 3-8 cms distal from #3 (black lead).

BIS Analyzer -The Hydra (Xitron Technologies, Model 4200):

- Turn the white power button to "On".
- Hit "Menu" button once and enter an ID number (use the last 5 digits of your student ID number). Hit "Enter" after putting in the ID number.
- In response to "OBJECT?", use the up and down arrow buttons until you see the option "ECF and ICF". Hit "Enter" to select this option.
- In response to "METHOD?", select the option "WB by WRIST -ANKL".
- In response to "MODE?", select the option "SINGLE MEAS."
- For gender, use the arrow keys to get either "MALE" or "FEMALE" and hit "ENTER".
- For height, use the arrow keys to increase or decrease the number to your height in cms, hit "ENTER" to save.
- For weight, increase or decrease the number and hit "ENTER" to save.
- Lastly, when the patient is all ready, hit the "START" button.
- The Hydra will say "MEASUREMENT In Progress..." For 20-30 seconds, and "DATA MODELING In Progress..." for another 30-60 seconds.
- If the Hydra takes longer than 3 minutes, hit "STOP" because something is wrong. If something does go wrong, simply turn the Hydra off and start all over again...

APPENDIX F

SOLUTIONS AND BUFFER RECIPES

1. Copper Chloride Solution

- Mix $[\text{CuCl}_2]$ with PBS to 300 μM
 - a. CuCl_2 : $170.5 \text{ g/mol} \times 0.0003 \text{ mol/L} = 0.05115 \text{ g/L CuCl}_2$
 - b. Weigh approximately 0.0025 – 0.0050 g of CuCl_2 and add to 100 mL beaker
 - c. Calculate volume of PBS to be added to beaker with CuCl_2
 - i. _____ g of $\text{CuCl}_2 \div 0.05115 \text{ g/L} =$ _____ L of PBS *

*multiply by 1000 to convert to mL (approximately 75mL of PBS)

2. PBS (Phosphate Buffer Saline) Buffer Recipe

0.01 M PBS, 0.16 M NaCl, pH 7.4

Prepare solutions A and B

- Solution A: 0.2 M NaH_2PO_4 (sodium phosphate monobasic)
 - o 23.992 g/L anhydrous NaH_2PO_4
 - o 2.3992g anhydrous NaH_2PO_4 + 100 mL distilled/deionized water
 - o (or) 1.199g NaH_2PO_4 + 50 mL water
- Solution B: 0.2 M Na_2HPO_4 (sodium phosphate dibasic)
 - o 28.392 g/L anhydrous Na_2HPO_4
 - o 5.678g Na_2HPO_4 /200 mL water distilled/deionized
 - o (or) 2.839g Na_2HPO_4 /100 mL water
- Make 5 times more solution B than solution A
- Mix together:
 - o 9.5 mL Solution A
 - o 40.5 mL Solution B
 - o 9.35 g NaCl
- Add the 50 mL (A+B+NaCl) solution to 850 mL of distilled/deionized water.
- Adjust the pH to 7.4 using pH analyzer/reader
 - If too basic add drops of HCl acid
 - If too acidic add drops of NaOH
- Bring total volume of solution to 1000 mL with deionized/distilled water
- Label, date, and cover the flask with parafilm

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