Health and lifestyle profiles across the lifespan: results from the Louisiana Healthy Aging Study

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HEALTH AND LIFESTYLE PROFILES ACROSS THE LIFESPAN: RESULTS FROM THE LOUISIANA HEALTHY AGING STUDY

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Department of Kinesiology

by

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B.S., Louisiana State University, 1995
M.S., Louisiana State University, 1999
December 2005
Dedication

I would first like to thank my family. This has been a long road with many bumps and obstacles and I truly appreciate all of your love and support.

I would also like to thank all my friends, especially, my girls. You have always stood by me over the years, no matter what. I want to thank you for always keeping me grounded and not holding the fact that I am a science nerd against me.

To Leonie, Leanne, and Enette, there are few women that I have met since I was a child that I consider good friends. You understand what I have gone through with all of this. Thanks for you ears and your shoulders. Thank you for reminding me that no matter how hard it got, I was going to make it. I am thankful for having such strong women to look up to.

I would also like to thank Eric. Thank you for teaching me to always strive for the best and never settle. Thank you for all of the opportunities that you have given me. I truly could not have done this without you.

To Melinda, you were there in the beginning. And I can tell you, that had I not met you when I did, I would not be writing this today. Thank you for all of your guidance and support.

Thank you Darlene, as busy as Eric keeps you, you never hesitated to help me with anything that I needed. Thank you for letting me confde in you. I truly appreciate that.

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Finally, the one thing I have learned from this project on healthy aging is that you have to surround yourself around good people who love, support, and always want the best for you. I have finally found that.

I dedicate this to all of you….
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Abstract

Aging and age related disease affects individuals differently. One possible explanation could be free radical production varies among individuals and this variation determines the aging process and the progression of disease. The purpose of this study was to test whether nonagenarians have a relatively low metabolic rate when compared to younger individuals and whether this low metabolic rate is associated with lower levels of oxidative stress and less incidence of disease. In addition, we predict that older individuals will have higher levels of physical activity and this will be associated with higher physical functionality. Resting metabolic rate (RMR), markers of oxidative stress to lipids, proteins, and DNA, components of the metabolic syndrome, and physical activity level were measured in 3 groups of individuals aged 20-34 (16M/25F), 60-74 (16M/11F), and ≥90y (23M/25F). RMR, adjusted for fat-free mass, fat mass, and sex was significantly lower in both of the older groups when compared to the younger group (p<0.007). Nonagenarians had significantly (p<0.01) lower DNA damage than the middle-aged subjects (60-74y). However, there were no significant relationships between RMR and any of the markers of oxidative stress. Nonagenarians had less prevalence of the metabolic syndrome than the aged individuals; however, this was not related to reduced levels of oxidative stress. Finally, nonagenarians were less physically active than the aged individuals and this was associated with impaired physical functionality. The current study confirms previous findings of an age related decline in RMR adjusted for body weight and body composition. In addition, nonagenarians appeared to be protected from an age-related increase in DNA damage and development of the metabolic syndrome. However, there was no relationship between the level of oxidative damage and RMR challenging the rate of living/ oxidative stress hypothesis.
Chapter One

Introduction

Aging can be defined as a progressive decline in the ability of an organism to resist stress, damage, and disease. It is characterized by an increase in the incidence of degenerative disorders including cancer, cardiovascular disease, decreased immune function, and diabetes. The aging process proceeds at a varied rate in different individuals. Consequently, some individuals age slower than others predisposing them to long life and retention of physical and cognitive capacity promoting the sense of well being associated with healthy aging.

Aging is characterized by a number of physiological changes, all of which contribute to a decreased ability to defend against disease ultimately resulting in death. However, in order to make it to extreme old age, “successful agers,” (individuals who have made it to extreme old age while still maintaining the ability to perform normal activities of daily life) markedly delay or even escape diseases that would otherwise be lethal at younger ages. This leads to questions concerning what factors have played a role in the apparent resistance to aging in these individuals.

Energy metabolism is indirectly related to lifespan. The Rate of Living Theory hypothesizes a direct relationship between metabolic rate and the rate of aging by demonstrating that larger animals live longer and have lower metabolic rate per unit of muscle mass (Greenberg 1999). The link between energy metabolism and aging may be oxidative stress. Adelman et al reported a significant relationship between species- specific metabolic rate and DNA damage (Adelman, Saul et al. 1988). In addition, Sohal and Weindruch (Sohal and Weindruch, 1999) demonstrated an inverse relationship between maximum lifespan potential and species- specific metabolic rate as well as free radical production. Oxidative stress is one of the prevailing
theories of aging. The theory states “aging and degenerative diseases associated with it are attributed basically to side attacks of free radicals on cell constituents and connective tissue.” This hypothesis is supported by numerous observations. For example, maximal lifespan is related to the amount of free radical production (Sohal and Allen 1985). Furthermore, overexpression of anti-oxidative enzymes or activation of defense mechanisms against oxidative stress retards aging and extends lifespan (Orr and Sohal 1994). Thus, the amount of oxidative damage increases as an organism ages and is postulated to be one of the major causal factors of aging and therefore lifespan.

Caloric restriction (CR), which is the only intervention to consistently extend average and maximum lifespan, is associated with a number of changes including a reduction in energy metabolism and a decrease in oxidative stress. One intriguing hypothesis is that CR lessens oxidative damage and repair by reducing energy flux and metabolism. It is not yet known if individuals who successfully reach “old age” have a reduced metabolic rate adjusted for respiring body mass and whether this is associated with lower levels of oxidative stress.

The purpose of this study and the major hypothesis tested in this project was that a relatively low metabolic rate (adjusted for body size and composition) is associated with “reduced” oxidative stress. We tested whether “healthy nonagenarians” have a relatively low metabolic rate when compared to middle age and young individuals and whether the low metabolic rate was associated with a low level of oxidative stress when compared to that of middle-aged individuals. We also measured predictors or biomarkers of the metabolic syndrome and cardiovascular disease in nonagenarians compared to younger individuals. We predicted that nonagenarians will have CVD profiles comparable to younger individuals and this will be associated with less oxidative stress. Regular physical activity has been proven to induce weight
loss, improve glucose and lipid profiles, as well as maintain bone density and muscle mass, improving the aging process. Although evidence does not support the idea that exercise increases lifespan, it may decrease morbidity and improve quality of life. Very little is known about physical activity in very old people. Therefore, we also measured levels of physical activity in nonagenarians using state of the art methods (doubly labeled water).

1.1 Specific Aim One

Measure and compare resting metabolic rate (RMR) and oxidative stress in three groups of non-diabetic subjects i.e. 20-34, 60-74, \( \geq 90 \) years.

It was hypothesized that those subjects \( \geq 90 \) years old have lower RMR (adjusted for fat free mass, fat mass, and sex) which will protect against aging by reducing oxidative stress. The low metabolic rate is associated with lower body temperature, and evidence of lower tissue oxidative stress when compared to middle age subjects (60-74y).

1.2 Specific Aim Two

Measure and compare markers of the metabolic syndrome (BP, fasting glucose and insulin, lipid profile, hemostasis factors, homocysteine) and markers of inflammation in three groups of non-diabetic subjects i.e. 20-34, 60-74, \( \geq 90 \) years.

It was hypothesized that because those subjects \( \geq 90 \) years old have lower levels of oxidative stress, they will have lower concentrations of markers of the metabolic syndrome than middle age subjects (60-74y).

1.3 Specific Aim Three

Measure and compare the level of physical activity by doubly labeled water in three groups of non-diabetic subjects i.e. 20-34, 60-74, \( \geq 90 \) years.
It was hypothesized that those subjects \( \geq 90 \) years old still have relatively high levels of physical activity which confer them to a high level of physical and cognitive functionality which will be measured in other projects in the study.
Chapter Two
Review of Literature

The purpose of this study is to test whether “healthy” nonagenarians have a relatively low metabolic rate when compared to middle age and young individuals and whether the low metabolic rate is associated with a low level of oxidative stress when compared to that of middle age individuals. The first objective was to measure and compare resting metabolic rate (RMR) and oxidative stress in three groups of non-diabetic subjects ages 20-34, 60-74, or ≥ 90 years of age. A second objective was to measure and compare levels of physical activity by doubly labeled water in the same three groups. The third objective was to measure and compare markers of cardiovascular disease and markers of inflammation in the same three groups.

2.1 Introduction

Aging can be defined as a progressive decline in the ability of the organism to resist stress, damage, and disease (Finch 1990). It is characterized by an increase in the incidence of degenerative disorders including cancer, cardiovascular disease, decreased immune function, and diabetes (Ames, Shigenaga et al. 1993). The aging process proceeds at a varied rate in different individuals. Consequently, some individuals age slower than others predisposing them to long life and retention of physical and cognitive capacity, promoting the sense of well being associated with healthy aging.

The oxidative stress hypothesis is one of the prevailing theories of aging. This theory states that oxidative damage caused by reactive oxygen radicals damages lipids, proteins, and DNA accelerating the aging process and increasing disease risk (Harmon 1956). The hypothesis is supported by numerous observations. For example, maximal lifespan is inversely related to the amount of free radical production (Sohal and Allen 1985). Furthermore, over-expression of
anti-oxidative enzymes or activation of defense mechanisms against oxidative stress retards aging and extends lifespan in some organisms (Orr and Sohal 1994). In contrast, mutations or deletions resulting in reduced expression of anti-oxidative enzymes increase oxidative damage but do not result in a reduction in lifespan (Van Remmen, Ikeno et al. 2003). However, there may be adaptations in these animals such as up regulation of other pathways, which may still protect them from the increase in oxidative damage.

Oxidative stress has been implicated in a number of disease states and is thought to contribute the aging process. Free radicals contribute to endothelial dysfunction and are involved in the pathogenesis and development of cardiovascular diseases, such as atherosclerosis (Vassalle, Petrozzi et al. 2004). Oxidative damage is elevated in individuals with hypercholesterolemia and hyperlipidemia (Araujo, Barbosa et al. 1995; Reilly, Pratico et al. 1998; Stojiljkovic, Lopes et al. 2002). Oxidative stress has also been implicated in a number of neurological disorders including Alzheimer’s, Parkinson’s, and Schizophrenia (Mecocci, MacGarvey et al. 1994; Milton 2004; Nishioka and Arnold 2004). In addition, oxidative stress is implicated in the development of insulin resistance and diabetes.

The study of oxidative stress is complex. Free radicals are produced throughout the body in any number of ways (Halliwell 1999). Although organisms have developed defense mechanisms to protect against oxidative insult, it seems that these systems become overwhelmed leading to accumulation of damage. Oxidative stress results from an imbalance between the pro-oxidative and antioxidative mechanisms. This is dependent on a number of factors including free radical production, strength of antioxidative defense, and rate of repair. This has made the study of oxidative stress difficult resulting in an overwhelming number of methods and techniques developed to assess “oxidative status” (Dotan, Lichtenberg et al. 2004).
There are a number of factors that are thought to contribute to an individual’s “oxidative status” including sex, body composition, age, smoking status, diet, and external environment. In addition, resting metabolic rate may have an effect on oxidative stress. The rate of living theory supports this idea. The theory postulates a direct relationship between metabolic rate and the rate of aging by demonstrating that larger animals live longer and have lower metabolic rate per unit of muscle mass (Greenberg 1999). The mechanism involved may be related to oxidative stress. Whether this relationship has an impact on individual lifespan is still being investigated, with some evidence of support for this coming from studies between species (Sohal, Svensson et al. 1990), but not within species (Speakman, Talbot et al. 2004). Furthermore, studies examining uncoupling proteins have indicated that this relationship is more complex than what was once thought (Speakman, Talbot et al. 2004).

2.2 Free Radicals

Free radicals are defined as any species with one or more unpaired electrons capable of living independently (Cheeseman and Slater 1993; Halliwell 1999; McBride J 1999). Oxygen is considered a di-radical because both of its electrons are unpaired (Cheeseman and Slater 1993). The oxygen paradox states that while oxygen is vital for survival, it is highly reactive and can initiate a series of reactions leading to tissue damage and death. Reactions with oxygen can produce a number of reactive oxygen species (ROS) including superoxide \( \left( \text{O}_2^\cdot \right) \), Hydrogen Peroxide \( \left( \text{H}_2\text{O}_2 \right) \), and the hydroxyl radical \( \left( \text{OH}^- \right) \) (Cheeseman and Slater 1993; Beckman and Ames 1998; Halliwell 1999; Droge 2002).

Earlier studies demonstrated that under normal conditions, the electron transport chain is the primary producer of superoxide anion. The electron transport chain is responsible for the transport of electrons through complexes in the chain creating a proton gradient. This proton
gradient produces the energy needed to generate ATP. Figure 2.1, adapted from Batandier et al. (Batandier, Fontaine et al. 2002) illustrates the electron transport chain and the potential sites of free radical production. Research indicates that electrons leak during their transfer from NADH dehydrogenase in Complex I to ubiquinone.

For example, Turrens et al revealed that inhibition of electron transport by rotenone or antimycin A resulted in increase in \( \text{O}_2^- \) production from reduction of NADH dehydrogenase (Turrens and Boveris 1980). Bovine heart mitochondria were isolated and measured for \( \text{O}_2^- \) production. Addition of NADH in the presence of rotenone as well as reverse electron transfer resulted in the production of \( \text{O}_2^- \). Leakage can also occur during transfer within Complex III, particularly at the point of cytochrome b resulting in the formation of superoxide. Studies by

**Figure 2.1.** Electron transport chain

antimycin A resulted in increase in \( \text{O}_2^- \) production from reduction of NADH dehydrogenase (Turrens and Boveris 1980). Bovine heart mitochondria were isolated and measured for \( \text{O}_2^- \) production. Addition of NADH in the presence of rotenone as well as reverse electron transfer resulted in the production of \( \text{O}_2^- \). Leakage can also occur during transfer within Complex III, particularly at the point of cytochrome b resulting in the formation of superoxide. Studies by
Cadenas et al (Cadenas, Boveris et al. 1977) isolating Complexes I and III of the electron transport chain point to the production of O$_2$•$^-$ and H$_2$O$_2$ from the NADH-ubiquinone reductase and ubiquinol cytochrome c reductase areas of the chain. Earlier measurements of oxygen consumption and free radical production in isolated mitochondria from heart and brain tissue of rats indicate that when succinate is used as a substrate in electron transport, H$_2$O$_2$ is produced unless ADP is introduced. Therefore, radicals were thought only to occur during state 4 respiration when ADP is absent (Boveris, Oshino et al. 1972). It was estimated that during state 4 respiration isolated (Chance, Sies et al. 1979) mitochondria generate 0.6-1.0nmol of H$_2$O$_2$/min/mg protein. This accounts for approximately 2% of the oxygen uptake under normal physiological conditions. However, recent studies indicate that when pyruvate or malate are used as substrates there is no difference in the production of H$_2$O$_2$ whether ADP (state 3 respiration) is present or not (Herrero and Barja 1997). These results point toward Complexes I and III to be responsible for free radical production under normal cellular conditions. Furthermore, free radicals are produced during both state 3 and state 4 respirations.

There are a number of other ways ROS can be produced. ROS can also be produced during peroxisomal β-oxidation of fatty acids, which generate H$_2$O$_2$ as a byproduct. Arnaiz et al examined the effect of fenofibrate on the metabolism of H$_2$O$_2$ and levels of antioxidant enzymes (Arnaiz, Travacio et al. 1995). Fenofibrate is a hypolipidemic drug that activates peroxisome proliferator receptors to increase fatty acid β-oxidation. Female Swiss mice were either given chow with added fenofibrate or identical chow with no fenofibrate added. Mice were killed by cervical dislocation after 1, 2, or 3 weeks of treatment. The liver was taken out and processed for analysis. Catalase and glutathione peroxidase content was measured. Hydrogen peroxide concentration and production rate was also measured. Fenofibrate treatment produced a 10-fold
increase in H$_2$O$_2$ production rate after two weeks of treatment. Furthermore, hydroxyl and alkyl radical concentrations were increased by 90% and peroxyl radical concentrations were increased by 37% in the treated animals compared to control. Another source of oxygen radicals is during metabolism of xenobiotic compounds by microsomal cytochrome P-450 enzymes. These enzymes are responsible for the oxidation of a wide range of substrates at the expense of oxygen (Beckman and Ames 1998). Strobel and Coon demonstrated the production of superoxide from cytochrome P-450 and oxygen (Strobel and Coon 1971). Finally, phagocytic cells attack pathogens with a mixture of oxidants and free radicals including O$_2^-$, H$_2$O$_2$, nitric oxide (NO$^*$), and hypochlorite. Ramos demonstrated this by investigating the production of OH$^-$ by human neutrophils and monocytes (Ramos, Pou et al. 1992). Myeloperoxidase catalyzes the reaction of H$_2$O$_2$ and chloride to form HOCl, which has potent bactericidal activity. It was hypothesized that the presence of O$_2^-$ during this reaction leads to the production of OH$^-$. Human neutrophils and leukocytes were isolated and levels of hydroxyl were measured using a combination of electron spin resonance and spin trapping. Indeed results support the ability of human neutrophils and monocytes to generate hydroxyl via the myeloperoxidase pathway. Although not normally a significant contributor of free radicals, it is nevertheless an unavoidable consequence of innate immunity.

2.3 Antioxidants

In an effort to defend itself against the attack of free radicals on the body, aerobic organisms have evolved an elaborate antioxidant defense system. These defense systems include enzymes that catalytically remove free radicals and other reactive species; proteins that minimize the availability of pro-oxidants; proteins that protect biomolecules from damage; and low molecular weight antioxidants that scavenge free radicals (Halliwell 1999). There are a number
of enzymes that are produced endogenously to act as antioxidants in the body. Superoxide Dismutase (SOD) is a family of enzymes responsible for dismutating superoxide to form hydrogen peroxide. Gregory and Fridovich first investigated the role of SOD in the presence of oxygen (Gregory and Fridovich 1973). Streptococcus faecalis and Escherichia coli were cultured and exposed to varying amounts of oxygen. Changing the conditions of growth from anaerobic to 5 atm of oxygen caused a 25-fold increase in the level of SOD. This was a response specific to oxygen rather than pressure because there was no effect of nitrogen. These results indicate the importance of SOD to organisms exposed to oxygen. There are three major isoforms of SOD; Copper/ Zinc SOD (CuZnSOD or SOD1) is located in the cytosol and contain copper and zinc in the active site; the Manganese SOD (MnSOD or SOD2) is located in the mitochondria and contains manganese in the active site of the enzyme; finally, there is extracellular SOD (ECSOD), which is located in extracellular compartments such as plasma (Ookawara, Haga et al. 2003). Catalase is another antioxidant enzyme that is responsible for the breakdown of \( \text{H}_2\text{O}_2 \) to ground state Oxygen and water. Otherwise, \( \text{H}_2\text{O}_2 \) reacts with a number of molecules including ferrous iron to produce the highly reactive hydroxyl radical. Kwong et al demonstrated this by ectopically inserting catalase into the mitochondrial matrix of flies, which is the main intracellular site of \( \text{H}_2\text{O}_2 \) formation and where catalase is normally absent (Kwong, Mockett et al. 2000). Mitochondrial release of \( \text{H}_2\text{O}_2 \) was decreased by approximately 90% in the transgenic lines when compared to levels in vector-only controls. Glutathione peroxidase (Gpx) is another family of enzymes responsible for removing \( \text{H}_2\text{O}_2 \) by coupling its reduction to \( \text{H}_2\text{O} \) with oxidation of reduced glutathione (GSH). Mills demonstrated in rat liver that exposure to \( \text{H}_2\text{O}_2 \) resulted in the induction of glutathione peroxidase (Mills 1960). In addition, Mills also demonstrated in rat erythrocytes that glutathione peroxidase might be responsible for protecting
hemoglobin from oxidative breakdown by reacting with hydrogen peroxide keeping it from reacting with hemoglobin (Mills 1957). Dietary antioxidants including vitamin A, E, and C are also important in defending against oxidative stress. For example vitamin C, also known as ascorbic acid can react with a number of radicals including superoxide and hydroxyl radicals. Vitamin E also known as $\alpha$-tocopherol can react with peroxyl radicals faster than they can react with other lipid molecules. Vitamin A, can also react with hydroxyl radical to produce much less reactive products (Halliwell 1999). Other protecting agents include heat shock proteins and other endogenous enzymes that are responsible for minimizing the availability of pro-oxidants such as ion irons, copper ions, and haem. For example, Heme-oxygenase (heat shock protein 32) is responsible for breakdown of haem to biliverdin, thereby reducing the availability of haem to be oxidized by free radicals (Halliwell 1999).

2.4 Oxidative Stress

Oxidative stress is the term used to describe damage to tissue that occurs when free radicals and pro-oxidants overwhelm defense mechanisms. However, this definition is not well defined. On the cellular level, what determines whether a cell dies or stays alive is dependent on the balance between the prooxidants and antioxidant defense mechanism. This is illustrated by Figure 2.2, adapted from Jotg B. Schulz, Jorg Lindenau, Jan Seyfried, and Johannes Dichgans., Glutathione, oxidative stress and neurodegeneration. Free radicals are produced in a number of ways and can affect all tissues. Oxidative stress is dependent on free radical production, susceptibility of tissue, and strength of the defense and repair system. Figure 2.3, adapted from Beckman and Ames illustrates these relationships (Beckman and Ames 1998).
The generation of oxidants leads to the induction of antioxidant defense and repair mechanisms. The interaction of these factors will determine the outcome for aging. Therefore, the assessment of “oxidative status” of an individual is complex resulting in the formation of more than 40 different indices of oxidative status and the development of more than 100 techniques (Dotan, Lichtenberg et al. 2004). This is illustrated in 2.4 adapted from Dotan et al (Dotan, Lichtenberg et al. 2004). Determining the potency or oxidizability of cells and tissues can assess oxidative stress. In addition, measurements can be made on the composition of oxidative stress including the promoters and inhibitors (free radicals and antioxidants) as well as the peroxidation products (damage to proteins, lipids, and DNA). Steady state measures of oxidative stress represent the equilibrium among the rate of ROS generation, the rate of oxygen scavenging, and the rate of repair (Heilbronn and Ravussin 2003).

However, one major reason as to why research in free radical metabolism and oxidative stress is so difficult is due to the reactive and unstable nature of free radicals. This instability

![Figure 2.2. Balance of Oxidative Stress](image)
makes the detection and measurement of free radicals very difficult. The only measurement that directly measures free radicals is electron spin resonance (ESR). Although this method has been used to measure radicals such as the ascorbate radical, it is not sensitive enough to detect highly reactive radicals such as superoxide and hydroxyl (Buettner and Jurkiewicz 1993). Therefore ESR has been combined with spin trapping to detect these radicals. A spin trap is a molecule that reacts with the radical producing a more stable product thereby trapping it so it can be measured (Halliwell 1999). Rosen et al used the spin trap 5,5-dimethyl-1-pyrole-N-oxide (DMPO) to detect levels of the hydroxyl radical in isolated leukocytes from humans (Rosen and Klebanoff 1979). Ide et al also used (DMPO) to detect superoxide production from electron
transport using transport inhibitors (Ide, Tsutsui et al. 1999). Finally, ESR combined with spin trapping was used to detect peroxyl radicals. When a fatty acid reacts with a OH\textsuperscript{−} radical it produces a carbon centered radical. This carbon-centered radical can combine with oxygen to form a peroxyl radical. North et al used a human monocytic cell line to measure lipid-derived radicals from a reaction between polyunsaturated fatty acids (PUFAs) and iron (North, Spector et al. 1992). An alternative to measuring free radicals directly is to measure products of damage by free radicals, otherwise known as fingerprinting (Halliwell 1999). ROS reacts in specific ways with DNA, lipids, and proteins. The products of these reactions can be measured and provide indirect information of oxidative attack.

**Figure 2.4.** Measures of Free Radical Production and Oxidative Stress
2.4.1 Lipids

Lipids are the molecules most susceptible to free radical attack. Bielski et al demonstrated that perhydroxyl radical (the conjugate acid of superoxide) could react with unsaturated fatty acids in the lipid membrane leading to damage of the membrane (Bielski, Arudi et al. 1983). Hydroxyl radicals or other highly reactive oxidants can react with lipids in the membrane by removing a hydrogen atom generating a carbon-centered radical that can rapidly combine with oxygen to form a peroxyl radical. Wagner et al demonstrated this by measuring oxygen consumption, lipid peroxidation, and degree of unsaturation in intact cells (Wagner, Buettner et al. 1994). Results of these experiments revealed that lipid peroxidation was exponentially related to degree of saturation of fatty acids. Peroxyl radicals are capable of removing hydrogen atoms from other fatty acids. This results in a cycle of lipid resulting in the formation of hydroperoxides (Gardner 1989). In addition, products of lipid peroxidation such as malondialdehyde (MDA) can also react with DNA bases and introduce mutagenic lesions. In an effort to determine the effect of MDA on DNA, Cai et al coincubated different deoxynucleosides with malondialdehyde (Cai, Tian et al. 1996). Addition of MDA to deoxynucleosides increased the number of DNA mutations mainly to the guanine base. Other products such as 4-Hydroxy-2-trans-nonenal (HNE) can react with thiol groups on proteins as well as amino groups on DNA. Uchida and Stadtman demonstrated this by incubating a protein mixture containing poly histidine insulin with HNE for two hours (Uchida and Stadtman 1992). This study demonstrated the ability of HNE to react with histidine residues in insulin to produce HNE modified insulin. The HNE modification was associated with a loss of 1.1 histidine residues per mol of insulin.
2.4.2 Proteins

Superoxide and H$_2$O$_2$ have little effect on proteins. However, OH$^-$ and singlet O$_2$ can react with proteins to generate a number of products such as amino acid peroxides and protein carbonyls. Proteins can bind metal ions, especially iron and copper. Exposure of the resulting products to H$_2$O$_2$ generates OH$^-$, which selectively damages amino acid residues at the binding site in a site-specific reaction (Halliwell 1999). However, because cellular proteins are constantly turning over, accumulation of damaged proteins would not be probable. It was not until we began to understand how cells regulate the turnover of individual enzymes, that the possibility of protein damage accumulation and implication in aging and disease became apparent (Stadtman 1992). It was also hypothesized that high molecular weight proteins were more susceptible to oxidation than others. For example, Yan et al investigated the oxidation of specific proteins and what affect it had in controlling the rate of the aging process in male houseflies (Yan, Levine et al. 1997). Mitochondria were isolated from the thoracic flight muscles of adult flies. Proteins were subjected to electrophoresis and immunochemical detection to distinguish specific modified proteins. Results indicate that aconitase, an enzyme in the citric acid cycle, is particularly susceptible to oxidation resulting in the production of carbonyl derivatives. This resulted in a decrease in the activity of the enzyme by 62% between 7 and 15 days of age. This same lab also determined that adenine nucleotide translocase (ANT) was also susceptible to oxidation by elevated carbonyl derivatives (Yan and Sohal 1998). In addition, the age related increase in ANT carbonyl content correlated with a corresponding loss in its functional activity. Thus it is apparent that oxidative damage to proteins can affect the function of important systems and may thereby regulate the aging process.
2.4.3 DNA

While DNA is a very stable molecule it can undergo spontaneous decomposition over a lifetime. Deamination can result in mutations. Free radicals can greatly accelerate this deamination. For example, OH⁻ can add on to adenine or guanine at positions 4, 5, or 8 in the purine ring. Hydrogens can also be removed from pyrimidines to produce a number of products including thymine glycol or dihydroxycytosines. Removal of hydrogens from the deoxyribose sugars by free radicals produces a number of carbon-centered radicals, which rapidly convert sugar peroxy radicals (Halliwell 1999). Heaton et al subjected feline and canine leukocytes to H₂O₂ to demonstrate this (Heaton, Ransley et al. 2002). Increased strand breakage was observed in cells subjected to oxidative stress. In addition, increased DNA damage occurs in individuals exposed to carcinogenic chemicals. Kim et al demonstrated that individuals exposed to a mixture of carcinogenic chemicals containing metals such as nickel and lead increased DNA damage following exposure (Kim, Mukherjee et al. 2004). Superoxide, nitric oxide, and hydrogen peroxide at physiologically relevant levels do not react with base sugars in DNA and RNA. However, the hydroxyl radical will react with sugars as well as purines and pyrimidines. Hiraoka et al investigated the effect of hydroxyl radicals on cytosine related compounds (Hiraoka, Kuwabara et al. 1990). Hydroxyl radicals were generated by X-irradiating an N₂O saturated aqueous solution and added it to a mixture of cytosine related compounds. The OH⁻ induced free radicals were measured by spin trapping and detected by ESR. Results indicate that the addition of OH⁻ generated four base radicals and one sugar radical. Masuda et al also examine the reactivity of hydroxyl radicals with nucleotides, polynucleotides and related compounds (Masuda, Shinohara et al. 1980). Rate constants were determined using a competition method using p-nitrosodimethylaniline. Rate constants were highest with guanosine
(rate constant=$8.0 \times 10^8$ M$^{-1}$ s$^{-1}$), which is known to be the most common oxidatively modified nucleotide.

2.5 Measurements of Damage

2.5.1 Lipid Peroxidation

Lipid peroxidation is the most extensively used marker of oxidative stress. Reactions between fatty acids and free radicals result in a number of products that can be measured as indices of lipid peroxidation. Isoprostanes are prostaglandin-like products resulting from the oxidation of arachidonic acid and are useful for the assessment of lipid peroxidation (Morrow, Hill et al. 1990). Bielski et al. revealed that arachidonic acid is a popular target of peroxidation due to the high number of double allylic hydrogens (Bielski, Arudi et al. 1983). Briefly, isoprostanes can easily be measured from plasma or urine using liquid chromatography combined with mass spectrometry. This method allows for the sensitive detection of specific isoprostanes produced during lipid peroxidation. Morrow et al. demonstrated that they were present in blood and urine and measurements of them in vivo have been validated against equal mixtures of compounds in urine and plasma generated in vitro (Morrow, Awad et al. 1992). Morrow et al. also validated the formation of these compounds in vivo using animal models of free radical-induced lipid peroxidation. Administration of diquat, an herbicide that results in the production of free radicals leads to an increase in isoprostanes in both blood and urine. Others have also examined the potential of isoprostanes as a marker of oxidative stress. For example, Delanty et al. measured urine concentrations of isoprostanes following coronary reperfusion, which is known to induce free radicals (Delanty, Reilly et al. 1997). Isoprostanes levels were measured in a canine model of coronary thrombolysis, in patients following acute myocardial infarction, and patients following coronary artery bypass surgery. Isoprostane excretion
increased during reperfusion in the canine model of coronary thrombolysis. Furthermore, there were significant differences in isoprostane levels between healthy individuals and those taking thrombolytic drugs. Levels are also increased following thrombolysis in humans.

### 2.5.2 Protein Carbonyls

Protein carbonylation is the most common marker of protein oxidation. Bonnes-Taourel examined protein carbonyl levels in brains of 2 month old mice compared to 25-month-old mice (Bonnes-Taourel, Guerin et al. 1993). Protein carbonyls increased with age in brain tissue, indicating accumulation of age-related cell damage (Agarwal and Sohal 1995). Under anaerobic conditions, radicals promote cross-linking through OH$^-$ facilitated –S-S- and –Tyr-Tyr- bonding. In the presence of O$_2$, the result is the fragmentation of the polypeptide chain. The residues, Proline, Arginine, and Lysine are particularly susceptible to oxidation by metal catalyzed oxidation systems, which produce H$_2$O$_2$ and Fe$^{2+}$ resulting in the production of carbonyl derivatives (Stadtman 1992).

### 2.5.3 DNA Damage

DNA damage can be assessed a number of different ways including techniques to measure strand breakage and baseless sites such as single cell gel electrophoreses or ARP (aldehyde reactive probe) assays. Singh et al first demonstrated the potential of single cell gel electrophoresis assay (comet assay) as a measurement of DNA damage (Singh, McCoy et al. 1988). Human lymphocytes exposed to X-radiation or treated with H$_2$O$_2$ displayed increased damage following exposure. Heaton et al also exposed canine and feline leukocytes to H$_2$O$_2$ to find similar increases in DNA damage (Heaton, Ransley et al. 2002). Fewer studies have utilized the aldehyde reactive probe (ARP) assay as a measurement of baseless sites in DNA. These are sites that have lost the purine or pyrimidine base. The aldehyde reactive probe binds to the
aldehyde group present in the baseless site. The baseless site is then tagged and can then be quantified using an ELISA like assay. Mohsin Ali et al used the ARP assay to detect oxidative damage in calf thymus DNA and HeLa cells following irradiation (Mohsin Ali, Kurisu et al. 2004). Results indicate an increase in baseless sites measured by ARP following irradiation.

Furthermore, by coupling ARP with repair enzyme glycosylases such as endonuclease III or 8-oxoguanine N-glycosylase (OGG1), glycosylated generated baseless sites can also be quantified giving an indication of DNA repair. Kow and Dare describe this method in detail for the detection of more specific DNA mutations (Kow and Dare 2000). The results of their research indicate that baseless sites can readily be measured by the use of the ARP reagent. In addition, the use of repair enzymes will further aid in assessing the various types of damage that are normal substrates of the glycosylase. Other methods have been developed to measure biomarkers of damage including DNA mutations such as 8-hydroxyguanosine (8-oxodG; damaged base) or the repair product, 8-hydroxydeoxyguanosine (8OhdG). This is the most common oxidative modification caused by the 8-hydroxylation of the guanine moiety. The hydrogen is replaced with OH on the guanine base. A number of techniques have been developed to measure this mutation as an indicator of DNA oxidative damage. As stated previously, Mohsin et al used a combination of the ARP assay with DNA glycosylase to measure baseless DNA sites due to specific mutations resulting from irradiation (Mohsin Ali, Kurisu et al. 2004). Others have utilized liquid chromatography and mass spectrometry to measure DNA mutations. Hamilton et al measured levels of 8-oxo-2-deoxyguanosine (8oxodG) in DNA isolated from tissues of rodents following exposure to radiation (Hamilton, Guo et al. 2001). 8oxodG was measured in nuclear DNA (nDNA) isolated from liver, heart, brain, kidney, skeletal muscle, and spleen and in mitochondrial DNA (mtDNA) isolated from liver. As expected, DNA
mutations were present in all tissues and in all mouse strains studied. In addition, DNA mutations were higher in mitochondrial fractions than nuclear fractions.

2.6 Factors Associated with Oxidative Stress

2.6.1 Environment

The external environment is a significant contributor to oxidative stress. Everything from sunlight and UV radiation to pesticides that is put on fruits and vegetables can cause oxidative stress. Jones et al demonstrated that ultra violet radiation was found to induce a substantial amount of oxidative stress in fibroblasts resulting in an increased release of superoxide anions and an increase in lipid peroxidation (Jones, McArdle et al. 1999). It is not surprising that chemotherapy and radiation therapy are associated with increased formation of reactive oxygen species and depletion of critical plasma and tissue antioxidants. Cetin et al demonstrated that following high dose chemotherapy, there were significant increases in levels of lipid peroxidation as well as Gpx, and SOD (Cetin, Arpaci et al. 2004). A single dose of UV radiation was found to result in a transient reduction in SOD activity followed by an increase in lipid peroxidation (Punnonen, Autio et al. 1991). Other factors that are known to induce oxidative stress are fine particulates such as residual oil fly ash (ROFA) or diesel exhaust particles (DEP). For example, Baulig et al studied whether diesel exhaust particles produce reactive oxygen species, which then induce a proinflammatory response in human bronchial epithelial cells. Using fluorescent probes, they detected ROS production in bronchial and nasal epithelial cells exposed to DEP (Baulig, Garlatti et al. 2003). Furthermore, Kim et al revealed that urinary levels of DNA damage were elevated in workers exposed to ROFA, which is a chemically complex mixture of compounds including metals (Kim, Mukherjee et al. 2004). Somewhat surprising, certain antibiotics, such as lincomycin, cephalothin, and erythromycin can
generate a significant amount of $O_2^*$ under various radiation conditions. That is why it is recommended to individuals taking the above medication to avoid UVR especially in tropical and sub tropical countries (Ray, Mehrotra et al. 2001). Finally, pesticides induce oxidative stress as a mechanism of their toxic action in the body (Abdollahi, Ranjbar et al. 2004).

2.6.2 Sex

A number of studies have examined the relationship between gender and oxidative stress. Generally males exhibit higher levels of oxidative stress than females. For example, Ide et al examined two markers of lipid peroxidation in plasma and urine to find that levels of both markers were higher in males than females (Ide, Tsutsui et al. 2002). Although, another study conducted by Block et al found higher levels of lipid peroxidation in women than men (Block, Dietrich et al. 2002). There are a number of reasons for discrepancies between studies. The studies by Block et al included smokers and nonsmokers in the analysis. Smoking is known to have an impact on levels of isoprostanes (Morrow, Frei et al. 1995). Furthermore, the age group included in Block et al was from 19-78 yrs while the individuals in Ide’s study were age matched at 25 yrs. These factors may have confounded results. In support of Ide et al, Fano et al demonstrated in older individuals (>70yrs), males have higher levels of lipid peroxidation in the vastus lateralis muscle than females (Fano, Mecocci et al. 2001). This same group also measured levels of DNA damage by 8-hydroxy-2’-deoxyguanosine (8OHdG), and protein carbonyls. Although they found no difference between males and females with regard to carbonyl levels, they did note DNA damage was higher in males than females in a group of older individuals. Furthermore, Loft et al measured urinary levels of 8OHdG and also found that men had higher levels than women (Loft, Vistisen et al. 1992). Finally, Borras et al examined free radical production in isolated mitochondria from male and female Wistar rats (Borras, Sastre et al.
Mitochondrial hydrogen peroxide production was measured along with levels of 8oxodG from liver and brain. Results indicate that hepatic peroxide production is significantly (p< .01) higher in males than females. This occurs whether succinate or pyruvate/malate are used as substrates. Hepatic mitochondria from males produce more than 40% more H2O2 than females. Furthermore, brain mitochondria from males produce more than 80% more H2O2 than females. To test if ovarian hormones may be responsible for differences in oxidant production, Borras et al also tested the effects of ovariectomy and estrogen replacement. Ovariectomy abolished the differences between men and women and estrogen replacement therapy restored these differences.

2.6.3 Body Composition

Body composition may also have an effect on oxidative stress. Keaney et al examined 2828 subjects from the Framingham Heart Study to determine clinical correlates associated with oxidative stress (Keaney, Larson et al. 2003). The data supports a relationship between obesity and urinary levels of isoprostanes with each extra 5 units of BMI associated with a 9.9% increase in urinary levels of isoprostanes. Block et al also found a positive relationship between plasma isoprostanes and BMI (Block, Dietrich et al. 2002). However, there was no relationship between MDA and BMI. In another study by Loft et al, multiple regression identified BMI was an independent predictor of 8OHdG excretion (Loft, Vistisen et al. 1992). There seems to be general agreement that with increasing BMI, body mass, or body fat, there is an increase in oxidative stress.

2.6.4 Smoking Status

The idea that exposure to smoke increases oxidative stress is no surprise. Data from the Framingham Heart Study reveal a positive association between smoking status and urinary levels
of isoprostanes (Keaney, Larson et al. 2003). This is supported by Ozbay and Dulger who compared the effects of smoking status on lipid peroxidation in 257 subjects (Ozbay and Dulger 2002). Their results revealed higher serum levels of lipid peroxidation in smokers. Harmon et al examined isoprostane levels in current smokers, ex-smokers, and never-smokers to find that current smokers had significantly higher levels of isoprostanes than either never-smokers (p<.0001) or ex-smokers (p=.007) (Harman, Liang et al. 2003). Furthermore, the ex-smokers had higher levels than the never-smokers (p=.002). Polidori et al also revealed that in otherwise healthy individuals, smoking cessation decreases lipid peroxidation (Polidori, Mecocci et al. 2003). However, once smoking is restarted oxidative damage increases back to initial levels. Chehne et al demonstrated that isoprostane concentrations in plasma, serum, and urine increased after restarting smoking (Chehne, Oguogho et al. 2001). Cigarette smoke is also thought to induce oxidative damage to DNA. Loft et al compared urinary 8OHdG excretion in a cohort that included both smokers and nonsmokers (Loft, Vistisen et al. 1992). The smokers had 50% higher levels of 8OHdG than nonsmokers. Park et al revealed a dose response (Park and Kang 2004). Others have shown the direct effects of smoke on cells. Carnevali et al studied the effect of cigarette smoke on human lung fibroblasts (Carnevali, Petruzzelli et al. 2003). Fibroblasts were exposed to various concentrations of cigarette smoke extract resulting in marked increase in DNA fragmentation compared to controls. Smokers may also have decreased resistance to induced oxidative damage. For example, Piperakis et al also demonstrated that when compared to nonsmokers, lymphocytes from smokers were less resistant to H₂O₂ induced DNA damage (Piperakis, Visvardis et al. 1998). There was more DNA fragmentation following H₂O₂ exposure in smokers than nonsmokers. Furthermore, results of the study indicate that smoking had more
of an effect on induced DNA damage than age. Older nonsmokers had less damage when exposed to H₂O₂ than younger smokers.

2.6.5 Age

Oxidative damage increases with age. Fraga et al evaluated endogenous oxidative damage to DNA as a function of age in 2 and 25 month old rats (Fraga, Shigenaga et al. 1990). Damage was assessed by measuring steady state levels of 8oxodG isolated from various tissues and compared to urinary excretion rate. Measurement in tissue is an index of damage and excretion into urine is an estimate of repair. Levels of 8oxodG increased with age in liver, kidney, and intestine but remained unchanged in brain and testes. Furthermore, urinary excretion rate decreased with age indicating a decline in repair. Hamilton et al also examined 8oxodG in isolated tissues of male and female mice and rats (Hamilton, Van Remmen et al. 2001). 8oxodG was measured in nuclear DNA isolated from liver, heart, brain, kidney, and skeletal muscle, and spleen. Isolated mitochondrial DNA was also measured in the liver. They also measured the ability of tissues from young and old mice to remove 8oxodG by exposing mice to a low dose of γ-irradiation then measuring the disappearance of 8oxodG. There was a significant age related increase in 8oxodG in nDNA all tissues studied. However, this was due to a decrease in ability to remove damaged lesions. Bonnes-Taourel et al measured markers of lipid and protein oxidation in brains of normal 2-month-old mice compared to that of 25-month-old mice (Bonnes-Taourel, Guerin et al. 1993). There were no significant age-related changes in lipid peroxidation, but there was an increase in protein carbonyls with age. Mecocci et al investigated oxidative damage to DNA, lipids, and proteins from 66 patients aged 25-93 years (Mecocci, Fano et al. 1999). Skeletal muscle biopsy samples from vastus medialis or lateralis were taken while the subject was having surgery for another reason. Samples were processed for the
assessment of oxidative damage to DNA, lipids, and proteins. There was an age-dependent increase in oxidative damage to DNA, lipids, and proteins. The increase was more significant for DNA and lipids than protein. There was also a direct association between DNA damage and lipid peroxidation ($r = 0.38$, $p < 0.001$). The accumulation of oxidative damage with age could be caused by a number of factors including increased free radical production, decreased antioxidant defense system, or a decrease in removal or repair. Although the exact mechanisms are not known, it is thought that oxidative stress is an important factor in the aging process and those individuals that are more resistant to the accumulation of oxidative damage will live longer.

### 2.6.6 Diet

The role of diet on the effect of oxidative stress has recently become of interest due to an interest in the effect of polyunsaturated fats on health. Polyunsaturated fatty acids (PUFAs) have gained much attention for their beneficial effects on insulin sensitivity (Ghoshal, Xu et al. 2000). However, a number of studies have demonstrated that PUFAs are highly susceptible to free radical mediated peroxidation (Bielski, Arudi et al. 1983; Wagner, Buettner et al. 1994). As stated previously, Bielski et al and Wagner et al demonstrated the susceptibility of PUFAs to free radical attack. Therefore, a number of researchers began examining what effect diets high in PUFAs have on oxidative stress. Vogel et al examined the effects of a diet of commonly consumed dietary animal fat on oxidative damage in the liver (Vogel, Danesvar et al. 2003). Thirty male Fischer rats were assigned to four groups including a control group and three experimental groups given varying amounts of lard. The rats were kept on the diet for 21 days. There was an increase in protein oxidation in the rats fed the lard. There were however, no differences in lipid peroxidation between the groups. The lard had no effect on the level of 8OHdG in urine or in DNA isolated from the liver or the colon. Slim et al also studied the
effects of different plant and animal fat on hepatic oxidative stress in rabbits compared to a diet low in fat (Slim, Toborek et al. 1996). As expected, when compared to a low fat diet, fat sources with higher levels of polyunsaturated fats resulted in a marked increase in lipid peroxidation in liver tissue. In contrast, saturated fats, which are known to be relatively inactive, result in no change in lipid peroxidation in the liver. Corn oil has the highest percent of linoleate (18:2) and resulted in the highest levels of lipid peroxidation. However, lard, which has lower levels of PUFAs does not induce DNA damage in the liver (Vogel, Danesvar et al. 2003). Similar results are also observed in DNA damage. Loft et al examined the effect of a 12-15 week dietary intervention containing various amounts of fat in Fischer rats compared to chow fed animals (Loft, Thorling et al. 1998). Animals were randomized into either a chow fed (3.4% fat) group or a group fed diets containing 21.8% corn oil or 19.8% coconut oil + 2% corn oil. In both males and females, the excretion of 8-OHdG was significantly higher in the groups fed corn and coconut oil compared to control groups (p < 0.05). These findings are supported by Djuric et al who measured DNA damage in plasma following a 20-week diet containing from 3-20% corn oil (Djuric, Lewis et al. 2001). In blood, levels of DNA damage increased with dietary fat. Levels in the blood were 68% higher in the animals fed 20% fat than those fed the 3% fat diet. The amount of fat in the diet also affects the susceptibility of DNA to H$_2$O$_2$ induced oxidative stress. Jenkinson et al measured resistance of DNA to induced oxidative stress in lymphocytes from 21 male, nonsmoking, healthy volunteers following either a 5% or 15% PUFA diet (Jenkinson, Collins et al. 1999). DNA damage induced by H$_2$O$_2$ was significantly higher following the 15% diet (p=. 008). Furthermore, DNA damage was significantly lower than baseline on the low fat diet (p<. 001).
On the other hand, diets low in fat and high in fruits and vegetables have been shown to decrease oxidative stress in high-risk individuals. In the Dietary Approaches to Stop Hypertension (DASH) study, Lopes et al studied the effects of a diet high in fruits and vegetables on markers of oxidative stress (Lopes, Martin et al. 2003). Obese hypertensive subjects were placed on the DASH diet for 4 weeks. Isoprostanes levels were reduced following the diet. Although similar results are not seen in healthy individuals. Dragsted et al observed the effect of a diet high in fruits and vegetables in healthy normal weight individuals. The 25-day diet did not have any effect on protein or lipid peroxidation (Dragsted, Pedersen et al. 2004). This is consistent with results from Miller et al implementing an eight-week diet rich in fruits and vegetables in 123 normal weight, healthy subjects (Miller, Appel et al. 1998). There was no difference in lipid peroxidation following the intervention. Diets low in fat and high in fruits and vegetables, while having a large impact in obese hypertensive individuals may not have any effect in already healthy individuals. However, diets high in polyunsaturated fat have detrimental effects on tissue due to oxidative stress.

2.6.7 Physical Activity

The beneficial effects of exercise on health have been consistently proven. Regular physical activity has been shown to induce weight loss, improve glucose and lipid profiles, as well as maintain bone density and muscle mass. However, exercise has long been associated with oxidative stress based on the assumption that the production of oxygen radicals is linearly related to the consumption of oxygen in the mitochondria (Adelman, Saul et al. 1988). This is complicated by the fact that individual results can differ depending on exercise type, duration, intensity, and individual training status. In addition, the effect of free radicals has been shown to differ depending on the tissue studied. Furthermore, while an acute bout of exercise may lead to
an increase in free radical production, adaptations may occur with chronic exercise that would result in decreased free radical production and/or strengthening the antioxidant defense system.

### 2.6.7.1 Acute Exercise

Studies performed in mice muscle in vivo and in vitro indicate that contraction results in an increase in free radical production as well as response activity to that increase. McArdle et al observed the effect of 15 minutes of contractile activity in mice (McArdle, Pattwell et al. 2001). Surface electrodes attached to the hindlimb-stimulated contraction. Superoxide production was measured ex vivo by reduced cytochrome c as well as in vivo using microdialysis. Muscle contractions were followed by a rapid release of superoxide anions from myocytes. Furthermore, this was followed by an increase in the activities of SOD and catalase. This same group also observed an increase in hydroxyl radicals following contractions (McArdle, van der Meulen et al. 2004). They hypothesized that during muscle contraction MnSOD is responsible for dismutating \( \text{O}_2^{\cdot-} \) to \( \text{H}_2\text{O}_2 \). However, in the presence of Iron, this may lead to the production of \( \text{OH}^- \). This was confirmed by the inhibition of SOD. Inhibition during contraction did not have an effect on \( \text{O}_2^{\cdot-} \) but did decrease the amount of \( \text{OH}^- \) produced. Mastaloudis et al examined the effects of acute exercise and compared it to a sedentary period (Mastaloudis, Leonard et al. 2001). Eleven athletes were studied during a 50km race and during a sedentary protocol 1 month later. Blood was obtained 30 minutes pre race, mid race, post race, 1-hour post race, and 24-hour post race. The same time points were used during the sedentary period. There was an increase in lipid peroxidation as measured by urinary isoprostanines immediately post race that returned to baseline 24 hours after the race. There were no changes in lipid peroxidation during the sedentary protocol. Similar results were seen in DNA fragmentation in untrained individuals. Hartmann et al measured fragmentation in DNA from blood during as well as following 45-
minute treadmill run (Hartmann, Plappert et al. 1994). Maximal fragmentation was seen six hours after the run. This returned to baseline 72 hours after the run. Cross-sectional studies indicate that free radical produced oxidative damage following acute exercise may be different in trained verses untrained individuals. Niess et al compared DNA fragmentation and lipid peroxidation following exercise in trained and untrained men (Niess, Hartmann et al. 1996). Subjects performed an incremental exercise test until exhaustion. Blood was drawn immediately prior, 15 post, and 24 hours after the test. Total running time between the two groups were not different however, running speed of the trained group was faster than the untrained. There were no differences in fragmentation between the groups 15 minutes following the test. However, 24 hours after the test, there was a significant increase in fragmentation in both groups with the untrained group being significantly higher (p<. 05). At rest and 15 minutes after the test lipid peroxidation was significantly higher in the untrained group.

The intensity of work and the tissue involved appear to play important roles in the oxidative stress that occurs with exercise. Kayatekin et al subjected mice to high intensity sprint exercise (Kayatekin, Gonenc et al. 2002). The animals performed 15 bouts of 30s duration at a speed of 35m/min at a slope of 5° with a 10 or 60s rest between bouts. Plasma lipid peroxidation increased immediately post exercise and returned to normal within 24 hours. Interestingly, no change in lipid peroxidation was noted in the liver at any point during recovery. Liu et al assessed the effects of 8 weeks of treadmill running on oxidative stress in the brain, liver, heart, kidney, and skeletal muscle at rest and following an acute bout of exercise (Liu, Yeo et al. 2000). The acute bout of exercise consisted of running at 1.6km/h until exhaustion. Acute exercise increased lipid peroxidation in the heart and skeletal muscle but did not have an effect on lipid peroxidation in the brain. Acute exercise also induced an increase in lipid peroxidation in liver
mitochondria. There were no changes in protein carbonylation following acute exercise. There was a small decrease in protein carbonyl in the liver and slow-twitch muscle following training. There was no change in nuclear 8oxodG following acute or chronic exercise in any of the tissue. On the other hand, chronic exercise decreased lipid peroxidation in brain mitochondria.

Human studies have been performed in a number of settings. For example, Duthie et al assessed whether running a half marathon caused oxidative damage in athletes (Duthie, Robertson et al. 1990). The subjects were seven healthy males (age=35±2yr). Blood was drawn 48 hours and 1 hour prior to the race and 5 minutes, 24, 48, 72, and 120 hours after the race. There were no increases in protein or lipid peroxidation at any point during recovery. There were also no significant changes in either α or γ tocopheral concentrations following the race. There was a significant increase in uric acid, a potent antioxidant in the blood, immediately after the race (p<.01). It should be noted that these subjects were highly trained and may have been adapted to exercise induced oxidative stress. Meijer et al examined the effect of submaximal exercise on oxidative stress in older adults (Meijer, Coolen et al. 2001). Thirty-four elderly subjects (age=62±1yrs) cycled at moderate intensity (50%max) for 45 minutes. Oxidative stress was measured in plasma before and after the exercise session. Exercise resulted in an increase in oxidative stress in plasma at 20 minutes and increased further 45 minutes after the exercise. Resistance training also leads to an increase in oxidative stress. McBride et al studied the effects of acute resistance exercise on markers of oxidative stress in 12 recreationally weight trained males 18=30 years of age (McBride, Kraemer et al. 1998). The resistant exercise protocol consisted of 8 exercise performed in a circuit fashion. A circuit of warm-up sets was performed at 50% IRM followed by heavy resistance exercise using each individual’s predetermined 10RM for each exercise on the first set. The weight was then adjusted based on the familiarization
workout to account for muscle fatigue. Blood was taken before and after the test to measure plasma lipid peroxidation levels. There was a significant increase in plasma levels of lipid peroxidation from pre exercise levels to 6 and 24 hours post.

2.6.7.2 Chronic Training.

Studies in animal models have revealed important information regarding the long-term effects of physical activity on oxidative stress. Radak et al examined the effects of long-term exercise training on oxidative status of lipids protein and DNA (Radak, Kaneko et al. 1999). Two groups of rats, one group 4 weeks of age, the other 14 months old underwent swim training 5 times a week for 9 weeks. Results indicate that while exercise training has no effect on lipid peroxidation, it decreased resting levels of damage to DNA and proteins in muscle. These benefits were seen in the older animals as well as the young. On the other hand, Alessio et al reported that regular treadmill running for 18 weeks had no effect on resting levels of lipid peroxidation in the liver or slow twitch muscle (Alessio and Goldfarb 1988). There was however a decrease in lipid peroxidation with training in red fast twitch muscle. Oztasan et al examined plasma levels of oxidative stress following 8 weeks of training in rats (Oztasan, Taysi et al. 2004). Plasma levels of lipid peroxidation were lower following training. There are number of possible reasons for differences in results betweens studies. There may be effects of different modes of exercise such as running compared to swimming. Furthermore, Radak and colleagues measured the gastrocnemius muscle, which is a mixed muscle and did not specify muscle type during analysis. Muscle type may also be a factor that affects oxidative damage. Different fiber types may affected differently by free radicals resulting in differences in oxidative stress.

The idea of chronic adaptation to exercise is also supported by research indicating that moderate exercise in mice decreases the age-associated development of oxidative stress.
Navarro et al regularly treadmill trained mice from 28 weeks of age to 78 weeks of age (Navarro, Gomez et al. 2004). Moderate exercise prevented the age related increase in protein carbonyls and lipid peroxidation. Kiren et al trained old Wistar rats for 4 weeks (Kirin 2004). The animals went through a 4-day pre-training period to get accustomed to swimming and then were swim trained for 4 weeks for 20 or 40 minutes a day at low, moderate, or high intensity. Both the 20 and 40-minute low or moderate intensity exercise decreased levels of lipid peroxidation. This was supported by Radak et al who also demonstrated that regular exercise attenuates the aged related increase in 8OHdG in rats (Radak, Takahashi et al. 2002). Furthermore, chronic training also prevented the age related decrease in antioxidant enzyme activities of SOD and catalase as well as the decrease in activities of important enzymes involved in oxidative phosphorylation (Navarro, Gomez et al. 2004). However, other studies have not been able to demonstrate a beneficial effect of chronic activity on antioxidant levels. Ji observed the effects of 8 weeks exercise training on antioxidants activities (Ji 1993). Male Wistar and Sprague Dawley rats were subject to graded treadmill running for 8 weeks. There was no change in MnSOD or CuZnSOD or catalase in the heart, liver, or skeletal muscle following training. However, there was an increase in glutathione peroxidase following training. Higuchi et al reported a decrease in SOD concentrations in skeletal muscle when adjusted for changes in mitochondrial enzyme activity following 12 weeks of treadmill running (Higuchi, Cartier et al. 1985). SOD concentration decreased per number of mitochondria indicating less of a need for antioxidant activity post training.

Chronic training may lead to adaptations that improve the response to subsequent exercise induced stress. Therefore trained individuals will respond better to exercise induced oxidative stress better than untrained. Alessio et al determined whether endurance training alters
lipid peroxidation during exercise (Alessio and Goldfarb 1988). Sprague-Dawley rats were assigned to one of four treatment groups, sedentary rest, sedentary acute exercise, trained rest, and trained acute exercise. The trained rats ran for 1h/day, for 5 days/wk, for 18 weeks. The acute exercise bout was running for 20 minutes at 20m/min up a 6% grade. There were significant differences between trained and untrained at rest in red skeletal muscle. There were also significant differences following exercise in the liver and red muscle. There was a significant increase in lipid peroxidation following acute exercise in the untrained group in the liver and white skeletal muscle. There was no increase in lipid peroxidation in the trained group. Information on antioxidant activity post exercise following training is not quite as clear. Alessio also measured SOD and catalase activity to find that liver catalase activity was similar between the trained and untrained rats at rest (Alessio and Goldfarb 1988). However, catalase activity increased in the sedentary group following acute exercise with no change in activity in the trained group. There were no differences in SOD activity at rest between groups, nor was there any change following acute exercise in liver or skeletal muscle. On the other hand Oztasan et al employed an exercise bout consisting of running 1.2km/hr up a 10% grade until exhaustion (Oztasan, Taysi et al. 2004). They reported that erythrocyte SOD activity decreased in the untrained but increased in the trained group following the exercise bout.

2.6.7.3 Fitness Levels

Cross sectional studies in humans indicate that oxidative stress is inversely related to fitness level. Robertson et al investigated the relationship between oxidative stress and regular physical training in sedentary individuals (Robertson, Maughan et al. 1991). Twenty-six males were included in the study. Maximum oxygen uptake was measured during an incremental treadmill test. There was an inverse relationship between maximum oxygen uptake and plasma
lipid peroxidation (p<.01). There was also a positive relationship between catalase and glutathione peroxidase with weekly training distance (p<.05).

Physical fitness also has an effect on oxidative stress following exercise elderly individuals (age=60±1yr). Meijer et al compared oxidative stress following a bout of exercise and physical activity level (PAL) (Meijer, Goris et al. 2002). Subjects cycled for 45 minutes at 50% VO2max. PAL was calculated by dividing total energy expenditure measured by doubly labeled water by resting metabolic rate measured by indirect calorimetry. Oxidative stress was measured using plasma levels of oxidized antipyrine. There was an indirect relationship between PAL and the increase in oxidative stress following the exercise stress. Toskulkao et al compared lipid peroxidation and antioxidant concentration in individual of different training levels (Toskulkao C 1996). Twenty-six healthy males (age=23±1) were divided into three groups: sedentary, short distance runners (100-m runners), and long distance runners (5,000-10,000-m runners). Subjects cycled at 70% maximum heart rate for 60 minutes. Blood was collected immediately before and 5 minutes, 24, and 48 hours post. Lipid peroxidation increased at 24 and 48 hours after the exercise test. Oxidative stress was significantly higher in the sedentary individuals when compared to the short (p<.01) and long distance runners (p<.001). At 24 hours post exercise there was significantly less oxidative stress between long distance runners when compared to the short distance runners (p<.001). In fact, results indicate a dose response effect of training on oxidative stress resulting from an exercise bout. SOD, Glutathione peroxidase, and Catalase activity decreased post exercise in the sedentary individuals. However, there was no change in any of the enzymes in the trained individuals. Thus trained individuals respond better to exercise induced stress than untrained.
Training studies provide a better description of changes that occur with improvement in fitness. Miyazaki et al evaluated whether high intensity endurance training would alleviate exercise induced oxidative stress (Miyazaki, Oh-ishi et al. 2001). Nine untrained male subjects (aged 19-21 yrs) participated in 12-weeks of training and performed an acute period of exhausting exercise on a cycle ergometer before and after training. The training program consisted of running at 80% maximal heart rate 60 minutes/day for 5 times/week for 12 weeks. Blood samples were collected at rest and immediately after the exercise bout. There was no difference in lipid peroxidation before or after training. Oxidative stress post acute exercise was lower after training than before. Dernbach et al examined the effects of rowing on indices of oxidative stress on already trained individuals (Dernbach, Sherman et al. 1993). Collegiate rowers underwent strenuous training for 4 weeks. Training was 40 minutes at 70% maximal O2 consumption as well as three 2,500m-, time trials. Total daily training time was 65 minutes at 70% VO2max and 38 minutes at ≥ 90% max VO2max. Blood was drawn on day 0, 6, 12, 18, 24, and 30. Muscle biopsies of the vastus lateralis were obtained on days 1, 10, 19, and 26. There were no differences in plasma lipid peroxidation levels in either men or women at any points during the training. There were also no differences in lipid peroxidation levels from muscle. Data concerning resistant training show mixed results. Meijer et al examined the effect of 12 weeks of exercise training in the elderly (Meijer, Coolen et al. 2001). Thirty-three subjects (age=60±1) trained twice a week for 1 hour on non-consecutive days for 12 weeks. There was no change in oxidative stress in any of the individuals before or after the training. Vincent et al studied the effects of 6 months of resistance exercise on basal and post exercise lipid peroxidation in the elderly (Vincent, Vincent et al. 2002). Men and women (n=62; Age=68±6) were divided into a control, low intensity training (50% of one repetition maximum (1RM), 13
reps/ exercise) or high intensity training (80% 1RM 8 reps/ exercise). Subjects trained 3 times per week for 6 months. Blood was collected pre and post training to measure plasma levels of lipid peroxidation. Following training, lipid peroxidation was lower in the low intensity and high intensity groups by 14% and 18% respectively, compared to the control. Post exercise peroxidation levels were also lower in trained groups compared to the controls. Differences in training i.e. training mode, intensity, duration, and frequency all play a role in whether exercise training decreases oxidative stress. Future studies examining these factors and what impact they have on oxidative stress are important to determine the best way to lower oxidative stress.

2.6.7.4 Effects of Exercise Training on Antioxidants

There is also the question of the effect exercise may have on antioxidant concentration and activity at rest and following exercise. Higuchi et al examined the effects a regular treadmill running on the levels of activity of SOD and Catalase in skeletal muscle of rats (Higuchi, Cartier et al. 1985). Three-month-old female Wistar rats were subjected to a 3-month long training program. Rats were trained on a treadmill; with work rates increased up to 31m/min up a 15% incline for 2 hours daily, 5 d./week. This is the most strenuous running program that rats can perform. Results indicate that total SOD (mitochondrial + cytosolic) increased in the runners in the soleus and red vastus lateralis muscles. There was also an increase in mitochondrial SOD in the soleus, red vastus, and white vastus lateralis in the runners. There were no changes, however in the sedentary group. Catalase did not change in either group in any muscle type. Tonkonogi et al investigated the influence of endurance training on antioxidant defense mechanisms (Tonkonogi, Walsh et al. 2000). Eight healthy subjects (4 males and 4 females) attended training sessions 4-times/week for 6 weeks. Training sessions consisted of 30 minutes of cycling at a constant workload of 70% VO2peak followed by five 2 min bouts at 100% VO2 peak.
interspersed with 4 min periods of exercise at 50% VO2 peak. Muscle biopsies were performed before and after training for analysis of SOD and glutathione peroxidase levels. There were no changes in any of the enzyme levels following 6 weeks of endurance training. Ellosua et al also trained previously inactive men and women for 16 weeks (Ellosua, Molina et al. 2003). Initially, the frequency of the training sessions was four 30-minute sessions per week. The frequency and duration of the sessions was gradually increased to 5 days per week and 50 minutes per day during the first 8 weeks, and continued at this level for another 8 weeks. Blood samples were taken before and after training for plasma antioxidant concentration. Training induced an increase in SOD, glutathione peroxidase, and glutathione reductase in the blood.

2.6.7.5 Exercise and DNA Repair

Finally, the effect of exercise on DNA repair mechanisms has also been studied. Accumulation of DNA damage involves three factors, production, removal, and susceptibility of tissue. Measurements of urinary excretion of 8OHdG are indicative of the repair mechanisms of DNA from the oxidation of guanine. Once the base is cleaved off and repaired, the cleaved portion is transported into the blood and excreted in urine. Poulsen et al studied the effects of vigorous exercise on DNA modification (Poulsen, Loft et al. 1996). Twenty-three healthy males (age=22±2) participated in a 30-day physical training program consisting of 8-11 hours of vigorous exercise, 6 days per week. There was a 30% increase (p<0.02) in 8OHdG following the exercise training. Although extreme, this may indicate both an increase in DNA damage and up regulation in repair following strenuous exercise. However, results in long distance runners demonstrated no difference in urinary levels of 8OHdG when compared to controls. Pilger et al investigated the influence of habitual long distance running on the urinary level of 8OhdG (Pilger, Germadnik et al. 1997). Long distance runners (27 men, 5 women, age 43±11 were
compared to a sedentary control group (28 men, 4 women, age 41±12). Twenty-four hour urinary concentration of 8OHdG was measured. There were no statistical differences in the level of 8OHdG between the runners and controls. Furthermore, there was no relationship between weekly running distance and 8OHdG excretion. This suggests that DNA repair is unaffected by exercise. However, Radak et al revealed that exercise might alter the activity of DNA glycosylases in skeletal muscle (Radak, Naito et al. 2002). Six trained male runners participated in a marathon race. Skeletal muscle biopsies of the vastus lateralis were performed four days before the race and 16-18 hours after. Results indicate that there was a significant increase in activity of the repair 8OHdG enzyme, hOGG1. These findings indicate that there is a DNA repair response following extreme exercise. Radak et al also demonstrated that 8 weeks of regular exercise training increased repair enzymes in gastrocnemius muscles in rats (Radak, Naito et al. 2002). Animals were assigned to two groups, sedentary or exercise. Exercise training resulted in an increase in 8OHdG repair enzyme content in muscle.

2.6.7.6 Antioxidants

Longevity studies have attempted to establish a relationship between antioxidant concentrations and maximum lifespan potential (MLSP). For example, Sohal et al compared maximum lifespan potential in six different mammalian species including the mouse, rat, guinea pig, rabbit, pig, and cow (Sohal, Sohal et al. 1990). Activities of SOD, catalase, and glutathione peroxidase were measured in the liver, heart, and brain. There was a direct correlation between SOD activity and MLSP in the liver (r=0.72, p<.001) and the heart (r=0.47, p<.01). There was also a direct correlation between catalase and MLSP in the heart (r=0.67, p<.0001). Finally, there was an indirect relationship between glutathione peroxidase and MLSP in the brain (r=-0.76, p<.0005). However, Tolmasoff et al were unable to reproduce the same results
(Tolmasoff, Ono et al. 1980). They measured cytosolic SOD activity in 2 rodent and 12 primate species including humans. They did not observe a relationship between SOD activity and MLSP. A number of studies have examined the effects of antioxidants on lifespan through transgenic research. For example, Van Remmen et al have investigated the effect of knocking out the SOD2 (mitochondrial) gene (Van Remmen, Ikeno et al. 2003). The result was a mouse heterozygous for the SOD2 gene (SOD2+/-). These mice have reduced SOD activity by 50% compared to wild type animals and have evidence for increase oxidative stress as assessed by elevated levels of 8OHdG in nuclear DNA from the liver, brain, heart, and spleen. In addition, the findings revealed elevated 8oxodG in mtDNA in the liver and the brain. Unexpectedly, these animals did not have a shortened lifespan or markers of accelerated aging such as cataract formation; immune response, etc compared to the wild type animals. On the other hand, multiple deficiencies in antioxidant enzymes can result in increased sensitivity to oxidative stress. Van Remmen et al examined mice that were deficient in MnSOD and glutathione peroxidase (Sod2+/--; Gpx1+/--) (Van Remmen, Qi et al. 2004). These mice demonstrate a 50% reduction in MnSOD activity and no detectable Gpx activity. Fibroblasts from the knockouts were more sensitive to induced oxidative stress than wild types. Furthermore, endogenous oxidative stress induced by cardiac ischemia/ reperfusion injury led to greater apoptosis in heart tissue from the knockouts than from the wild types. They did not measure lifespan in these studies. Overexpression of antioxidants leads to increase in lifespan. Orr and Sohal investigated the effects of simultaneous expression of Cu-Zn SOD and catalase in Drosophila melanogaster (Orr and Sohal 1994). Transgenic flies carrying three copies of each of these genes exhibited a one-third extension of lifespan, lower amount of protein damage, and delayed loss in physical performance. Parkes et al who generated transgenic Drosophila which express human SOD1 specifically in adult motor
neurons support this (Parkes, Elia et al. 1998). The overexpression of SOD1 in a motor neuron extends normal lifespan by 40% and rescues the lifespan of the SOD null mutant. Finally, Melov et al augmented the antioxidant systems of Caenorhabditis elegans with synthetic SOD/catalase mimetics (Melov, Ravenscroft et al. 2000). Treatment to increase catalase and SOD activity resulted in an increase in lifespan by 44%. Additionally, treatment to prematurely aging worms resulted in normalization of their lifespan, which was a 67% increase.

2.6.7.7 Antioxidant Supplementation and Exercise

There are number of dietary antioxidants including Vitamin E, C, the carotenoids, and selenium. Whether or not dietary supplementation reduces oxidative damage is questionable. Kannon et al examined the effect of vitamin B6 on free radical production, mitochondrial membrane potential, and lipid peroxidation in isolated cells (Kannan and Jain 2004). U937 monocytes were cultured with vitamin B compounds including pyridoxine, pyridoxal phosphate and pyridoxamine and H2O2 either alone or together for 2 hours. The results indicate that treatment with H2O2 increased free radical production and membrane potential. However, treatment with B compounds resulted in a marked decrease in free radical production and membrane potential. Furthermore, supplementation with B compounds resulted in a decrease in lipid peroxidation induced by H2O2. Lee et al examined the effects of α- Lipoic acid and coenzyme Q10 on lifespan in mice (Lee, Pugh et al. 2004). Lipoic acid is an antioxidant that acts as a cofactor for the enzymes pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. Coenzyme Q10 acts as electron carrier and proton translocator in the electron transport chain as well as directly scavenging radicals and regenerating α-tocopherol. Results indicate that there was no effect of supplementation of either of the antioxidants on lifespan but there seemed to be a change in gene expression indicating a state of reduced oxidative stress similar to what is seen
with caloric restriction. This is in agreement with research by Midaoui et al who examined the
effect of α-Lipoic acid supplementation in a rat model of hypertension (Midaoui and de
Champlain 2002). The Sprague-Dawley rats used in this study, when chronically glucose fed
will become hypertensive, and insulin resistant. Incidentally, these animals also have elevated
superoxide production. Supplementation with α-Lipoic acid resulted in a decrease in
superoxide production following glucose ingestion. Supplementation also resulted in increased
glutathione peroxidase activity in erythrocyte and plasma. Furthermore, α-Lipoic acid resulted
in a decrease in blood pressure and an increase in insulin sensitivity. Vitamin E supplementation
has also proven beneficial in another model of disease, lipoprotein E deficient mice. Mice
deficient in lipoprotein E develop atherogenesis as well as an increase in oxidative stress.
Pratico et al examined the effect of vitamin E supplementation on lipid peroxidation (urinary
isoprostane levels) and aortic lesions and isoprostanes in the arterial walls (Pratico, Tangirala et
al. 1998). Oral supplementation resulted in significantly reduced isoprostane generation and a
decrease in aortic lesions and isoprostane levels in the arterial wall. While supplementation may
prove beneficial in deficiency states, there may not be an added benefit under normal conditions.
There has been interest in the effect of supplementation on exercise induced oxidative stress and
longevity. Holloszy examined the effects of voluntary exercise with or without antioxidant
supplementation on lifespan (Holloszy 1998). Male Long Evans rats were assigned to four
groups: sedentary control, sedentary supplemented, running control, and running supplemented.
Supplemented animals received ascorbic acid and α-tocopherol for the duration of their life.
Holloszy demonstrated that antioxidant supplementation had no effect on maximum lifespan in
either sedentary or exercising rats, although he did not examine the effect on oxidative damage.
Vitamin E supplementation may decrease the amount of free radical interaction with cellular
membranes resulting from exercise. McBride et al investigated the effect of vitamin E supplementation on exercised induced oxidative stress resulting from resistance training (McBride, Kraemer et al. 1998). Subjects were supplemented with 1200 IUs of vitamin E once a day for 2 weeks. Plasma lipid peroxidation was significantly lower in the supplemented group following the exercise bout. Rokitzki et al used a combination of α-tocopherol (400IU/day) and ascorbic acid (200mg/day) to supplement 24 trained long distance runners (Rokitzki, Logemann et al. 1994). Supplemented individuals and controls were matched for age, weight, running time, and maximal oxygen uptake. Subjects were supplemented for 4.5 weeks before running a marathon. The control group was given placebo for 4.5 weeks. Blood was collected for assessment of catalase and glutathione activity as well as markers of lipid peroxidation immediately before and after the race as well as 24 hours after the race. There were no differences in plasma lipid peroxidation between groups at any time points during the study. There were also no changes in catalase or glutathione peroxidase activity between the groups.

2.6.7.8 Exercise and the Elderly

There is also an interest in antioxidant supplementation in the elderly. Jessup et al supplemented 59 elderly individuals (age-76±4yrs) 800IUs of vitamin E daily for 16 weeks (Jessup, Horne et al. 2003). There was a decrease in lipid peroxidation in individuals following supplementation both at rest and following an exercise bout. In addition, subjects who underwent endurance exercise training (two 1 hour bout of exercise/week) in addition to supplementation had reduced lipid peroxidation following the exercise session. This is in contrast with Meijer et al who also investigated the effect of supplementation on exercise induced oxidative stress in elderly subjects (Meijer, Goris et al. 2001). Twenty-two elderly subjects (age=60±1yrs) were supplemented with 100mg α-tocopherol, 200mg ascorbic acid, and
2mg β-carotene per day for 12 weeks. There was no effect of supplementation on the exercised induced oxidative stress.

2.6.7.9 Exercise and Antioxidant Deficiency

Antioxidant deficiency however may result in increased oxidative stress following exercise. Watson et al investigated the effects of short-term restriction of high antioxidant foods on oxidative stress markers and antioxidant defenses during acute exercise (Watson, Callister et al. 2003). Antioxidant rich foods were restricted in 17 athletes exercised to exhaustion and blood antioxidant and oxidative stress markers were examined. Participants followed habitual diets high in antioxidants prior to an initial exercise test and then followed a two-week restricted-antioxidant (R-AO) diet before a second exercise test. During both exercise tests blood samples were taken pre-exercise, post sub-maximal exercise, post exhaustion and with one hour recovery. The R-AO diet reduced antioxidant intake 3-fold when compared to habitual-antioxidant (H-AO) intakes. Isoprostanes were significantly increased following sub-maximal exercise (38%), exhaustive exercise (45%) and one-hour recovery (31%) on the R-AO diet compared to the H-AO diet. Isoprostanes during exercise when following the H-AO diet remained relatively unchanged from rest. Total antioxidant capacity and circulating antioxidants were not significantly affected by the R-AO diet, but tended to be lower. Exercise performance was not affected by the R-AO diet. Athletes may require a higher antioxidant intake than regular individuals although athletic performance does not seem to be effected on an antioxidant restrictive diet.

2.6.8 Energy Expenditure

Total daily energy expenditure (TDEE) can be divided into three main components. Basal or resting metabolic rate (BMR or RMR) is the energy required to maintain the integrated
systems of the body and maintain body temperature. RMR is the energy expended by a subject resting in a relaxed state in the morning in the fasting state under comfortable, ambient conditions. It is the sum of the sleeping metabolic rate (SMR) and the energy cost of arousal. RMR accounts for approximately 60-70% of total daily energy expenditure (Tataranni and Ravussin 1995). The second component of TDEE is thermogenesis, which is defined as the

**Figure 2.5. Components of Energy Metabolism**

increase in metabolic rate in response to a stimulus such as food, cold or heat, psychological influences, or in response to drug administration. The most common form of thermogenesis is in response to food intake. Diet induced thermogenesis and accounts for approximately 10% of TDEE (Ravussin 1993). Finally, physical activity level is the most variable component of
energy expenditure. It can be divided into 2 main components. Energy spent for exercise and energy expended on activity that is not exercise i.e. spontaneous physical activity or also known as non-exercise activity thermogenesis (NEAT) (Ravussin, Lillioja et al. 1986; Levine, Eberhardt et al. 1999). Physical activity can account for as little as 20% in sedentary individuals to 50% in very active individuals. Figure 2.5, adapted from Ravussin et al (Ravussin 1993) illustrates the components of energy expenditure as well as the methods to measure them.

Different organs in the body contribute differently to oxygen consumption. For example, adipose tissue, which comprises 21-33% of body weight in a reference male and female, only accounts 4-5% of total oxygen consumption. The majority of the resting energy expenditure (60%) takes place in organs such as liver, kidney, heart, and brain, which account for only 5-6% of body weight. Muscle on the other hand, which is the largest tissue in the body accounts for approximately 20-25% of total energy expenditure (Zurlo, Larson et al. 1990). However, oxygen consumption by muscle can increase 10-20 fold during periods of intense activity.

It has been suspected that energy expenditure may affect oxidative stress. In particular, that variations in resting metabolic rate and physical activity level influence individual levels of oxidative stress. It has been hypothesized that individuals, who have a higher resting metabolic rate, will produce more free radicals and accumulate more oxidative damage. Adelman et al reported levels of background DNA damage in four species and compared the levels to species-specific metabolic rate (Adelman, Saul et al. 1988). Metabolism and oxidative stress were compared in the mouse, rat, monkey, and human. Measurements of energy metabolism and weight adjusted DNA oxidative stress were taken for 6 consecutive days. There was a significant relationship between species-specific metabolic rate and DNA damage. While there
may be a relationship between metabolic rate and oxidative stress, there are a number of factors that affect resting metabolic rate that must be considered.

2.6.8.1 Factors affecting Metabolic Rate

Early observational studies in animal models identified a number of factors that seem to affect oxygen consumption. Pennycuik compared the effects of a number of factors on metabolic rate of the mouse (Pennycuik 1967). Male and female mice were kept in chambers to measure metabolic rate. Mice were exposed to varying degrees of temperature to assess adaptation to differing environments. Observations on the effect of various temperatures in mice indicate that mice acclimatized to higher ambient temperatures had a lower rate of oxygen consumption than those acclimatized to lower temperatures. Hairless mice also consumed more oxygen than mice with hair. Other factors such ear and tail length also varied with metabolism. Furthermore, mice of different genetic backgrounds were found to have significantly different metabolic rates. Morrison and Middleton studied body temperature and metabolism in the pigmy marmoset (Morrison and Middleton 1967). These studies revealed that females had RMR values more than 10% lower than males. Results also indicated that metabolic rate was maintained over a wide range of ambient temperatures (30-40°C).

2.6.8.2 Body Composition

Body composition consistently appears to be a factor affecting both resting metabolic rate and total energy expenditure. Studies in animals helped to determine the relationship between body mass and energy expenditure. Goldstone et al examined RMR in baboons (p. ursinus) and compared to estimated body surface area (Goldstone, Savage et al. 1967). Although RMR did not correlate with estimated surface area, it did correlate with body weight, specifically weight^{9229}. This is supported by results from Banerjee and Bhattacharjee that demonstrated
RMR was least correlated with surface area and highest with fat free mass (Banerjee and Bhattacharjee 1967). The development of more advanced techniques such as dual energy X ray (DEXA), has allowed for the determination of separate body compartments, specifically bone mass, fat free mass, and fat mass. Ravussin et al assessed factors affecting resting metabolic rate in 177 Pima Indian and Caucasian adults (Ravussin, Lillioja et al. 1986). They found that FFM explained 67% of the variation in RMR. Fontvieille et al also determined in Pima Indian and Caucasian children, that FFM and FM explained 86% of the variation in RMR (Fontvieille, Dwyer et al. 1992). They also found a significant correlation with weight (p<.0001). Similar results were also seen with sleeping metabolic rate (SMR) (Webb 1981). Segal et al also noted the influence of lean mass on RMR (Segal, Lacayanga et al. 1989). They performed two studies to assess the relationship between FFM and RMR. The first study had two groups of men, one lean, and one obese. The men were matched for absolute body fat but had different FFM and total weight. Absolute RMR was higher in the lean group. However, once adjusted for FFM, there were no longer differences between the groups. The second study had two groups of men of different weights. They were matched for percent fat but had different amounts of FFM, FM, and total weight. RMR was higher in the group that weighed more but again after adjustments for FFM were made, there were no longer any differences. This is further supported by Rodriguez et al who measured resting energy expenditure and body composition in 116 lean and obese children (Rodriguez, Moreno et al. 2002). Again, RMR was higher in the obese but not after adjustment for lean mass. Larger studies indicate that both fat (.004) and fat free (.0001) mass determine RMR (Sharp, Bell et al. 2002). Many of these conflicting results are due to the difficulty in accurately identifying and measuring metabolically active body components. Fat-free body mass (FFM) is often considered the metabolically active compartment and is widely
used to adjust between-subject differences in resting energy expenditure for body composition. The use of FFM as the metabolically active portion of body weight makes the assumption that the body cell mass (BCM) component, which is more difficult to measure, maintains a relatively constant relationship to FFM within and between subjects. However, as Gallagher et al (Gallagher, Visser et al. 1996) has demonstrated BCM does not maintain a fixed relationship to FFM, as often assumed, but varies significantly and independently of FFM with age, adiposity, and gender. In addition, body mass can be divided into two main components; high metabolically active tissue, which includes organs such as brain, and liver, and low metabolically active tissue which includes bone and adipose tissue. The proportion of body mass of these components changes with increasing body size. Therefore, with greater size, the proportion of both body mass and fat free mass as high-metabolic rate organs tissues decreases and the proportion of body mass as low-metabolic rate organs increases. Heymsfield et al used magnetic resonance imaging that enables researchers to quantify the volumes of all major heat-producing tissues and organs in healthy subjects (Heymsfield, Gallagher et al. 2002). They concluded that the magnitude RMR/fat free mass ratio will decrease with increasing body mass which is due to the changes in proportion of high and low metabolically active tissues. These studies indicate that the relationship between resting metabolic rate and body composition is much more complex than a linear relationship across body size.

In addition, Zurlo et al investigated skeletal muscle metabolism and the effect on resting energy expenditure (Zurlo, Larson et al. 1990). In this study they measured resting energy expenditure in a metabolic chamber in 17 Caucasian males (age=30±6). Forearm oxygen uptake was measured by capacitance plethysmography and blood gas analysis and was adjusted for volume. Forearm oxygen uptake correlated with RMR (r=. 72) and may account for some of the
variability in resting metabolic rate. In this same study, muscle biopsies were performed on the vastus lateralis muscle. In vitro oxygen consumption was measured by an oxygen polarograph. Electron microscopy was also used to determine mitochondrial morphology. Volume density of mitochondria and volume fraction of the cell occupied by the mitochondria were calculated. There was no relation between mitochondrial morphology and any measures of energy expenditure (Kirkwood, Zurlo et al. 1991).

2.6.8.3 Sex

A number of studies have addressed the issue of whether or not sex is an independent determinant of resting metabolic rate. Earlier studies compared resting metabolic rate using indirect calorimetry and body composition by skinfold measurements and total body water. Banerjee and Bhattacharjee analyzed factors that affected RMR in 43 college students (21 male; 22 female; age between 18-20yrs) after a 14 hour fast (Banerjee and Bhattacharjee 1967). Absolute RMR was higher in the males than the females. However, when expressed in terms of fat free mass, there was no longer a difference between males and females. Cunningham performed a reanalysis of factors affecting RMR on the classical studies by Harris and Benedict (Cunningham 1980). Lean body mass was calculated from weight, height, and age. This reanalysis also demonstrated that once adjustment for lean body mass is made, the influence of sex adds little to the estimation of RMR. However, larger trials with more advanced techniques have revealed mixed results. Ferrero et al examined RMR in 235 individuals (114 males; 121 females age=34±14) (Ferraro, Lillioja et al. 1992). Body composition was measured by underwater weighing and RMR by indirect calorimetry (hood) following an 11 hour fast. Percent fat was calculated using Siri’s formula. Males had significantly higher RMR even after adjustment for body composition. Another study by this same group found males to have a
higher adjusted RMR (Fontvieille, Ferraro et al. 1993). RMR was investigated in 245 individuals (63 white males, 59 white females; 68 Pima males; 55 Pima females) by indirect calorimetry. Body composition was also measured by underwater weighing. The results of this study indicate that males had significantly higher RMR than females even after adjustment for body composition. However Klausen et al did not find this relationship (Klausen, Toubro et al. 1997). They investigated RMR in 313 individuals (78 males age 34±11 and 235 females age 36±11). Body composition was measured by bioelectrical impedance and RMR was measured in a respiratory chamber following a 12 hour fast. There were no significant differences in RMR between males and females following adjustment for body composition. Similar results have been found in older people. Starling et al studied RMR in 99 older individuals (48 men and 51 women age 69±8yrs) (Starling, Toth et al. 1998). They also found that there was no difference between men and women after adjustment for body composition. However, these same studies by Starling et al found significant differences between men and women in African Americans (Starling, Toth et al. 1998). However, Blanc et al examined RMR in 288 individuals (72 white men; 72 black men; 77 white women; 67 black women) aged 70-79 yrs to find that body composition explained the differences between men and women in both African Americans and Caucasians (Blanc, Schoeller et al. 2004). Finally, Gallagher et al hypothesized that women have less body cell mass, which is the active component of FFM and this may be a cause of the lower BMR (Gallagher, Visser et al. 1996). This is supported by Buchholz et al who examined factors affecting RMR (Buchholz, Rafii et al. 2001). Body composition was measured by total body water. While RMR was no longer significant once adjusted for FFM, it did remain significant after adjustment for body cell mass.
2.6.8.4 Age

A number of cross sectional studies has revealed that absolute resting metabolic rate declines as a function of age. McGandy et al measured basal oxygen consumption in 252 men aged 20-99 yrs using indirect calorimetry (McGandy, Barrows et al. 1966). Their results indicate a decline in RMR per decade of approximately 52 cal per day, although this assumes a constant body weight. Once adjusted for body weight, the decline in RMR plateaus after the age of 60. Calloway and Zanni also investigated the relationship between age and resting metabolic rate in six healthy men aged 63-77 yrs (Calloway and Zanni 1980). The men were confined to a metabolic unit for 47 days and received a defined formula diet. RMR of the older men was 13% below the rate of a younger group (1622±189 vs. 1875±220 kcal/day). However, once adjusting for whole body potassium content this difference disappeared. They concluded that the there is no decline in RMR with age once adjustments for body composition are made. However, there were only six subjects in this study. This has been supported by Tzankoff and Norris who showed that after 45y of age there was a progressive reduction of RMR, which was related to a concomitant reduction in skeletal muscle mass (measured by 24-hour urinary creatinine excretion) (Tzankoff and Norris 1977). Conversely, there have been a number of cross sectional studies that have demonstrated a decline in RMR with age independent of changes in body composition. For example, Fukagawa et al compared RMR and age in a group of old men (n=24; 69-89yrs) to a group of young men (n=24; age 18-33yrs) (Fukagawa, Bandini et al. 1990). Body composition was assessed by bioelectrical impedance and total body water. RMR was lower in the old men than the young (1.04±0.02 vs. 1.24±0.03 kcal/min, p<0.001) and remained lower even after adjusting for FFM (p<0.001). This has been supported by a number of other cross sectional studies (Pannemans and Westerterp 1995; Visser, Deurenberg et al.
1995; Klausen, Toubro et al. 1997; Hunter, Weinsier et al. 2001) as well as longitudinal research. Keys et al measured basal metabolic rate on young men (age=18-26yr) and repeated 19 years later (Keys, Taylor et al. 1973). RMR was also repeatedly measured over a period of 22 years on men initially aged 44-56 yrs. In the younger men, basal metabolic rate decreased 3% in 19 years, but these men gained approximately 10.6 kg so the actual decline in BMR was 9% per unit of body weight. The older men only gained 1 kg and there were no changes in BMR with age. They concluded that although there is a decline in adjusted RMR with age, it might not be as dramatic as once thought, the decline only being 1-2% per decade. In addition, Vaughn et al compared 24h chamber energy expenditure and its different components (resting metabolic rate, thermogenesis, physical activity, and sleeping metabolic rate) in young and old subjects (71±6y) (Vaughan, Zurlo et al. 1991). After adjustment for fat free mass, RMR was the only significantly lower measure in old as compared to young subjects. Piers et al attempted to determine if age related decline in metabolic rate was related to changes in the quality of lean tissue mass (Piers, Soares et al. 1998). Body composition was assessed by DEXA and divided into 4 compartments, bone mineral mass, fat mass, appendicular lean tissue mass, and nonappendicular lean tissue mass. Measurements were performed on 38 young (n = 38, 18-35 yr, weight=65.9±10.9kg) and 24 older (n = 24, 50-77 yr, 72.6±10.8kg) subjects. There were significant differences in absolute RMR between the two groups, which persisted even after adjustment for body composition. In fact, older subjects had significantly lower RMR after adjustment for appendicular lean tissue mass which accounted for 78% of the variance in RMR. However, there is also the question of whether the decline in RMR with age is related to reduced physical activity. Poehlman et al examined the influence of age and habitual physical activity level on resting metabolic rate (Poehlman and Danforth 1991). There were four groups in the study defined by age and habitual
activity level assessed from a questionnaire; an active younger group, an active older group, a sedentary younger group, and a sedentary older group. The active groups were comprised of runners with a frequency of running at least 3 times per week (24+6km/wk) for the younger subjects and 28+7km per week for the last 12 years for the older subjects. RMR adjusted for FFM was lower in the sedentary men relative to the other three groups. This is supported by Van Pelt et al who hypothesized that the decline in RMR with age is due to changes in physical activity level (Van Pelt, Jones et al. 1997). Sixty-five, healthy, weight stable women aged 21-35 or 50-72 yrs were included in the study. Resting metabolic rate was measured in 12 premenopausal and 15 postmenopausal sedentary women and 13 pre and 15 postmenopausal distance runners. RMR was adjusted for FFM and FM. Adjusted RMR was 10% (P<0.001) lower in the postmenopausal vs. premenopausal sedentary women. However, there were no differences in RMR between the active women. This was also observed in a similar study performed in a group of 137 weight stable males (Van Pelt, Dinneno et al. 2001). RMR was adjusted for FFM. While RMR was lower in both the sedentary and active older men compared to the younger men, adjusted RMR was not significantly different in the subgroups of physically active men when adjusted for exercise volume or food intake. Therefore, physical inactivity may also contribute to the relationship between RMR and aging. There are a number of possible mechanisms responsible for the decline in RMR with age. Changes in the quantity and quality of lean tissue can affect RMR. For example, decreases in number of myocytes or liver volume can result in a decline in RMR. Other possibilities are increase in rate of degenerative changes including increased lipid deposition, tubular dilation, lipofuscin deposits, decreased protein turnover, or decline in Na/K channel activity (Lie and Hammond 1988; Olivetti, Melissari et al.)
In addition, a decline in physical activity with age may also add to the decrease in resting metabolic rate seen with aging.

2.6.8.5 Race

A number of studies that have looked at the effect of sex on RMR have also looked at the effect of race. Studies by Fontvieille comparing resting metabolic rate and body composition in Pima Indian children and Caucasian children (Fontvieille, Dwyer et al. 1992). Forty-two Caucasian children (21 males; 21 females) and 43 Pima Indian children (22 males; 21 females) were studied. RMR was conducted after 10 hours of fasting and body composition was assessed by bioelectrical impedance and skinfold thickness. Results indicate that after adjusting for body size, the effect of race was removed. However, this same group also reported that there was a race difference between RMR/SMR ratio, which is a measurement of activation of CNS and SNS, and its effect on energy expenditure. Others have concentrated on the race differences between African Americans and Caucasians. Sharp et al investigated factors affecting metabolic rate in 395 adults (95 white men; 100 black men; 106 white women; 94 black women) between 28-40 years of age (Sharp, Bell et al. 2002). Again, RMR was measured by indirect calorimetry but body composition was measured by Dual Energy X-ray Absorptiometry (DEXA) and CT-scan. RMR was higher for Caucasians even after adjustment for body composition. The difference was the same for men and women. This is supported by Blanc et al who measured RMR and body composition in African American (n=139) and Caucasian (n=149) men and women (Blanc, Schoeller et al. 2004). Body composition was also measured by DEXA. RMR adjusted for FFM was lower in African Americans than Caucasians in both men and women (p<.001). Jones et al also examined racial differences in RMR of 44 women (22 AA women age 43±17yrs; 22 C women age 41±15yrs) (Jones, Shen et al. 2004). In this study, however, body
composition was assessed by DEXA and magnetic resonance imaging (MRI). By combining these two measurements it is possible to calculate four tissue compartments, adipose tissue, skeletal muscle, bone mineral mass, and residual mass (brain, liver, kidney, heart, and GI). The tissue is then grouped into either skeletal mass (adipose tissue, skeletal muscle, and bone) or residual mass (brain, liver, kidney, heart, and GI). Residual mass is much more metabolically active at rest than skeletal mass. Skeletal mass was higher in AA than C women. Results indicate that lower RMRs in AA women than C women are related to corresponding differences in proportions of heat producing tissues and organs, and these differences increase as a function of body weight.

2.6.8.6 Physical Activity

There are many factors concerning the relationship between physical activity, fitness level, and RMR. For example, early studies measured food intake and body weight in an effort to estimate energy expenditure in athletes. Edwards et al studied 8 Harvard football players in an effort to estimate the impact of intense physical activity on daily energy expenditure (Edwards, Thorndike et al. 1989). Food intake and body weight was measured on three occasions for several days during the football season. Results from these studies estimated that average energy requirements of the players was approximately 5,600 kcal during the periods studied. Others have looked at the residual effects of exercise on resting metabolic rate. Passmore and Johnson, measured oxygen consumption in 10 men following prolonged periods of moderate exercise (Passmore and Johnson 1960). The men performed a 10-mile walk on a treadmill at 4 miles/hr. Oxygen consumption was measured up to 8 hours following the walk. Oxygen consumption did not return to baseline for at least 7 hours following the walk. However, they did not study beyond 7 hours. Bielinski et al also investigated the residual effect of exercise on RMR in 10
healthy males (Bielinski, Schutz et al. 1985). Their results indicate that RMR remains elevated (+4.7%) up to 20 hours after 3 hours of exercise at 50% VO2max. On the other hand Freedman-Akabas et al found no difference in RMR following exercise (Freedman-Akabas, Colt et al. 1985). Twenty-three subjects (age 25-45 yrs) were studied after a 30min rest period 4 hours after breakfast and up to three hours after 20 minutes of exercise. They observed no difference in RMR following the exercise bout than during the sedentary period. However, their exercise session was much shorter than other studies indicating that the exercise bout may have been insufficient to produce residual effects. Since then, the focus has been whether or not regular physical activity elevates resting metabolic rate independent of the residual effects of the exercise bout. For instance, Tremblay et al performed a series of studies to test this effect. They first compared resting metabolic rate in a cohort of 59 individuals comprising 10 trained (age=34) and 39 un-trained (age=21) individuals (Tremblay, Fontaine et al. 1986). These individuals were of similar body weight (trained= 68.5±1.4 vs. untrained =68.0±1.4), however, fat free mass (62.5±1.2 vs. 59.3±1.0) and percent fat (8.4±0.7 vs. 12.4±0.9 59.3) were significantly different. The absolute RMR was 11% higher in the trained group than in the untrained group. When comparing regression lines for RMR verses FFM between the two groups, the line for the trained was higher than the untrained. They then went on to train 8 moderately obese women for 11 weeks to test the effect of training on RMR. The women were trained for 5 hours a week at 50% VO2 max. Results indicate that there was an 8% increase in RMR from baseline after adjustment for changes in body composition. Finally, they examined the effect of a 3-day interruption of exercise training on resting metabolic rate in trained individuals. Eight endurance-trained athletes were tested before and after three days of cessation of their regular training programs. The athletes were competitive runners and cross country
skiers. Mean training time was approximately 10 hours a week. Individuals were tested 16 hours after a 90-minute exercise bout and following a 3-day interruption of training. A significantly lower RMR was observed in subjects following 3 days of exercise cessation in comparison to the morning after the 90-min exercise bout (1.19±0.04 vs. 1.27±0.04 kcal/min, p<0.05) (Tremblay, Nadeau et al. 1988). Poehlman et al also compared RMR in highly trained and untrained individuals (Poehlman, Arciero et al. 1988). The highly trained males in this study were distance runners running approximately 100-160km/wk. Their results demonstrate that both absolute and adjusted RMR was higher in the trained than untrained subjects. They also compared RMR and fitness level in a group of males with a wide range of VO2max. Twenty-eight nonobese males were grouped into highly trained (training distance 80-120km/wk), moderately trained (running 3-4 times/wk), and untrained (no regular activity). RMR was measured 24 hours after the last exercise session. There was a significant correlation between VO2max and RMR (r=.77; p<0.01). This persisted after adjustment for FFM. Furthermore, the highly trained individuals had a significantly higher RMR than either the moderately trained or untrained groups. However, Schulz et al used a respiratory chamber to measure the effect of exercise training on the components of 24 energy expenditure including RMR, SMR, and thermic effect of food where only spontaneous physical activity was allowed (Schulz, Nyomba et al. 1991). Results from 20 highly trained male endurance athletes (25±6 y, 70±8 kg body weight, 64±7 kg fat free mass, 70±8 ml/kg/min) were compared to 43 untrained males who were matched for age (28±6), body weight (73±13kg), and fat free mass (62±8 kg). No significant differences were found with respect to 24-h EE, RMR, or SMR between trained and untrained subjects before or after adjusting for differences in body composition. A similar study conducted by the same group in elite female runners also indicated that there was no effect of intense
physical activity on any measures of sedentary energy expenditure, including RMR (Schulz, Alger et al. 1992). Tremblay et al also investigated the contribution of β-adrenergic stimulation to the increase in RMR observed in exercise-trained individuals (Tremblay, Coveney et al. 1992). Nine trained (Age=30±5yrs; Weight=66.5±7.7kg; VO2max=67.8±8.6mL/kg/min) and 8 sedentary (Age=26±6yrs; Weight=67.1±5.8kg; VO2max=52.8±7.7 ml/kg/min) individuals were subjected to two testing sessions during which RMR was measured before and for 3 hours after oral administration of propranolol (β-adrenergic blocker) or placebo. Untrained subjects were requested to not perform any vigorous exercise for 48 hours before the test. The trained individuals were instructed to maintain their usual training program including an exercise bout of at least 60 minutes the day before the test. Resting metabolic rate was higher in the trained group before the administration of propranolol and throughout the placebo test in comparison with the sedentary control. Propranolol decreased RMR in the trained group; however there was no difference in the untrained group. These results indicate that β-adrenergic stimulation may be involved in the exercised induced increase in RMR. This is supported by Poehlman and Danforth who demonstrated that endurance training increases metabolic rate and norepinephrine (NE) appearance rate in older individuals (Poehlman and Danforth 1991). Nineteen older individuals (13M; 6F) cycled 3 times per week for 8 weeks. The subjects began exercising the first week to expend 150kcal at 60% VO2max. The duration and intensity was incrementally increased so that by the beginning of the eighth week, the participants were exercising at 85% expending 300kcal per session. Norepinephrine kinetics was assessed under steady state conditions using the tritiated isotope dilution method. Labeled NE was infused for 60 minutes and blood samples were drawn 50, 55, and 60 minutes later for determining steady state conditions of plasma NE and calculation of appearance and clearance rates. Training increased
VO_{2\text{max}} by 14% and resulted in a 21% increase in NE appearance with no changes in clearance. Moreover NE appearance was correlated with the increase in RMR (r=.57; p=0.05). Lennon et al also demonstrated that moderate daily exercise was able to attenuate the decline in RMR resulting from caloric restriction (Lennon, Nagle et al. 1985). Seventy-eight subjects aged 20-49 (38M; 40F) were randomized into one of three groups, control (diet only), a diet-exercise group which selected their own activities, and a diet-prescribed exercise group which adhered to a program of jogging exercise for 12 weeks. RMR was significantly greater in the diet-prescribed group than either of the other groups (p<0.05). When separated by sex, the diet prescribed females had a higher RMR than control. There were no differences in males.

2.6.8.7 Genetics

Heredity is another factor that has been shown to contribute to basal metabolic rate. Earlier studies tried to control environment in an effort to uncover what effect genetics might have on energy metabolism. Edholm et al used such a design to investigate food intake and energy expenditure on 12 cadets at a military establishment (Edholm 1955). All the food eaten by each individual cadet was weighed every day for 2 weeks. Expenditure was measured during a number of activities including sleeping, sitting, dressing, standing, walking, cycling, sport, and drill. The energy expended on any one task, especially a basal one such as sitting, lying, or standing, often varied very much from one man to the next. Booyens et al attempted to demonstrate that in a group of healthy and outwardly homogenous group of people, there are some who expend considerably less energy than others (Booyens and McCance 1957). Energy expenditure was measured in 36 individuals (22M; 14F) while lying, sitting, and standing. There was a great deal of inter-subject variation for the group in all three positions. Three subjects had rates more than 30% below, and one a rate of more than 24% above the average for the group.
These early studies indicated that even after adjustment for body composition and sex, there is still considerable inter-individual variation in resting metabolic rate.

A second approach to examining heritable characteristics of energy expenditure is Twin and Familial studies. Fontaine et al examined the genetic effect in RMR with data obtained in 20 monozygotic (MZ) and 19 dizygotic (DZ) male twin pairs (Fontaine, Savard et al. 1985). Resting metabolic rate was measured after a 12-hour overnight fast. Subjects were requested to refrain from exercise for 48 hours before the test. Body composition was assessed by hydrostatic weighing using Siri’s equation. Results revealed that the intraclass coefficient between monozygotic twins was between 0.45 and 0.81 suggesting that heredity may contribute to BMR.

In another study by this same lab, resting metabolic rate was measured in 37 pairs of male monozygotic twins and 21 pairs of male dizygotic twins (Bouchard, Tremblay et al. 1989). The heritability of RMR reached approximately 40% of the variance remaining after adjustments for age, gender, and fat free mass. Bogardus et al investigated the effect of familial dependence on resting metabolic rate. They studied resting metabolic rate in 130 adult southwestern American Indians (74 men and 56 women) from 54 families. Results indicate that family membership accounted for an additional 11% (p<0.0001) of variance in RMR after adjustment for FFM, age, and sex.

2.6.8.8 Sympathetic Nervous System

There are a number of other factors that are thought to contribute to resting metabolic rate. Studies indicate that the sympathetic nervous system (SNS) is related to the three major components of energy expenditure, RMR, thermic effect of food, spontaneous physical activity. Saad et al measured the relationship between 24-hour energy expenditure measured in a respiratory chamber and 24 hour urinary norepinephrine excretion in 36 Caucasians and 33 Pima
Indians (Saad, Alger et al. 1991). Twenty-four hour energy expenditure correlated significantly with twenty-four hour norepinephrine excretion in Caucasians (p<0.001) bit not in Pimas independent of fat free mass, fat mass, and age. Physiological levels of epinephrine infusion result in increases in resting metabolic rate. Staten et al investigated the effect of very low epinephrine infusion rates (0.1, 0.5, and 1.0 μg/min) on metabolic rate (Staten, Matthews et al. 1987). Five young men (age=26+4yr, weight=70+7kg) were studied on four separate occasions to test the effects of epinephrine on metabolic rate. Epinephrine was infused for 4 hours with the appropriate infusion rate. For the control, saline was infused for 4 hours. A significant increase in RMR was noted with the lowest infusion rate. This resulted in an increase in RMR, which correlated with plasma levels of epinephrine. This indicates that the SNS may be partly responsible for regulating RMR under eucaloric conditions. Jung et al investigated the effect of propranolol on RMR in obese patients on a normal energy intake and in response to slimming (Jung, Shetty et al. 1980). Ten obese females (age=29+5 yr; weight=91.3+3.7kg, 58% above ideal body weight) were studied for 31 days. Subjects were placed on a weight maintenance diet for 10 days followed by a low energy diet (LED) for 21 days. The diets consisted of the same percentage of protein and fat with a reduction in carbohydrate. The women were assigned to one of two groups, a control group (n=4) and a group administered propranolol (n=6) from days 4-10 on the weight maintenance diet. Four of the six women receiving propranolol were re-administered it on day 14 during the low energy diet. Propranolol administration resulted in a drop in serum T₃ and an increase in reverse T₃ on the maintenance diet. T₃ remained low in the subjects who stopped taking propranolol. In addition, the women who began taking it again on the LED had an even further fall in T₃ during the LED. As expected, there was a drop in RMR in the controls on the low energy diet. Propranolol administration reduced resting metabolic rate
on the weight maintenance diet by 8.6% but had no effect on the low energy diet. Once the drug was stopped, the fall in RMR was maintained as the subjects were transferred to the low energy diet with no rebound in RMR seen. Those individuals who further received the drug during the LED saw only a 2% reduction in RMR, 2% less than the controls on the low energy diet. These effects however, may be race specific. Spraul et al examined sympathetic outflow to skeletal muscle and its relationship to energy expenditure (Spraul, Ravussin et al. 1993). Muscle sympathetic nerve activity (MSNA) was measured by microneurography in 25 Pima Indian (age=26±6yr, weight=82±19kg) and 19 Caucasian (age=29±5yr, weight=81±13kg) males. MSNA was lower in the Pimas compared to the Caucasians (p<.0007). Resting metabolic rate adjusted for FFM, FM, and age were significantly correlated with MSNA in Caucasians but not the Pimas. In addition, the effects of the SNS may also be independent of the thyroid. Welle et al also administered Nadalol, another β-adrenergic blocker for 1 week in five men and compared to changes in RMR (Welle, Schwartz et al. 1991). Nadalol is a non-selective blocker that does not affect T₃ production. After 6-10 days of treatment, RMR declined by 7% (p<.01) with no significant changes in serum T₃ or thyroxin (T₄) concentrations. Finally, research (reported earlier) indicates that the increase in RMR in trained individuals may be due to β-adrenergic stimulation (Tremblay, Coveney et al. 1992).

2.6.9 Caloric Restriction

Since 1935, calorie restriction (CR) is the only intervention that has consistently been shown to increase lifespan in a number of species including fish, yeast, flies, worms, mice, and rats (McCay 1935; Sohal and Weindruch 1996; Roth, Lane et al. 2002). CR induces a number of effects including reducing body fat, reducing fasting glucose and insulin, increasing insulin sensitivity, decreasing lipids, decreasing body temperature and total energy expenditure, and
slowing growth and reproduction (Kemnitz, Weindruch et al. 1993; Lane, Ingram et al. 1999; Roth, Lane et al. 2002).

One of the ways that CR is thought to extend lifespan is by a reduction in oxidative stress. Studies in mice demonstrate a 30% decrease in 8oxodG mutations in calorie-restricted animals (Sohal, Ku et al. 1994; Drew, Phaneuf et al. 2003). Similar results have also been observed in lipid peroxidation markers in rat kidney (Kim, Yu et al. 2002). In addition, a number of studies performed in rats indicate that there is a decrease in protein carbonylation in the brain. Reduced carbonyl accumulation was also observed in the liver of rats subjected to 25-40% CR (Youngman, Park et al. 1992). Similar results have also been observed in non-human primates. Rhesus monkeys subjected to caloric restriction exhibit down regulation of genes involved in oxidative stress (Kayo, Allison et al. 2001). Furthermore, they observed a decrease in levels of oxidative damage to proteins (Zainal, Oberley et al. 2000; Kayo, Allison et al. 2001).

One of the theories of caloric restriction is that it reduces oxidative damage by decreasing energy flux and metabolism (Sacher 1977; Sacher and Duffy 1979). CR results in a reduction in weight and reduction in metabolic rate. Whether there is a reduction in metabolic rate that is independent of changes in body composition is still under investigation. Studies performed in mice indicate that there is a decrease in absolute basal metabolic rate following prolonged caloric restriction. However, after adjusting for changes in FFM the changes in BMR are no longer evident (McCarter and Palmer 1992). However, many adjust RMR for FFM by simply dividing one from the other but it has been questioned that this is mathematically incorrect because the regression line between EE and body size does not pass through the zero intercept (Poehlman, Melby et al. 1991; Ramsey, Roecker et al. 1997; Blanc, Schoeller et al. 2003). When data has been normalized the appropriate way a reduction in resting metabolism been demonstrated in a
number of species following caloric restriction (Ramsey, Roecker et al. 1997; Blanc, Schoeller et al. 2003).

There have been number of studies that have addressed the effect of caloric restriction on energy metabolism in humans. Early studies were performed in lean men with a 50% reduction of food intake. This resulted in a decrease in RMR adjusted for body surface area, body weight, or cell mass (Keys 1950). Other studies performed in obese individuals indicate that following severe energy restriction; there is a reduction in resting metabolic rate even after adjustment for body composition (Keesejy 1989; Vansant, Van Gaal et al. 1989; Fricker, Rozen et al. 1991; Astrup, Gotzsche et al. 1999). In fact, the new resting metabolism is significantly less (≈15%) than in never-obese persons (Weigle, Sande et al. 1988; Weigle and Brunzell 1990; Leibel, Rosenbaum et al. 1995). However, these earlier studies were extreme states of caloric restriction that would be considered malnutrition. Individuals from Biosphere 2 who participated in 2 years of caloric restriction had decreases in adjusted 24- hour energy expenditure and spontaneous physical activity in a respiratory chamber when compared to 152 control subjects (Weyer, Pratley et al. 2000). Whether or not this reduction in metabolism independent of the changes in body composition has an effect of lifespan is still under investigation.

2.7 Aging

There are a number of changes that occur as organisms age. Body composition changes dramatically with aging. Poehlman et al studied 427 healthy men with an age range of 17-90 yrs to examine changes in body composition across the lifespan (Poehlman, Toth et al. 1995). Body fatness was estimated from body density as measured by hydrostatic weighing. Fat mass increased, and the rate was greater in women (r=.61; slope=0.25 kg/y; p<.01) than men (r=.43; slope=0.16 kg/y; p<.01). Interestingly, the increase in fat mass was strongly associated with
decreases in maximal oxygen consumption and physical activity. After adjusting for these
variables, the increase in fat mass declined from 17% to 3% per decade in men and from 26% to
5% in women. The Fels Longitudinal Study reported body composition data in 102 men and 108
women (Guo, Zeller et al. 1999). Body composition was determined by underwater weighing
and physical activity was assessed through questionnaires. Data was collected biennially from
1976 to 1996. There was a significant decrease in FFM and increases in total body fat, percent
fat, weight, and BMI with age. While age was associated with changes in body composition and
physical activity level, investigators were unable to account for all of the changes that occurred
with age, especially the decline in fat free mass. A number of studies have examined the
changes that specifically occur to skeletal muscle with age. Larsson et al examined muscle from
vastus lateralis of 55 untrained, healthy male subjects from 22-65 years of age (Larsson, Sjodin
et al. 1978). They determined fiber type and enzyme activity of ATPase, myokinase, and lactate
dehydrogenase. Individuals were divided in two groups, a younger group and an older group.
Results indicate that fiber type distribution changed towards a decrease in percentage of type II
fibers, both in type IIA and IIB. There were no differences in the activity of ATPase or
myokinase, while the activity of lactate dehydrogenase was highest in the younger group. Lexell
et al also compared muscle fiber composition in a group of older individuals to a group of
younger subjects (Lexell, Henriksson-Larsen et al. 1983). Muscle biopsies were taken from the
vastus lateralis of 12 previously healthy males (n= 6, age=30±6, range 19-37yrs and n=6,
age=72±1, range 70-73yrs) who had died a sudden accidental death. The size of the muscle from
the older individuals was 18% smaller (p<0.01) and the total number of fibers was 25% (p<0.01)
less than those of the young individuals. There was, however, no difference in fiber types
between the two groups. This is supported by Coggan et al who also demonstrated that type I
fibers occupy a larger percent of total muscle area in the older men and women because Type IIa and IIb fibers were 13-31% smaller (p<.001) in the older group (Coggan, Spina et al. 1992). They also found that activities of succinate dehydrogenase, citrate synthase, and β-hydroxyacyl-CoA dehydrogenase were approximately 25% lower (p<.05) in the older subjects. Similar results were observed by Pastoris et al who examine biochemical alterations due to aging in human skeletal muscle of sedentary subjects (Pastoris, Boschi et al. 2000). The activities of a number of enzymes were measured in muscle biopsies of 76 sedentary subjects (32 males and 44 females) between the ages of 15 and 91 years. There were age related decreases found in hexokinase and lactate dehydrogenase in the rectus abdominus, citrate synthase in the vastus lateralis, and a decrease in ATP and creatine phosphate concentration in the glutemus maximus. This is further supported by Balagopal et al who examined myosin heavy chain from skeletal muscle of 24 subjects aged 20-92 years (Balagopal, Rooyackers et al. 1997). They observed an age related decline in myosin heavy chain synthesis rate (p<.01) which may contribute to the declining muscle mass and contractile function in older individuals.

Similar changes in muscle mass are also seen in the myocardium. Olivetti et al compared heart muscle from 67 subjects who died form causes other than cardiovascular disease (Olivetti, Melissari et al. 1991). The age range was 17-90 years. Results indicate that the process was characterized by a loss of 38 million and 14-million myocyte nuclei/year in the left and right ventricles, respectively. The changes that occur in muscle mass with age are thought to be responsible for changes in maximal oxygen consumption. In 1975, Robinson et al studied the physiological response of men (age=18-22 years) to aerobic (5.6km/h, up 9% grade) and maximal treadmill work (Robinson, Dill et al. 1975). They were then reexamined 20 and 25 years later. They found that by the ages 40-44y, mean VO_{2max} had declined by 25%. Fleg and
Lakatta examined changes in muscle mass and VO\textsubscript{2max} in 184 nonobese volunteers, ages 22-87 years from the Baltimore Longitudinal Study (Fleg and Lakatta 1988). Subjects performed a VO\textsubscript{2max} test to determine maximal oxygen consumption and 24–hour urinary creatinine excretion was used as an index of muscle mass. A positive correlation was found between VO\textsubscript{2max} and creatinine excretion in men (r=0.64, p<0.001) and women (r=0.47, p<0.001). There was a decline in VO\textsubscript{2max} with age; however this disappeared once adjusted for the decrease in creatinine excretion. This was supported by Proctor and Joyner who also compared VO\textsubscript{2max} to muscle mass, particularly appendicular muscle mass measured by DEXA (Proctor and Joyner 1997). Measurements were made on 32 endurance trained young men (age=24±4yr) and women (26±4yr) and compared to a group of older men (64±4yr) and women (61±8yr). Subjects performed a VO\textsubscript{2max} test and body composition was determined by DEXA. Older men and women had approximately the same body weight as the younger individuals, but they were shorter and had 6-10% less FFM. There was an age- associated reduction in appendicular mass of 2.6kg in men and 2.8kg in women. These differences abolished the differences in VO\textsubscript{2max} between the groups.

Many researchers have investigated the effect of age on the electron transport system. Kwong et al studied the activities of the 4 complexes of the electron transport chain in mitochondrial isolated from brain, heart, skeletal muscle, liver, and kidney from 2.5 month old and 28-30 month old mice (Kwong and Sohal 2000). Their major finding was that although there was no common pattern to the changes among the complexes, post-mitotic tissues such as brain, heart, and skeletal muscle were more adversely affected than others. Furthermore, from young to old, there was a shift in the activity ratios among different complexes that would tend to hinder the ability of mitochondria to effectively transfer electrons down the chain. Desai et al
also studied the influence of age on electron transport chain activity (Desai, Weindruch et al. 1996). Electron transport activity was studied in gastrocnemius muscle from 10- and 20- month old mice. Activities of the complexes I, III, and IV decreased by 54-74% from 10 to 20 months. Complex IV contains high and low affinity-binding sites. The Km for the high affinity sites was not affected by age, but the km for the low affinity sites was approximately two-fold higher in the older mice. Similar age related trends are noted in humans. Boffoli et al investigated oxygen consumption and enzyme activity of isolated mitochondria from vastus lateralis of 63 healthy individuals undergoing orthopedic surgery (Boffoli, Scacco et al. 1994). There was a negative relationship between state 3-coupled respiratory activity and age (r=-0.682, p<.001). There were also negative relationships between age and NADH-cytochrome c reductase (r=-0.605, p<0.001), succinate-cytochrome c reductase (r=0.518, p<0.001), and cytochrome c oxidase (r=-0.421, p<0.01). The changes in the electron transport chain activity that occur may contribute to decreases in skeletal muscle function with aging. Recent studies by Petersen et al using nuclear magnetic resonance spectroscopy indicate that with aging there is a 40% reduction in mitochondrial oxidative phosphorylation activity (Petersen, Befroy et al. 2003). These changes may also have an impact on age related changes in resting energy expenditure. Furthermore, changes in the components of metabolism may be responsible for the increase in free radical production associated with age.

2.8 Centenarians

Studies in individuals that have lived to extreme old age can provide valuable information regarding aging and longevity. There are a limited number of studies in this special population (Koenig 2001). Studies in centenarians indicate that there is a genetic commonality in these individuals protecting them from aging and disease (Barzilai, Atzmon et al. 2003).
Healthy centenarians can be considered examples of successful agers. The compression of morbidity hypothesis predicts that in order to achieve extreme old age, centenarians markedly delay or even escape diseases that would otherwise be lethal at younger ages. Evert et al studied 424 centenarians from the New England Centenarian Study started in 1998 (Evert, Lawler et al. 2003). Participants filled out healthy history questionnaires to get information concerning disease history. They obtained lifetime diagnosis of 10 major lethal illnesses including but not all, hypertension, heart disease, diabetes, osteoporosis, and Parkinson’s, as well as alcohol and tobacco use. The authors concluded that the centenarians fit into one of 3 categories, survivors, delayers, or escapers. Twenty four percent of males and 43% of females fit into the survivor profile, those who had been diagnosed with an age associated illness and survived the illness past the age of 100 y. Forty four percent of the males and 42% of the females fit into the delayer category, delaying the onset of disease until at least 80yrs of age. Finally, 32% of the males and 15% of the females were considered escapers, attaining their 100th year of life without being diagnosed with disease. These results suggest there may be multiple routes to achieving exceptional longevity. Studies involving offspring of centenarians indicate that they too may also be protected against disease. Terry et al studied offspring of to see if they also had they same delay or escape of disease as their parents (Terry, Wilcox et al. 2003). Health history was obtained in the offspring of 192 centenarians who were also enrolled in the New England Centenarian Study. Controls of the offspring consisted of offspring whose parents were born in the same year as the centenarians but died at an average life expectancy. Prevalence of a number of age-related illnesses was compared between the two groups. Centenarian offspring had a 56% reduction in relative prevalence of heart disease of heart disease; a 66% reduced prevalence of hypertension, and a 59% reduced prevalence of diabetes. Offspring had more favorable lipid
profiles than controls, specifically HDL and LDL cholesterol levels. Barzilai et al examined biological and genetic factors that are associated with a human longevity phenotype (Barzilai, Gabriely et al. 2001). In this study, 213 individuals (age=98±5yr) from the Ashkenazi Jewish population and their offspring were studied. Controls were spouses of the offspring and participants from the Framingham Offspring Study. Blood samples were taken to assess lipids and lipoprotein subclass levels and particle size by nuclear magnetic resonance. High-density lipoprotein (HDL) and low-density lipoprotein (LDL) particle size were significantly higher in the probands compared with both control groups. The phenotype was also typical of the offspring but not the spouses of the offspring. This phenotype was associated with the absence of hypertension and cardiovascular disease as well as the metabolic syndrome. Studies in centenarians also reveal that they have lower levels of oxidative stress and higher levels of antioxidant scavengers than “aged individuals”. Klapcinska et al assessed the antioxidant defense mechanisms in a group of centenarians (1M, 15F; aged 101-105yr) and compared to a group of young females (aged 20-22 yrs) (Klapcinska, Derejczyk et al. 2000). Glutathione peroxidase levels in centenarians were very close to values found in the young individuals (37.3±15.7 vs. 54.5±8.3 μg/L). Uric acid concentrations were also significantly higher in centenarians than in young adults (p<0.01). Mecocci et al assessed plasma levels of a number of antioxidants including SOD, GPX, vitamins C, E, and A, uric acid, and carotenoids in a group of centenarians and compared to two younger populations. Thirty-two healthy centenarians were evaluated along with 34 elderly subjects age 60-79years and 24 adults aged less than 60 years. Centenarians were characterized as having the highest levels of vitamins A and E, whereas the activity of SOD, which decreases with age, increased in the centenarians. In fact, SOD activity showed a positive correlation to age when centenarians were included in the analysis. Paolissi et
al investigated the degree of oxidative stress in a group of centenarians (n=22, age=103±0yr) and compared to a group of aged subjects (n=30, age=79±1yr) and a group of adults less than 60yrs (n=30, age=43±2yr) (Paolisso, Tagliamonte et al. 1998). Lipid peroxidation was evaluated along with plasma concentrations of vitamin E and C. Lipid peroxidation products were lower in the centenarians than in the aged subjects (p<0.02). Furthermore, levels of vitamin E (p<0.05) and C (p<0.01) were significantly higher in the centenarians than the aged subjects. Centenarians appear to be less susceptible to oxidative damage that may help to make them more resistant to age related disease.

2.9 Resting Metabolic Rate, Oxidative Stress, and Aging

The Free Radical Theory of Aging states that the production of free radicals form normal metabolic processes increases and accumulates with age eventually leading to disease and ultimately death. Therefore it has been hypothesized that individuals who have a higher resting metabolic rate will produce more free radicals and accumulate more oxidative damage resulting in aging and death. There is a definitive relationship between free radical generation and maximum lifespan potential. Sohal et al demonstrated that there was a negative correlation between maximum lifespan potential and mitochondrial free radical production (H2O2 and O2•-) production in the liver of 8 different species of animals with different lifespans (Sohal, Svensson et al. 1990). This has also been supported by Ku et al who also measured rates of mitochondrial free radical production in kidney and the heart (Ku, Brunk et al. 1993). They also found an inverse association between mitochondrial O2•- and maximum lifespan potential. In addition, free radical production was directly related to species-specific metabolic rate and state 4-mitochondrial respiration. Cutler also demonstrated that there is an inverse relationship between tissue susceptibility to oxidation and lifespan (Cutler 1985). Furthermore, Adelman et al
demonstrated a direct relationship between oxygen consumption and DNA damage (Adelman, Saul et al. 1988). Therefore, it has been inferred that there must be connections among an organism’s metabolism, the accumulation of oxidative stress, and lifespan potential. However, these relationships have not been entirely elucidated. Pigeons and rats have similar metabolic rates and body mass, but eight-fold difference in lifespan. This could be due to the lowered rate of ROS generation in isolated mitochondria in pigeons compared to rats (Barja 2002). Furthermore, researchers have not been able to establish a relationship between resting metabolic rate and longevity within a given species (Sacher and Duffy 1979; Promislow and Haselkorn 2002; Speakman, van Acker et al. 2003). In direct contrast, Speakman et al demonstrated that in a group of outbred mice, there was a positive association between absolute and adjusted metabolic rate and maximal lifespan (Speakman, Talbot et al. 2004). However, most of the studies have been performed in flies, worms, and mice and required predicting resting energy expenditure or lifespan. Research needs to be conducted in humans to clarify these issues.

2.10 Summary

Oxidative stress has an impact on disease risk and the aging process. There are a number of factors that have an effect on an individual oxidative status. Factors affecting oxidative stress include gender, body composition, smoking status, diet, physical activity level, and the strength of the defense mechanism. There is still much debate as to whether the connection between energy expenditure and oxidative stress in humans plays a role in the aging process. The development of measurements to establish an oxidative stress index would be beneficial to the assessment of oxidative stress on aging and lifespan. Furthermore, the assessment of energy metabolism and oxidative stress in long-lived individuals such as nonagenarians compared to younger individuals would provide valuable information on these relationships.
Chapter Three

Aging, Energy Metabolism, and Oxidative Stress

3.1 Introduction

There are currently over 300 theories attempting to explain the aging process (Austad 1998). However, even after a century of research, there is no agreement regarding the cause or mechanisms of biological aging. Two complementary, closely related theories that have received much attention are the Rate of Living Theory and the Free Radical / Oxidative Stress Hypothesis of Aging. Combined, these theories state that free radicals produced during normal cellular metabolism, react with biomolecules to produce oxidative damage, which accumulates with age (Harman 1956; Greenberg 1999). There is support for both of these theories. For example, free radical production and metabolic rate are inversely related to lifespan (Sohal and Allen 1985; Adelman, Saul et al. 1988; Greenberg 1999). Furthermore, calorie restriction increases lifespan while reducing both metabolic rate and oxidative damage (DeLany, Hansen et al. 1999; Hagopian, Harper et al. 2005).

Pearl’s (1928) rate of living theory states that the duration of life among species varies inversely with the rate of energy expenditure (Greenberg 1999). At the time, the mechanism relating energy expenditure to the aging process was unknown. However, in 1956, Harman (Harman 1956) postulated that free radicals produced during normal cellular metabolism can damage cell constituents leading to abnormal cell function and eventually cell death. This theory provided for the first time a direct link between energy metabolism, aging, and lifespan. More than 40 years later, Barja and Herrero (Barja and Herrero 2000) demonstrated an inverse relationship between maximum lifespan and mitochondrial or nuclear 8-oxo7,8-dihydro-2’deoxyguanosine (8oxodG) from the heart and brain of six mammalian species ranging in
maximum lifespan from 3.5 to 46 y. In addition, calorie restricted animals exhibit a decrease in oxidative stress accompanied by an increase in lifespan (Sohal, Agarwal et al. 1994; Holloszy 1997). Early studies demonstrated a positive relationship between free radical production and species-specific metabolic rate (Cutler 1985; Ku, Brunk et al. 1993) and an inverse relationship with both of these to maximum lifespan (Sohal and Weindruch 1996). In contrast with the above theory, a recent study by Speakman et al (Speakman, Talbot et al. 2004) reported a positive relationship between oxygen consumption and maximum lifespan in a group of outbred mice. Furthermore, in mice subjected to calorie restriction, energy expenditure was found to be higher than predicted based on the change in body weight (Selman, Phillips et al. 2005). However, unlike in humans, it is notoriously difficult to measure metabolic rate in mice. Unfortunately, there are very few studies that have investigated the relationship between energy expenditure and oxidative stress in humans (Loft, Astrup et al. 1994), and even less that have looked at the effect of age on this relationship.

The effect of age on RMR has been extensively studied in humans, but its relationship with oxidative damage or its impact on lifespan is unknown. A consensus as to whether RMR is lower in elderly individuals has not been reached. McGandy et al (McGandy, Barrows et al. 1966) measured basal oxygen consumption in 252 men aged 20-99 y and reported a decline in RMR of approximately 52 kcal/d for each decade of life. In a longitudinal study, Keys et al (Keys, Taylor et al. 1973) observed a 1-2% decline in RMR that was independent of the decline in FFM. However, studies utilizing whole body potassium or creatinine excretion to measure metabolically active tissue also attribute the decline in RMR with age to a decrease in the amount of metabolically active tissue (Tzankoff and Norris 1977; Calloway and Zanni 1980). In addition, RMR in very old individuals (>90y) was not different from predicted based on fat free
mass and similar to that of individuals aged 73y (Rothenberg, Bosaeus et al. 2000). However, using the state of the art method (respiratory chamber), Vaughan et al (Vaughan, Zurlo et al. 1991) demonstrated that RMR was the only component of total energy expenditure that was lower in older individuals when compared to young, after adjustment for fat free mass and fat mass.

To a large extent, aging research performed in humans consists of cross sectional studies comparing young individuals to extremely old individuals. Studies in centenarians reveal that these long living individuals may be protected against the age-related accumulation of oxidative stress. Klapcinska et al (Klapcinska, Derejczyk et al. 2000) assessed antioxidant enzyme activity in a group of centenarians to find that glutathione peroxidase concentration in this population was comparable to that of a group of young individuals (aged 20-22y). In addition, lipid peroxidation in centenarians was comparable to that of younger individuals (<50y) (Paolisso, Tagliamonte et al. 1998). Hyland et al (Hyland, Duggan et al. 2002) demonstrated that nonagenarians had levels of DNA damage similar to that of a middle aged group (40-60 yrs) and higher levels of antioxidant enzymes. They concluded that nonagenarians could be protected from an age related accumulation of oxidative damage through higher antioxidant activity. However, the effects of antioxidant administration do not seem to alter lifespan and calorie restriction does not necessarily result in any changes to antioxidant activity (Halliwell 1999). To our knowledge no one has examined both RMR and oxidative damage in the oldest old. Therefore, the purpose of this study was to test whether “healthy” nonagenarians have relatively low metabolic rates when compared to middle age and young individuals and whether such a low metabolic rate is associated with a relatively low level of oxidative stress.
3.2 Methods

3.2.1 Subjects

Subjects included in this study represented a subset from a population-based study called the Louisiana Healthy Aging Study. Data was collected in three groups of individuals aged 20-34 (16M / 25F), 60-74 (16M / 11F), and ≥90y (23M / 25F). Randomly selected subjects (voter registration lists and Medicare Beneficiary Enrollment Data File from Center of Medicare and Medicaid Services) were invited to participate in the study. Only subjects with elevated fasting (12h fast) blood sugar (>125 mg/dl) and thyroid disease were excluded. Subjects provided written informed consent. The study was approved by the IRB of the Pennington Biomedical Research Center.

3.2.2 Metabolic Tests

Whole body composition was measured by DEXA (Hologics, QDA 4500A, Bedford, MA). RMR was measured for 30 minutes in the morning following a 12-hour fast using a Deltratrac II metabolic cart (Sensormedics, Yorba Linda, CA). Participants were required to rest in a reclined position for thirty minutes before the start of the test. Subjects remained under the hood for 30 minutes and the last 20 minutes were used to calculate energy expenditure. The cart was calibrated before each test using room air and a known calibration gas concentration with 96% oxygen and 4% carbon dioxide.

3.2.3 Lipid Damage by Urinary Isoprostanes

The method for the measurement of urinary isoprostanes was previously described (Davies, Zackert, et al. 2005). Briefly, urine samples were prepared by adding 10ng of \([^{18}\text{O}_2]15\text{-F}_2\text{-IsoP-M}\) to 3 ml water, then 0.25 ml of urine and sufficient 1 N HCl to acidify the sample to pH 3. Samples were then loaded on a C18 Sep-Pak cartridge (Waters, Milford, MA) which had
previously been equilibrated with 5 ml methanol and 5 ml pH 3 water. The sample was washed with 10 ml pH 3 water and then 8 ml heptane and eluted with 10 ml heptane/ethyl acetate (1:1). A small amount of sodium sulfate was added to the sample to remove residual water. The eluant was then reloaded on a silica Sep-Pak cartridge (Waters) pre-equilibrated with 5 ml ethyl acetate, washed with 5 ml ethyl acetate and eluted with 5 ml ethyl acetate/methanol (1:1). The sample was then dried under N₂ gas. After resuspension in 100 μl methanol, the sample was transferred to a Spin X filter tube, centrifuged for 30 seconds, and the filtrate transferred to an autosampler vial. HPLC was carried out on a 30ml sample using a Surveyor MS Pump from ThermoFinnigan (San Jose, CA) with a mobile phase consisting of solvent A (5 mM ammonium acetate with 0.1% acetic acid) and solvent B (acetonitrile/methanol (95:5)). F₂-IsoP-M were chromatographed on a Magic C18AQ, 3 μm 100 Å column (Michrom BioResources, Auburn, CA). Mass Spectrometry was performed using ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer (San Jose, CA) equipped with standard electrospray ionization. The interassay coefficient of variation was 13% while the day to day coefficient of variation was 5%.

3.2.4 Protein Damage by Protein Carbonyls

The carbonyl content in proteins was determined using a modified 2,4-dinitrophenylhydrazine (DNPH) assay according to the method of Mates (Mates, Perez-Gomez et al. 2000). Briefly, serum was treated with acidified DNPH. Proteins were then precipitated by trichloric acid and centrifuged. The pellet was dissolved in guanidine hydrochloride and insoluble material was removed by additional centrifugation. The carbonyl content was calculated from peak absorbance at 355-390 nm, using an absorption coefficient ε of 22,000 M⁻¹ cm⁻¹ (Beckman, Brea, CA). Results were expressed as nmol per mg protein. Total protein
concentration was determined by the method of Bradford (Bradford 1976; Waterman 1978). The intraassay coefficient of variation is 4.7%. The Interassay coefficient of variation is 8.5%.

3.2.5 DNA Damage by Single Cell Gel Electrophoresis (Comet Assay)

DNA fragmentation was measured by Single Cell Gel Electrophoresis as described by Deutsch et al (Deutsch, Kukreja et al. 2001). Briefly, whole blood cells were washed with phosphate-buffered saline and dimethylsulfoxide and centrifuged. The blood pellet was suspended in low melting point agarose and spread onto two commercially available slides (Trevigen, Gaithersburg, MD) and the slides were treated as per manufactures instructions. Two slides were prepared for each blood sample, one for the Comet assay and one for the Fpg (E.coli Formamidopyrimidine-DNA Glycosylase) FLARE (Fragment Length Analysis using Repair Enzymes) assay. The Fpg enzyme solution was added to the agarose on the slide for the FLARE assay and placed at 37°C for 55 minutes. The slide for the Comet assay was placed in a drawer at room temperature during this time. All slides were then immersed in alkaline buffer, washed and electrophoresed for 10 minutes at 17 volts. The slides were then fixed, stained with SYBR green dye (Trevigen) and viewed under a UV microscope (Nikon Microphot FXA, Hamamatsu high resolution 512 lines, Image I AT software, FITC 3 filter). The extent of DNA damage was determined by calculating the comet moment, which is the integrated density in the comet tail multiplied by the distance from the center of the nucleus to the center of mass of the tail of 25 cells using freely available software (Herbert M Geller; http://www2.umdnj.edu/~geller/lab/comet-Scoring-Macro.txt).

Because the rapidly changing intensities of individual cells are difficult to control (Lovell, Thomas et al. 1999), a large variance within each experiment is unavoidable. Therefore, a normalizing and variance-stabilizing logarithmic transformation was then applied to the
calculated tail moments. Although there are no agreed upon reference values for the comet assay, data from our laboratory revealed an intraclass correlation coefficient of 0.949.

3.2.6 Statistical Analysis

Data in the text and tables are provided as means ± SEM. Statistical analysis was performed using SPSS Version 12 (SPSS Inc, Chicago, IL). Multiple regression analysis was used to adjust resting metabolic rate for FFM, FM, and sex. The residuals represent the difference between predicted and actual measured values. Analysis of variance with post hoc Bonferroni adjustment was used to assess differences between groups. For the Comet and FLARE Assay, data was log transformed before an analysis of variance with multiple comparisons was applied. Linear regression models were used to assess associations between RMR and markers of oxidative stress. Multiple regression analysis was also used to determine which factors best-predicted indices of oxidative damage.

3.3 Results

3.3.1 Body Composition

A total of 116 subjects were included in the analysis. Isoprostane and protein carbonyl data was only available on 70 individuals because of delay of analysis in the lab. Subject characteristics by age and sex are displayed in table 3.1. The three groups will be referred to as young (20-34y), aged (60-74y), and nonagenarians (≥ 90y). The nonagenarians weighed significantly less than the young and aged groups (p<0.001) resulting in significantly lower BMI (p<0.001). While there were no differences in percent body fat between the three groups, nonagenarians had significantly less fat free mass (p<0.001) and fat mass (p<0.02) than the two younger groups.
### Table 3.1. Subject Characteristics

<table>
<thead>
<tr>
<th>Mean±SEM [Range]</th>
<th>20-34 y</th>
<th>60-74 y</th>
<th>≥ 90 y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16M / 25F</td>
<td>16M / 11F</td>
<td>23M/25F</td>
</tr>
<tr>
<td><strong>Age (y)</strong></td>
<td>27±1 [21-34]</td>
<td>70±1 [61-74]</td>
<td>92±1 [90-97]</td>
</tr>
<tr>
<td></td>
<td>27±1 [21-34]</td>
<td>69±1 [64-74]</td>
<td>92±1 [90-98]</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>88.2±5.1 [61.2-135.7]</td>
<td>90.5±3.3 [70.8-128.2]</td>
<td>71.7±1.5 [56.4-93.3]</td>
</tr>
<tr>
<td></td>
<td>72.9±4.2 * [50.1-127.3]</td>
<td>74.8±3.2* [50.2-94.9]</td>
<td>60.4±2.2* [36.5-88.5]</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td>174. +1 [154-185]</td>
<td>177+1 [165-187]</td>
<td>170+1 [162-181]</td>
</tr>
<tr>
<td></td>
<td>163+1* [148-182]</td>
<td>160+1* [143-169]</td>
<td>157+1* [147+172]</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>28.3±1.4 [21.4-41.4]</td>
<td>28.9±0.9 [26.0-41.3]</td>
<td>25.2±0.4 [20.4-34.4]</td>
</tr>
<tr>
<td></td>
<td>27.1±1.5 [18.3-49.5]</td>
<td>28.8±0.8 [24.5-33.5]</td>
<td>24.7±0.9* [16.1-38.7]</td>
</tr>
<tr>
<td><strong>Percent Fat (%)</strong></td>
<td>21.6±2.4 [9.4+41.2]</td>
<td>26.8±1.4 [17.8-43.7]</td>
<td>26.6±0.9 [17.6-35.8]</td>
</tr>
<tr>
<td></td>
<td>34.2±1.7* [19.0-49.5]</td>
<td>40.0±1.0* [34.1-45.8]</td>
<td>35.4±1.1* [25.8-47.7]</td>
</tr>
<tr>
<td><strong>Fat Free Mass (kg)</strong></td>
<td>62.6±4.7 [49.0-79.8]</td>
<td>66.0±2.2 [51.8+85.3]</td>
<td>52.6±1.0 [41.0-64.4]</td>
</tr>
<tr>
<td></td>
<td>46.7±1.7* [34.9-67.4]</td>
<td>44.7±1.6* [30.9-53.8]</td>
<td>38.5±1.2* [26.9-46.3]</td>
</tr>
<tr>
<td><strong>Fat Mass (kg)</strong></td>
<td>20.1±3.5 [6.9-55.9]</td>
<td>24.5±2.1 [16.9-56.0]</td>
<td>19.2±0.9 [11.8-29.6]</td>
</tr>
<tr>
<td></td>
<td>26.6±2.7 [10.8-60.3]</td>
<td>30.1±1.8 [19.3-41.1]</td>
<td>21.8±1.5 [9.6-42.2]</td>
</tr>
</tbody>
</table>

* Males significantly different from females p<0.05.
3.3.2 RMR

Absolute resting metabolic values are displayed in table 3.2. There were significant differences in absolute RMR between the three age groups (p<0.001). In addition, RMR was significantly related to fat free mass and sex (Figure 3.1, p<0.001).

Figure 3.1. Relationship between resting metabolic rate and fat free mass. Long dashed line-young group; Dotted line- Aged group; Solid line-nonagenarians. Regression line for the total group: RMR (kcal/d) =335 + 20.3 (FFM (kg)); (r=0.99, p< 0.000). The regression lines are significantly different from one another (p<0.007) but do not have significantly different slopes.

As shown in table 3.2 and figure 3.1, adjusted RMR was significantly different between the three groups (p<0.001). In addition, there were significant differences in T3 (p<0.001) and T4 concentrations between the three groups (p<0.001).
Table 3.2. Resting Metabolic Rate and Markers of Oxidative Stress

<table>
<thead>
<tr>
<th>Mean +/- SEM</th>
<th>20-34 yrs 16M / 25F</th>
<th>p value Young to Aged</th>
<th>60-74 yrs 16M / 11F</th>
<th>p value Aged to Nonagenarian</th>
<th>90 yrs 23M/25F</th>
<th>p value Young to Nonagenarian</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMR (kcal/day)</td>
<td>1544±44</td>
<td>NS</td>
<td>1475±47</td>
<td>p&lt;0.001</td>
<td>1185±24*</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>RMR (Adj for FFM, FM, sex*)</td>
<td>83±20</td>
<td>p&lt;0.001</td>
<td>-15±23</td>
<td>NS</td>
<td>-57±31</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>T3 (ng/ml)</td>
<td>164±6</td>
<td>p&lt;0.001</td>
<td>126±4</td>
<td>NS</td>
<td>134±5</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>T4 ng/ml)</td>
<td>8.8±0.3</td>
<td>p&lt;0.05</td>
<td>7.9±0.2</td>
<td>NS</td>
<td>7.4±0.2</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Isoprostanes (ng/mg Cr)</td>
<td>52.1±7.7</td>
<td>NS</td>
<td>49.8±7.2</td>
<td>NS</td>
<td>46.7±3.5</td>
<td>NS</td>
</tr>
<tr>
<td>Protein carbonyls (nmol/mg)</td>
<td>0.6±0.1</td>
<td>NS</td>
<td>0.6±0.1</td>
<td>NS</td>
<td>0.8±0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Comet (au)</td>
<td>17.4±0.1</td>
<td>NS</td>
<td>17.8±0.1</td>
<td>NS</td>
<td>17.4±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>FLARE (au)</td>
<td>17.1±0.1</td>
<td>p&lt;0.003</td>
<td>17.7±0.2</td>
<td>p=0.02</td>
<td>17.2±0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

T3=triiodo-thyronine; T4=thyroxine, Cr=Creatinine
* Expressed as residual values, i.e. measured minus predicted value or the mean of the entire group.
3.3.3 Markers of Oxidative Stress

Values for oxidative stress to lipids, proteins, and DNA are displayed in Table 3.2 and Figure 3.2. Across age groups, males had significantly lower urinary isoprostane concentrations than females (34.8±2.7 vs. 57.0±5.5ng/mg cr; p<0.001, data not shown). However, as illustrated in figure 3.3, the lower values in males were mostly due to the significant correlation between isoprostane concentrations and percent body fat (r=0.34; p<0.003). After adjustment for percent fat, isoprostane concentrations were similar in both sexes. On the contrary, serum protein carbonyl concentrations were higher in males than females (0.9±0.1 vs. 0.6±0.1 nmol/mg; p<0.03, data not shown) whereas there were no significant difference in DNA fragmentation between males and females.

There were no significant differences in urinary isoprostane or serum protein carbonyl concentrations between the three age groups. There were also no significant differences in spontaneous DNA damage (comet) between the three groups, whereas aged subjects had greater damage as measured by the FLARE assay than the two other groups (p<0.003).

Contrary to our hypothesis, there were no relationships between absolute or adjusted RMR and any of the markers of oxidative stress for the entire population or within each sex. In addition, multiple regression analysis revealed that RMR, adjusted for FFM, FM, and sex was not a determinant of the variation in urinary isoprostanes, serum protein carbonyls, or DNA fragmentation.
Figure 3.2. Markers of oxidative stress in the three different age groups. There were no significant differences in isoprostanes, protein carbonyls, or DNA fragmentation by the Comet assay between the 3 age groups. There was a significant difference in DNA damage measured by the FLARE assay.
3.4 Discussion

To our knowledge, the Louisiana Healthy Aging Study is the first study to examine the relationship between RMR and oxidative stress in humans. The results from this study strengthen previous research that RMR declines with age even after adjustment is made for body weight and body composition. In addition, the study confirms an accumulation in oxidative damage to DNA with age and that long-lived individuals may be protected against this age related increase. The current study, however, does not support the theory that energy metabolism is directly involved in the aging process through the production of free radicals and accumulation of oxidative damage to lipids, proteins, and DNA.
Free radicals are produced in a number of ways. Under normal conditions, the electron transport chain is a significant contributor to the production of the superoxide anion, precursor to other harmful reactive oxygen species (ROS), hydroxyl (OH•) and hydrogen peroxide (H2O2) (Halliwell 1999). As electrons are transported through the electron transport chain, some leak into the mitochondrial matrix where they react with oxygen to generate ROS (Batandier, Fontaine et al. 2002). The primary sites of free radical production occurs during the transfer of electrons from Complex I to Coenzyme Q and within complex III (Cadenas, Boveris et al. 1977; Turrens and Boveris 1980). Approximately 0.2-2.0% of oxygen consumed results in the formation of free radicals (Chance, Sies et al. 1979; St-Pierre, Buckingham et al. 2002). Accumulation of oxidative stress is dependent on the rate of free radical production, susceptibility of tissues to damage, and the level of defense against these free radicals as well as the activity of the repairing system (Beckman and Ames 1998). Although organisms have developed elaborate antioxidative defense mechanisms, it is apparent that the defense mechanisms are not perfect, and oxidative stress accumulates (Dotan, Lichtenberg et al. 2004). Free radicals can damage lipids, proteins, and DNA. Lipids are the most susceptible to free radical attack. Free radicals react with unsaturated fatty acids located in the membrane leading to the formation of a carbon centered radical (Wagner, Buettner et al. 1994). This initiates a cycle of lipid peroxidation resulting in the formation of hydroperoxides (Gardner 1989). Furthermore, products of lipid peroxidation can also react with proteins and DNA (Uchida and Stadtman 1992; Cai, Tian et al. 1996). Free radicals can also directly interact with proteins generating a number of products such as amino acid peroxides and protein carbonyls (Halliwell 1999). Oxidative damage to DNA can result in the formation of genetic mutations resulting in
altered cell function (Halliwell 1999), and can induce the formation of several base adducts implicated in mutagenesis, carcinogenesis, and neurological disorders (Bohr and Dianov 1999).

There is still considerable controversy over whether oxidative damage increases with age. A number of studies in animals report an increase in oxidative damage to DNA and protein with age in tissue such as the liver, heart, brain, kidney, skeletal muscle, and spleen (Fraga, Shigenaga et al. 1990; Bonnes-Taourel, Guerin et al. 1993; Hamilton, Van Remmen et al. 2001). Roberts and Reckelhoff (Roberts and Reckelhoff 2001) demonstrated a 20 fold increase in plasma isoprostane concentrations in 24 mo old rats compared to 3 mo old rats. In addition, young animals appear to be better equipped at repairing or removing oxidatively damaged tissue (Hamilton, Van Remmen et al. 2001). Whether RMR declines with age is not clear in animals. Recently, Speakman et al (Speakman, Talbot et al. 2004) demonstrated in a group of outbred mice that those in the upper quartile of metabolic intensity had 17% greater resting oxygen consumption and lived 36% longer than animals in the lower quartile. In addition, Selman et al (Selman, Phillips et al. 2005) demonstrated that total energy expenditure was actually higher than predicted in mice subjected to calorie restriction. However, the measurement of RMR in animals, especially rodents, is notoriously difficult when compared to measurement in humans in whom strict cooperation can be obtained during the procedure. Therefore, it may not be possible to apply conclusions from animal studies to humans when it comes to aging and energy metabolism.

Studies in humans are too scarce to conclude whether an accumulation of oxidative damage also occurs with aging. In animals, it is consistently reported that oxidative damage to DNA and proteins increase with age. In humans, Mecocci et al (Mecocci, Fano et al. 1999) reported that oxidative damage to DNA and proteins was increased in skeletal muscle of older
patients. In another study, similar results were obtained when comparing individuals 61-85y to individuals 21-40y (Gianni, Jan et al. 2004). On the other hand, data regarding lipid peroxidation has been less consistent. Studies using malondialdehyde (MDA) as a marker of lipid peroxidation have indicated an increase in lipid peroxidation as a result of increasing age (Mecocci, Polidori et al. 2000; Mutlu-Turkoglu, Ilhan et al. 2003). On the contrary, lower MDA concentrations have been reported in centenarians compared to individuals aged 70-99y (Paolisso, Tagliamonte et al. 1998). Data from the Framingham Study actually observed a decrease in isoprostanes (a more reliable marker of lipid peroxidation) in individuals ranging from 33-88y (Keaney, Larson et al. 2003).

There is no consensus whether the decline in RMR with age is due to a decline in fat free mass and an increase in fat mass or whether there is a decline in RMR with age independent of changes in body weight and body composition that is per unit of tissue weight. Earlier observations of a decline in RMR with age (McGandy, Barrows et al. 1966) were later supported by reports of lower RMR in older individuals when compared to young even after adjusting for fat free mass (Fukagawa, Bandini et al. 1990). In fact, Rothenberg et al (Rothenberg, Bosaeus et al. 2003) found that nonagenarians had significantly lower adjusted RMR when compared to individuals aged 71-73y. Others have reported that the decline in RMR may be due to the decline in cell mass only (Calloway and Zanni 1980). The decline in physical activity with age has (Van Pelt, Dinneno et al. 2001) also been implicated as a potential cause for the reduction in RMR with age, even if there is no clear relationship between RMR and maximal oxygen consumption, an index of the level of physical activity (Schulz, Nyomba et al. 1991).
In the current study, RMR was lower in each of the older groups. The lower RMR in the aged group could not be explained by differences in body composition since the aged group had similar amounts of fat mass and more fat free mass than the young group. RMR was even lower in the nonagenarians even after accounting for these differences in body composition. A possible reason for the decline in metabolic rate with age may be a decline in serum T3 concentrations. While the nonagenarians and the aged group had similar T3 concentrations, both were lower than the young group. Similarly, serum T4 concentrations were also lower in the two older groups. Other mechanisms could include lower sympathetic nervous system activity or lower overall energy intake. Although neither of these were measured in the current study, previous studies have indicated that sympathetic nervous system activity is related to energy expenditure (Saad, Alger et al. 1991) and that the decline in RMR in older men may directly due to a decline in energy intake (Van Pelt, Dinneno et al. 2001).

There were no differences in any markers of oxidative stress between the three groups. However, the aged group had significantly higher levels of glycosylase-induced damage than either the young group or the nonagenarians. Treating cells with Fpg gives an indication of 8-oxodG adducts, the most common adduct caused as a result of reactions with free radicals. Results from the current study indicate that individuals who reach extreme old age may be protected against the age related accumulation of oxidative stress. However, the fact that the aged group did not have higher levels of isoprostane and carbonyl concentrations contradicts the much-accepted theory that oxidative stress accumulates with age. Although not significant, the nonagenarians tended to have lower concentrations of isoprostanes compared to the younger groups. This decline in isoprostane concentrations with age may be partially due to the decline in fat mass beyond the age of 60y. It is recognized that the accumulation of oxidative stress is
greater in post mitotic tissues such as skeletal muscle and brain (Hamilton, Van Remmen et al. 2001) than in other cell types with faster turnover rates such as blood, which may partially account for the fact that there were no differences detected between groups with regards to protein carbonyls. Given that cells from tissue such as skeletal muscle and brain are more metabolically active, the use of whole blood to assess oxidative damage is a limitation of the current study. A recent study (Wolf, Fasanella et al. 2005) in rats reported that 8-OhdG significantly increased with age in peripheral lymphocytes as well as heart, skeletal muscle, brain, liver, or intestine. The current study did not isolate lymphocytes but used whole blood to assess DNA damage. However, previous validation data indicates no difference in amount of DNA damage whether using whole blood or isolated lymphocytes (unpublished data). In addition, the previous study in rats was longitudinal in nature and the current study’s cross sectional design may not have been successful at detecting age-related changes in oxidative damage. Finally, oxidative stress accumulation is dependent on free radical production and removal. The current study did not directly measure free radical production or antioxidant activity, so it is impossible to determine if older individuals produce more free radicals but also have an enhanced capacity to defend against them.

Mitochondria isolated from male mice produce more free radicals than mitochondria isolated from female mice of the same age (Borras, Sastre et al. 2003). In the current study, isoprostane concentrations were higher in females than males. Keaney et al (Keaney, Larson et al. 2003) also demonstrated that isoprostane concentrations were higher in females than males, however, isoprostane concentrations may be affected by the amount of body fat. In the current study, males had higher serum protein carbonyl concentrations than females. When body fat and age are held constant, males produced more free radicals, had lower antioxidant enzyme activity...
(Borras, Sastre et al. 2003), and had higher levels of oxidative damage than females (Fano, Mecocci et al. 2001; Ide, Tsutsui et al. 2002). Although, not entirely elucidated, estrogen may have a protective antioxidant effect, reducing oxidative stress in females (Ide, Tsutsui et al. 2002; Borras, Sastre et al. 2003).

Contrary to our hypothesis, RMR was not a significant determinant of urinary isoprostane concentrations, serum protein carbonyl concentrations, or DNA fragmentation. Loft et al (Loft, Astrup et al. 1994) found a positive relationship between 24-hour oxygen consumption and urinary excretion of 8-oxodG in women. However, free radical production does not necessarily increase in proportion to oxygen consumption because free radical leak is not always constant. In addition, free radical production is highest in the resting state when ATP demand is low (Turrens 1997). Therefore, one would expect a direct relationship between markers of oxidative stress and RMR rather than total energy expenditure, which is the sum of RMR, the thermic effect of food, and the energy needed to sustain physical activity. The fact that the current study, with an age range of 21-98y, did not observe a significant relationship between RMR and any markers of oxidative stress may indicate that factors, other than total oxygen consumption, may be more important in predicting levels of oxidative stress. While our data does not directly support the rate of living / oxidative stress hypothesis it does not refute it either. The cross sectional study design lends itself to limitations in that in the younger age groups, it is impossible to know who will live beyond 90y. Prospective studies are needed to examine if individuals with a lower metabolic rate throughout life have less oxidative damage and therefore live longer.

3.5 Conclusion

In summary, the current study confirms previous findings that there is an age related decline in RMR that cannot be fully explained by changes in fat free mass or fat mass.
Interestingly, nonagenarians appear to be protected from the age related increase in oxidative damage to DNA, but the reduced RMR could not be directly implicated in the mechanism of oxidative stress since no relationship was found between metabolic rate and oxidative damage.
Chapter Four

The Impact of Oxidative Stress on the Metabolic Syndrome and Risk Factors for Cardiovascular Disease

4.1 Introduction

The average life expectancy in the United States is 77 years (Oeppen and Vaupel 2002) and in the western world the chance of living to 100 is 1 in 10,000 (Perls, Wilmoth et al. 2002). Individuals achieving longevity appear to delay or even escape various age related diseases such as NIDDM and cardiovascular disease (CVD) that are typically fatal at much younger ages (Perls T 2000). These individuals may therefore possess genetic and physiological traits that contribute to their successful aging.

Cardiovascular disease is one of the leading causes of death in the westernized world (Kahn, Buse et al. 2005). The development of CVD is associated with the metabolic syndrome, a clustering of risk factors for CVD including obesity, dyslipidemia, hypertension, insulin resistance, and glucose intolerance (Ferrannini, Haffner et al. 1991; Ferrannini, Haffner et al. 1991; 1999). Recently, evidence has demonstrated that oxidative stress is related to each of the components of the metabolic syndrome (Davi, Guagnano et al. 2002; Redon, Oliva et al. 2003; Lin, Chen et al. 2005) and is directly implicated in the progression of CVD (Vassalle, Petrozzi et al. 2004).

In 1988, Reaven et al (Reaven 1988) proposed the existence of a unique pathophysiological condition known as the “Insulin Resistance Syndrome” or “Syndrome X”. Since then, the condition now termed the Metabolic Syndrome has been defined primarily by The World Health Organization (WHO) (1999), the Third Report of the National Cholesterol Education Program’s Adult Treatment Panel (NCEP-ATP III) (2001; Grundy, Brewer et al. 2002).
2004), and more recently by the International Diabetes Federation (IDF). The components of the metabolic syndrome include insulin resistance, obesity, hypertension, and lipid abnormalities (1999). Although there are many similarities, differences exist between the definition and classification systems. For example, the WHO includes the presence of microalbuminuria as well as insulin resistance, whereas the other two classifications do not. In addition, the most recent classification from the IDF places more emphasis on central obesity and includes the treatment of conditions such as hypertriglyceridemia, or elevated blood sugar as components. The ATP III is the most widely used classification (Kahn, Buse et al. 2005), however, the inherent similarities between it and the IDF classification may allow these two methods to be used interchangeably.

The incidence and prevalence of CVD increase with age. The American Heart Association reports that 84% of individuals diagnosed with CVD are over the age of 65. Aging is associated with alterations in body composition (Roubenoff and Hughes 2000), a rise in serum cholesterol (Keys, Fidanza et al. 1952; 1987), impaired glucose tolerance, and insulin resistance (Ferrannini, Haffner et al. 1991; Ryan 2000) all which negatively effect cardiovascular health. Interestingly, individuals with exceptional longevity are seldom affected by the age-related alterations in CVD risk. For example, cross sectional reports indicate that centenarians have FFM (Paolisso, Gambardella et al. 1995), glucose tolerance, insulin sensitivity (Paolisso, Gambardella et al. 1997), and lipoprotein profiles (Barzilai, Atzmon et al. 2003) similar to individuals of much younger ages. The mechanisms responsible for this younger profile in “successful agers” are currently unknown, however, genetic factors have been proposed as a possible explanation. Studies examining physiological aspects of these successful agers can provide important information regarding the aging process.
CVD is caused by the development of atherosclerosis (Ross 1999). The initiating event in the progression of atherosclerosis is believed to be the development of endothelial dysfunction (Ross 1999). Potential causes of endothelial dysfunction include elevated levels of oxidatively modified LDL; generation of reactive oxygen species (ROS) and oxidative stress, hypertension, and diabetes. The injured endothelium responds to these insults by developing procoagulant instead of anticoagulant properties, and by releasing a number of cytokines and growth factors. The release of these factors leads to the sequestration and accumulation of lymphocytes and macrophages from the blood and the migration and proliferation of underlying smooth muscle cells. Consistent with this paradigm is the recognition that in addition to the well-known CVD risk factors including lipids, lipoproteins (Paolisso, Gambardella et al. 1997) and blood pressure, other factors are of importance. Among those, elevated concentrations of hemostasis factors (factor VII, fibrinogen, PAI-1), and C-reactive protein (CRP), are related to increased incidence of CVD (Ridker, Buring et al. 1998; Ridker, Glynn et al. 1998; Koenig, Sund et al. 1999) and predictive of future CVD events (Ridker, Brown et al. 2004). Furthermore, recent evidence also supports a direct effect of ROS on these markers (Furukawa, Fujita et al. 2004) thereby exacerbating the proatherogenic environment.

Oxidative stress has been implicated in the development of metabolic syndrome and CVD (Figure 4.1) (Hansel, Giral et al. 2004). Oxidative stress is higher in individuals with hypertension (Redon, Oliva et al. 2003), hypercholesterolemia (Davi, Alessandrini et al. 1997), and diabetes (Lin, Chen et al. 2005) and it has been proposed that oxidative stress is directly
involved in each of these conditions. In fact, medications to treat conditions such as hypertension and hypercholesterolemia have antioxidant effects thereby reducing oxidative stress while also treating the primary condition (Wassmann and Nickenig 2003; Lee, Lee et al. 2005).

Figure 4.1. Oxidative stress and risk factors for CVD are implicated in the development of endothelial dysfunction. The endothelium responds by creating a pro atherogenic environment through the actions of Fibrinogen, CRP, Factor VII, PAI-1, and Homocysteine. PAI-1: Plasminogen Activator Inhibitor 1, CRP: C-reactive protein.

Hansel et al demonstrated that individuals with the metabolic syndrome had elevated isoprostane concentrations and attenuated antioxidant activity of small, dense, HDL subtractions, known to be protective against systemic oxidative stress (Hansel, Giral et al. 2004). Reactive oxygen species (ROS) serve as precursors to the formation of oxidized low-density lipoproteins, essential to the formation of atherosclerotic lesions (Sigurdardottir, Fagerberg et al. 2002). It is
consistently reported in both animals and humans that oxidative damage accumulates with age (Fraga, Shigenaga et al. 1990; Hamilton, Van Remmen et al. 2001; Gianni, Jan et al. 2004). On the other hand, studies in long-lived humans demonstrate they have less accumulation of oxidative stress (Paolisso, Tagliamonte et al. 1998). This is evident in Okinawa, a population known for its successful agers; these individuals contain low blood levels of free radicals (Weindruch and Sohal 1997). Interestingly, these individuals also possess low cholesterol and low homocysteine levels when compared to Westerners, factors which help reduce their risk for coronary heart disease by up to 80% (Alfthan, Aro et al. 1997). It has been proposed that a low rate of free radical production results in less oxidative stress and protection from age-related disease (Barbieri, Rizzo et al. 2003).

The purpose of this study was to cross-sectionally examine the relationship between age and risk factors for CVD in 3 groups of subjects aged 20-34, 60-74, and ≥90y. We hypothesize that nonagenarians will have levels of risk factors and CVD markers that are comparable to aged subjects (60-74y) and this will be related to less accumulation of oxidative stress.

4.2 Methods

4.2.1 Subject Characteristics

Subjects included in this analysis were participants in a population-based study called the Louisiana Healthy Aging Study. Data was collected in three groups of individuals aged 20-34, 60-74, and ≥90y. Randomly selected subjects (voter registration lists and Medicare Beneficiary Enrollment Data File from Center of Medicare and Medicaid Services) were invited to participate. A complete medical history and physical was performed in all the volunteers. Only subjects with elevated fasting blood glucose (≥126 mg/dl) and thyroid disease were excluded.
Subjects provided written informed consent. The study was approved by the IRB of the Pennington Biomedical Research Center.

4.2.2 Body Composition Assessment

Anthropometric measurements were taken while the subject was in a hospital gown. Weight was measured to the +0.1kg with an electronic scale (Detecto, Webb City, MO) that was checked daily with a standard 25kg weight. Height was measured to the ±0.5 cm with a wall-mounted stadiometer (Holtain; Crymych, Dyfed, UK), and BMI was calculated as weight divided by height squared. Waist and hip circumferences were measured while the subject was in the standing position using a standard tape measure. Waist circumference was measured by locating the natural waist (the narrowest part of the torso) or midway between the ribcage and the iliac crest. Hip circumference was measured at the level of the trochanter (the maximal extension of the buttocks). Waist to hip ratio (W/H) was also calculated. Whole body composition was estimated by Dual Energy X-ray Absorptiometry (DEXA) (Hologics, QDR 4500A, Bedford, MA).

Blood pressure was measured in a quiet room with minimal temperature fluctuations. All measurements were taken twice on the participant’s right arm with a manual sphygmomanometer (Baum Co, Coplaque, NY) after a 5 min rest.

4.2.3 Blood Chemistry

Fasted blood samples (12y fast) were drawn from an antecubital vein. All serum lipids were analyzed using a Beckman-Coulter Synchron CX7 (Brea, CA). Serum cholesterol and triglycerides were measured by enzymatic assay using commercially available kits. HDL-C was measured according to the manufacturer’s instructions (Sigma Chemical Co.) and LDL-C was calculated using the Friedewald equation (Friedewald, Levy et al. 1972). The coefficient of
variation for this assay is less than 2.0%. Glucose was analyzed using a glucose oxidase electrode (Synchron CX5, Beckman, Brea, CA), whereas insulin was assayed by immunoassay on a DPC 2000 (Diagnostic Product Corporation, Los Angeles, CA). The Homeostasis Model Assessment (HOMA) to assess insulin resistance was computed using the formula (fasting glucose x fasting insulin)/22.5 (Lansang, Williams et al. 2001). Factor VII and fibrinogen were assayed on an Instrumentation Laboratory ACL 3000+ (Lexington, MA). Factor VII activity was assayed as described by the manufacturer by determining the ability of test plasma to correct the clotting time of factor VII-deficiency plasma and expressed as percent relative to serial dilution of pooled plasma. Fibrinogen was measured following a standard protocol (Clauss 1957). CRP was measured by automated immunoassay with chemiluminescent detection on a DPC-Immulite instrument (Los Angeles, CA). Plasminogen activator inhibitor 1 (PAI-1) was measured by Elisa using a kit from American Diagnostica (Stanford, CT). Homocysteine was measured by immunoassay on the DPC 2000 (Diagnostic Product Corporation, Los Angeles, CA).

4.2.4 Markers of Oxidative Stress

Urinary isoprostanes were measured as previously described (Davies, Zackert et al. 2005) using Liquid Chromatography with Mass Spectrometry. The serum protein carbonyl content was determined using a modified 2,4-dinitrophenylhydrazine (DNPH) assay according to the method of Mates et al (Mates, Perez-Gomez et al. 2000) and DNA fragmentation was measured by Single Cell Gel Electrophoresis (Comet assay) as described by Deutsch et al (Deutsch, Kukreja et al. 2001).
4.2.5 Metabolic Syndrome Classification

Metabolic syndrome was defined by the ATP III criteria if at least 3 or more of the following criteria were present: 1) Waist circumference that is >102 cm in men and >88 cm in women, 2) Triglycerides ≥150 mg/dl, 3) HDL cholesterol that is <40 mg/dl for men and <50 mg/dl for women, 4) Blood pressure ≥130/85 mmHg or documented use of antihypertensive therapy, 5) Fasting glucose concentration ≥110 mg/dl. In the present study, classification of the metabolic syndrome did not consider whether subjects were taking medication to control triglyceride or HDL levels, however, analysis was also performed using the IDF classification which includes treatment of hyperlipidemia and elevated fasting glucose in the components of the metabolic syndrome.

4.2.6 Statistical Analysis

Data in the text and tables are provided as means ± SEM. Statistical analysis was performed using SPSS version 12 (SPSS Inc, Chicago, IL). Analysis of variance with post hoc Bonferroni adjustment was used to assess differences between groups. For the Comet and FLARE Assay, data was log transformed. Pearson correlation coefficients were used to assess relationships between markers of cardiovascular disease and components of the metabolic syndrome with markers of oxidative stress. Multiple regression analysis was used to determine if any markers of oxidative stress were predictive of the development of the metabolic syndrome.

4.3 Results

Subject characteristics in the three age groups are displayed in table 4.1. The three groups will be referred to as young (17M/ 27F), aged (17M/ 14F), and nonagenarian (29M/ 30F). Data for Factor VII, fibrinogen, CRP, and homocysteine was available on 69, 83, 108, and 108 subjects respectively. In all three age groups, males weighed significantly more than females.
(p<0.001) and had higher W / H ratio (p<0.001). As expected, females had significantly higher percent body fat than males (p<0.001).

4.3.1 Age and Body Composition

Nonagenarians weighed significantly less (p<0.001) and had significantly lower BMI (p<0.02) than the two younger groups. There were no significant differences in percent body fat between the three groups. The aged group had significantly larger waist circumference than both the young group and the nonagenarians (p<0.001). Fifty four percent of the young group, 97% of the aged group, and 56% of the nonagenarians were classified as overweight, BMI ≥25. In addition, 30% of the young group, 25% of the aged group, and 7% of the nonagenarians were classified as obese, BMI ≥30.

4.3.2 Age and Metabolic Syndrome Classification

According to the ATP III classification, 4.3% of the young group, 31.3% of the aged group, and 27.3% of the nonagenarians were classified as having the metabolic syndrome. Figure 4.2 illustrates the average number of components of the metabolic syndrome across the 3 groups. Individuals in the aged group had a significantly greater number of components of the metabolic syndrome than the two other groups (p<0.001). Classification of the metabolic syndrome was also performed using the IDF classification which includes treatment for hyperlipidemia as a component. When medications to treat hyperlipidemia were included as a component for the metabolic syndrome the differences between the groups remained significant (p<0.001).

4.3.3 Age and CVD risk factors

Values for the components of the metabolic syndrome and risk factors for CVD are displayed in tables 4.2 and figure 4.3. There were significant differences in systolic blood
pressure (4.3, panel A, p<0.001), total serum cholesterol (4.3 panel E, p=0.05), triglyceride concentrations (4.3, panel B, p<0.008), and fasting glucose concentrations (4.3, panel C, p<0.001). There were, however, no significant differences in diastolic blood pressure, plasma LDL-C, or HDL-C between the groups. Interestingly, there were also no significant differences in fasting insulin concentrations or HOMA index between the three groups. While there were no differences in concentrations of CRP, PAI-1, and Factor VII activity, there were significant differences in plasma fibrinogen (4.2, panel E, p<0.05) and homocysteine (4.2, panel F, p<0.001) concentrations between the three groups.

![Figure 4.2](image)

**Figure 4.2.** Age-related differences in components of the metabolic syndrome. All three groups were significantly different from each other (p<0.001).
**Table 4.1.** Subject Characteristics

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<td>[87.0-112.0]</td>
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</tr>
<tr>
<td>[65.0-132.0]</td>
<td>[72.0-112.0]</td>
<td>[67.0-109.0]</td>
<td></td>
</tr>
<tr>
<td>W / H ratio</td>
<td>0.89±0.01</td>
<td>1.00±0.01</td>
<td>0.97±0.01</td>
</tr>
<tr>
<td></td>
<td>0.82±0.01</td>
<td>0.86±0.02*</td>
<td>0.88±0.01*</td>
</tr>
<tr>
<td>[0.80-0.97]</td>
<td>[0.92-1.09]</td>
<td>[0.85-1.15]</td>
<td></td>
</tr>
<tr>
<td>[0.70-0.94]</td>
<td>[0.76-1.00]</td>
<td>[0.75-1.08]</td>
<td></td>
</tr>
</tbody>
</table>

*p* Males significantly different than females (*p*<0.001)
Table 4.2 Metabolic Syndrome Components and Markers of CVD

<table>
<thead>
<tr>
<th>Mean ± SEM</th>
<th>Young 20-34 y</th>
<th>P value Young to Aged</th>
<th>Aged 60-74 y</th>
<th>P value Aged to Nonagenarian</th>
<th>Nonagenarian ≤ 90 y</th>
<th>P value Young to Nonagenarian</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mmHg)</td>
<td>118±2</td>
<td>p&lt;0.001</td>
<td>140±4</td>
<td>p&lt;0.001</td>
<td>142±3</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>75±1</td>
<td>NS</td>
<td>78±1</td>
<td>NS</td>
<td>75±1</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>175±5</td>
<td>NS</td>
<td>191±7</td>
<td>NS</td>
<td>192±5</td>
<td>0.05</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>102±11</td>
<td>p&lt;0.05</td>
<td>146±16</td>
<td>NS</td>
<td>126±10</td>
<td>NS</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>102±4</td>
<td>NS</td>
<td>113±6</td>
<td>NS</td>
<td>113±4</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>53±2</td>
<td>NS</td>
<td>50±3</td>
<td>NS</td>
<td>55±2</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>89±1</td>
<td>p&lt;0.001</td>
<td>100±2</td>
<td>NS</td>
<td>101±1</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Insulin (uU/mL)</td>
<td>10.1±1.1</td>
<td>NS</td>
<td>10.4±0.8</td>
<td>NS</td>
<td>9.8±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>HOMA</td>
<td>2.3±0.3</td>
<td>NS</td>
<td>2.6±0.2</td>
<td>NS</td>
<td>2.5±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.3±0.1</td>
<td>NS</td>
<td>0.5±0.1</td>
<td>NS</td>
<td>0.4±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Factor VII (%)</td>
<td>108±5</td>
<td>NS</td>
<td>114±4</td>
<td>NS</td>
<td>106±4</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>357±19</td>
<td>p=0.11</td>
<td>408±17</td>
<td>p&lt;0.001</td>
<td>461±12</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>PAI-1 (ng/mL)</td>
<td>35.0±11.0</td>
<td>NS</td>
<td>33.2±4.7</td>
<td>NS</td>
<td>28.0±4.1</td>
<td>NS</td>
</tr>
<tr>
<td>Homocysteine (umol/L)</td>
<td>7.0±0.4</td>
<td>p=0.16</td>
<td>9.0±0.6</td>
<td>p&lt;0.002</td>
<td>12.2±0.7</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 4.3. Age-related differences in components of the metabolic syndrome and risk factors for CVD.
4.3.4 Oxidative Stress and CVD risk factors

There were no significant differences in urinary isoprostane or plasma protein carbonyl concentrations between the three groups. The aged group had the highest levels of oxidative damage to DNA (p<0.003) and this is discussed in detail elsewhere (Frisard, M in preparation). Unexpectedly, there were no significant correlations between oxidative damage to lipids, proteins, and DNA and any markers of cardiovascular disease within the study cohort. In addition, multiple regression analysis revealed that markers of oxidative stress were not related to the incidence of the metabolic syndrome or markers of CVD in this population.

4.4 Discussion

The results of this study indicate that risk factors for CVD in nonagenarians are not elevated and comparable to younger individuals. Our findings support the hypothesis that like centenarians, nonagenarians may be protected from age related disease, a factor that may contribute to their longevity. Contrary to our hypothesis, oxidative stress was not related to components of the metabolic syndrome and markers of CVD.

Studies in centenarians reveal that these individuals appear to be resistant to the development of age-related diseases such as CVD and NIDDM (Perls T 2000). While CVD risk factors worsen with age (1987; Ryan 2000) centenarians display a favorable lipid profile (Barzilai, Atzmon et al. 2003), preserved glucose tolerance, and insulin sensitivity (Paolisso, Gambardella et al. 1997). Although centenarians are susceptible to the age related changes in body composition (Paolisso, Gambardella et al. 1995), this does not appear to affect their “risk for longevity”. One of the most interesting traits of centenarians is that they appear to be protected from oxidative stress and this has been proposed as partially responsible for their successful aging (Klapcinska, Derejczyk et al. 2000).
4.4.1 Age and Body Composition

Nonagenarians in the current study weighed less than the two younger groups but all three groups had similar percent body fat. Body weight increases with age until age 60y and then begins to decrease (Waaler 1988; Erens 1999) as individuals get closer to 100y. The current findings are in agreement with Paolisso et al who observed a decrease in body weight in individuals >100y when compared to individuals aged < 50y and 75-100y (Paolisso, Gambardella et al. 1995). Although, they also observed a positive association between age and percent fat and W / H ratio in their population, which was not observed in the current study. It is evident that the rate of obesity is increasing in westernized societies. The prevalence of obesity from NHANES III conducted between 1988 and 1994 was 23% increasing to 31% in 1999-2000. To examine if the lack of differences in body composition measured between the groups was the result of a possible birth cohort effect, we compared the means from the current data to data collected in the National Health Examination Survey I (NHES I) conducted from 1960-1962. Interestingly, the individuals in the young and aged group in the current study were on average 15kg heavier compared to subjects from the NHES 1 (http://www.cdc.gov/nchs/about). Therefore, our results should be interpreted with caution because Newschaffer et al (Newschaffer, Bush et al. 1992) demonstrated that cross sectional studies are often affected by birth effects. With this in mind, the differences observed in our own study may be due to poor health in the young individuals and not from disease resistance of the nonagenarians. Prospective studies are needed to address this issue. Furthermore, there were almost twice as many females as males in the young group. Since females generally have more body fat than men, differences in body fat between the groups may have been the result of gender effects. The
elevated mean level of body fat in the young versus older group may actually be due to the increased number of female subjects. Finally, sophisticated techniques including computed tomography and magnetic resonance spectroscopy would provide more specific information on body fat distribution changes with age.

4.4.2 Age and Metabolic Syndrome Classification

The aged group (60-74y) had the greatest number of components of the metabolic syndrome. These differences remained when medication to treat hypertension and hyperlipidemia were included in the components for the metabolic syndrome. These results are interesting considering that the nonagenarians had the highest incidence of hypertension. Meigs et al (Meigs, D'Agostino et al. 1997) used factor analysis to determine if there is a single etiology accounting for the mutual occurrence of the components of the metabolic syndrome. They proposed that there were three factors underlying the clustering of factors for the metabolic syndrome, hyperinsulinemia, obesity, and dyslipidemia. Hypertension was linked only through associations with obesity indicating that hypertension may not be a central characteristic of the metabolic syndrome. In the current study, if hypertension is removed as a component of the metabolic syndrome, the differences between the aged and nonagenarians increased and the significance between the young group and the nonagenarians disappeared. Taken together, these results signify that hypertension may not be a specific component of the metabolic syndrome but part of the normal aging process.

Previous studies have reported that individuals who reach extreme old age have unique lipoprotein profiles, a trait also observed in their offspring when compared to age-matched controls (Barzilai, Gabriely et al. 2001; Barzilai, Atzmon et al. 2003). Although, lipoprotein particle size was not measured in the current study, nonagenarians had fasting plasma lipids
comparable to younger individuals. A major limitation to the present study is a lack of exclusion
criteria regarding blood pressure and hyperlipidemia medication. More nonagenarians (78%)
were taking medication to treat blood pressure than those in the aged group (47%). In contrast, a
higher percentage of the aged group (34%) compared to the nonagenarians (20%) were taking
medication to treat hyperlipidemia. The fact that a large number of the older individuals were
taking medication could mask potential differences detected between the groups and lead to
conclusions that the older subjects were healthier than they actually were. Furthermore, the
exclusion of diabetes in the current study may have led to selection bias since elevated fasting
glucose is one of the components of the metabolic syndrome. The inclusion of individuals with
diabetes could lead to a higher prevalence of the metabolic syndrome in the older groups.

4.4.3 Age and CVD markers

In addition to the widely recognized risk factors for CVD, there are a number of
emerging novel biomarkers becoming more widely accepted. For example, the American Heart
Association has established reference values for CRP (Pearson, Mensah et al. 2003). Clinicians
are also examining the potential of elevated fibrinogen, homocysteine, and PAI-1 concentrations
to predict future CVD events (Pearson, Mensah et al. 2003). In the current study, values of CVD
markers were all within the normal range. Except for fibrinogen and homocysteine
concentrations, nonagenarians had comparable levels of CVD markers to the younger groups
indicating that nonagenarians have CVD risk profiles that are comparable to younger individuals.
It is interesting to note that we found no significant differences in CRP concentrations between
the groups. CRP has been relied upon as a highly sensitive marker of inflammation (Ridker,
Brown et al. 2004). The American Heart Association and Centers for Disease Control (Pearson,
Mensah et al. 2003) have recently issued guidelines for high sensitivity (hsCRP). Levels of
hsCRP of <1mg/L, 1 to 3mg/L, and >3mg/L should be interpreted as low, moderate, and high risk, respectively. The values of hsCRP measured in the present study were much lower than the issued guidelines; however the population in the current study was a relatively healthy population with no incidence of diabetes and few cases of untreated cardiovascular disease. The overall health of the population, especially in the nonagenarians may explain the reason for the low hsCRP levels. In addition, we found no differences in PAI-1 concentration and Factor VII activity. PAI-1 is the primary inhibitor of fibrinolysis and Factor VII is a clotting factor that promotes thrombosis. Each of these markers have been demonstrated to be predictive of future coronary events (Kannel, Wolf et al. 1987; Ridker, Glynn et al. 1998; Thogersen, Jansson et al. 1998) and in fact, CRP and PAI-1 may be better markers of future disease than fibrinogen or homocysteine (Ridker, Brown et al. 2004). The higher homocysteine concentrations may not have had a consequential effect in the nonagenarians unless CRP and PAI-1 are also elevated.

4.4.4 Oxidative Stress and CVD risk factors

Oxidative stress is directly related to the progression of atherosclerosis (Wassmann and Nickenig 2003). Therefore, it was expected that individuals with higher levels of oxidative damage would also have more risk factors for CVD. The finding that oxidative stress was not related to cardiovascular risk does not support the hypothesis that oxidative stress is implicated in the development of the metabolic syndrome and CVD. However, this study was limited by a lack of exclusion criteria regarding medication to control specific components of the metabolic syndrome. Thus, the results may have been affected by the fact that the older subjects were taking medications to treat high blood pressure and hyperlipidemia. These medications have previously been shown to alter markers of oxidative stress thereby masking possible relationships.
4.5 Conclusion

In conclusion, nonagenarians in our study had cardiovascular risk profiles that were comparable to aged individuals indicating that their longevity may occur as a result of their resistance to disease. Furthermore, these individuals have less incidence of metabolic syndrome than aged individuals although the reduced incidence is not directly related to less accumulation of oxidative stress.
Chapter Five

Physical Activity Level and Physical Function in the Elderly

5.1 Introduction

The number of individuals aged 65 and older accounts for just over 12% of the population in the United States. In 1997, more than half of the older population (54.5%) reported having at least one disability, with at least 1/3 reported having at least one severe disability affecting their daily life. This loss of independence is thought to be partially due to a decrease in physical activity with age (Powell, Thompson et al. 1987). However, in large populations, accurate measurement of physical activity is challenging and very little data is available on physical activity level in extremely old individuals (Washburn, Janney et al. 1990). Furthermore, studies in very old individuals indicate that they may be protected from “aging” and this may be due to higher than expected levels of physical activity (Paolisso, Gambardella et al. 1995). More importantly, the assessment of physical functionality in the elderly may provide more specific information on the severity of functional impairment as well as steps for the prevention and treatment of age-related disability.

Aging is associated with a decrease in muscle mass and physical functionality, an accumulation of body fat, and consequently increased risk for diseases of the metabolic syndrome (Roubenoff and Hughes 2000). This in turns results in frailty, reliance on others, and reduced quality of life causing a vicious cycle (Verbrugge and Jette 1994). Maintenance of independence in the elderly relies on a number of factors including strength and endurance, both of which are determined by physical activity level or aerobic fitness level (Cress, Buchner et al. 1999). Physical activity (PA) is inversely associated with mortality risk in epidemiological
studies (Davis, Neuhaus et al. 1994; He, Ogden et al. 2001; Rockhill, Willett et al. 2001) and usually (McGandy, Barrows et al. 1966) although not always (Segebartt, Nieman et al. 1988) declines with age. McGandy et al (McGandy, Barrows et al. 1966) reported that the reduction in physical activity could account for approximately 58% of the decrease in total energy expenditure with age. Doubly labeled water (DLW) is considered the reference method for the measurement of total energy expenditure under free-living conditions (Schoeller and van Santen 1982). In addition, DLW studies allow the calculation of activity energy expenditure (AEE) by determining the difference between TEE, and the sum of RMR and the thermic effect of food (TEF). From these studies it has been clearly shown that physical activity adjusted for body size does in fact decline with age (Black 1996) and by using these calculations it is estimated that physical activity level is responsible for 46% of the decrease in TEE (Black 1996). Furthermore, sedentary energy expenditure measured in a metabolic chamber was not reduced in elderly subjects compared to younger subjects after adjustment for body size (Vaughan, Zurlo et al. 1991) also suggesting that lower EE reported in free living conditions is related to lower activity levels. Finally, comparisons of nonagenarians and individuals aged 73y revealed that all of the reduction in TEE observed in the older individuals was a result of decreased activity (Rothenberg 2002).

Tests of physical function in the elderly have gained popularity as alternatives to traditional laboratory measures of physical fitness (Wood, Hondzinski et al. 2003). These tests have a number of advantages over traditional measures of fitness because they assign greater meaning to one’s ability to perform activities of daily living (Wood, Hondzinski et al. 2003), are more insightful to the site and severity of functional impairment, are less susceptible to floor and ceiling effects (Cress, Buchner et al. 1996), and are applicable to a wider range of age and fitness
levels than more traditional fitness tests (Wood, Hondzinski et al. 2003). The Reduced Continuous Scale-Physical Function Performance Test (CS-PFP (10)) is an adaptation of the full CS-PFP(10) designed by Cress et al (Cress, Buchner et al. 1996) and is used in several physical domains and applied to a broad spectrum of abilities. The test consists of a series of activities required for independent living. The test has been validated against other measures of physical fitness including maximal oxygen consumption and measures of upper and lower body strength (Cress, Buchner et al. 1996). However, whether the CS-PFP (10) predicts free-living physical activity is currently unknown.

The benefits of regular physical activity on health require the accurate assessment of physical activity level at the population level (Mohan, Gokulakrishnan et al. 2005). While doubly labeled water remains the gold standard for assessment of energy expenditure and physical activity level, the cost makes it impractical for wide scale use. Therefore it is important to validate alternative methods for the estimation of physical activity level. The MTI (Manufacturing Technology Inc., formerly Computer Science Applications, Fort Walton Beach, FL) is a uniaxial accelerometer that records acceleration of magnitudes ranging from 0.05 to 2 G and frequencies of 0.25 to 205 Hz, thereby filtering out movements not made by the subject (Swartz, Strath et al. 2000). The monitor is compact, lightweight, and can be worn for extended periods of time for more complete assessment of physical activity level (Nichols, Morgan et al. 2000). The data is collected as a series of counts representing the intensity of activity at that time interval (Melanson and Freedson 1995). This activity monitor has been shown to be a valid tool in assessing activity level in children (Janz 1994) and adults (Melanson and Freedson 1995). However, there is very little data on the accuracy of these monitors to accurately estimate physical activity in older individuals, especially nonagenarians (Washburn, Janney et al. 1990).
In the present study, we compared levels of physical activity and physical functionality in two groups: a group of nonagenarians and a group of elderly subjects aged 60-74y. We hypothesize that relatively healthy nonagenarians still have “high levels” of physical activity, which confer them to a high level of physical functionality as measured by the CS-PFP (10). In addition, we validated the MTI accelerometer in a group of elderly subjects against doubly labeled water, the gold standard assessment of physical activity.

5.2 Methods

5.2.1 Subjects

The subjects included in this study represent a subset from a population based study called the Louisiana Healthy Aging Study. Data was collected in 2 groups of individuals aged 60-74 and ≥90y. Randomly selected subjects (voter registration lists and Medicare Beneficiary Enrollment Data File from the Center of Medicare and Medicaid Services) were invited to participate in the study. Subjects were excluded if they had elevated fasting blood glucose (>125 mg/dl), diagnosed diabetes, unstable cardiovascular disease, thyroid disease, or mental health problems requiring drug treatment. Those subjects who are ≥90y were excluded if they had a heart attack or stroke in the last three months, severe high blood pressure, blood vessel aneurysm, taking certain medications used for myasthenia gravis, or have uncontrolled asthma, an asthma like condition, or emphysema / Chronic Obstructive Pulmonary Disorder. Subjects provided written informed consent. The study was approved by the Institutional Review Board at the Pennington Biomedical Research Center.

5.2.2 Study Design

Subjects aged 60-74y were asked to report to the Pennington Center for a screening visit to assess eligibility for the study. If eligible, they were asked to return to the Center for an 8-h
test day. Eligible nonagenarians were visited by a representative who explained the study and assessed initial inclusion criteria. If the subjects were interested and eligible, a nurse practitioner returned to the subject’s home to draw blood for screening assessment, administered a series of physical activity questionnaires, and scheduled the test day. Nonagenarians were transported to and from the Pennington Center for the 8-h test day. Subjects reported to the inpatient unit at the Pennington Center the morning of the test day where all measurements were taken in the same order for each subject. Upon arrival to the Pennington Center, baseline urine was collected for the doubly labeled water measurement. The subjects were then given the doubly labeled water which was followed by the RMR measurement. Subjects were fed both breakfast and lunch at the center and this was followed by physical function testing. The purpose of this study design was to ensure that the subjects were fully aware of what would be involved if they agreed to participate in the study.

5.2.3 Body Composition

The participants were weighed in a hospital gown. The measurement was taken to ±0.1kg with an electronic scale (Detecto, Webb City, MO) that was checked daily with a standard 25-kg weight. Height was measured to ± 0.5cm with a wall mounted stadiometer (Holtain, Crymych, Dyfed, United Kingdom), and body mass index was calculated as mass/height$^2$ (kg/m$^2$). Body composition was measured by DEXA (Hologics, QDA 4500A, Bedford, MA). Subjects were asked to lie on their backs in a hospital gown and asked to remove all metal. The components of total body mass (lean mass, bone, and fat mass) were used to calculate percentage body fat.
5.2.4 Resting Metabolic Rate

Resting Metabolic Rate was measured using a Deltratrac II metabolic cart (Sensormedics, Yorba Linda, CA). Participants were measured in the morning following an overnight fast. The participants were required to rest for thirty minutes before the start of the test. Subjects remained under the hood for 30 minutes and the last 20 minutes were used to calculate energy expenditure. The cart was calibrated before each test using room air and a known calibrant gas concentration (96% oxygen and 4% carbon dioxide).

5.2.5 Doubly Labeled Water

Upon arrival to the Pennington Center on the morning of the test day, a baseline urine sample was collected before any other measurements were performed. Soon after, subjects were dosed (mixture of 0.2g of 10% enriched H$_2^{18}$O and 0.12g of 99% enriched $^2$H$_2$O per kg of estimated total body water; Cambridge Isotopes, Cambridge, MA). Total body water was estimated from body weight based on the assumption that 55% of body weight is total body water. The dose was followed by 100-ml of tap water used to rinse the dose container. Following two voids, two more urine samples were collected at 4.5 and 6h after dosing. On the mornings of day 13 and 14, subjects were instructed to discard their first morning urine and collect the second urine of the day. The H$_2^{18}$O abundance was measured in duplicate on a Finnigan MAT 252 dual inlet Gas Isotope Ratio Mass Spectrometer (DeLany, Schoeller et al. 1989). The $^2$H$_2$O abundance was also measured in duplicate also with a Finnigan MAT 252 dual inlet Gas Isotope Ratio Mass Spectrometer using a Finnigan H/D device at 500° (DeLany, Schoeller et al. 1989). The enrichments of the post dose samples were compared to the enrichment of the baseline sample. The $^2$H and $^{18}$O isotope elimination rates ($k_D$ and $k_O$) were calculated using linear regression following a log transformation. Total body water was
determined at enrichment time zero, obtained from the regression line of the H$_2^{18}$O isotope. The rate of CO$_2$ production was calculated using the equations of Schoeller et al (Schoeller 1988) and later modified (Racette, Schoeller et al. 1994) as follows:

$$r_{CO_2} \text{ (moles/d)} = (N/2.078) \left(1.007k_O-1.041k_D\right) - 0.0246r_{GF},$$

where $r_{CO_2}$ is the rate of carbon dioxide production; $N$ is total body water calculated from $N_O/1.007$; $k_O$ and $k_D$ represent the elimination rates of oxygen-18 and deuterium, respectively from total body water; and $r_{GF}$ is the rate of fractionated gaseous evaporative water loss, which is estimated to be $1.05N \left(1.007k_O - 1.041k_D\right)$. Total energy expenditure (TEE) was calculated by multiplying $r_{CO_2}$ based on the estimated food quotient of the diet and estimated changes in body energy stores:

$$TEE \text{ (kcal/d)} = 22.4 \ r_{CO_2} \left(3.9/RQ + 1.10\right)$$

where TEE is the total energy expenditure and $RQ$ is the estimated respiratory quotient (estimated to be 0.88). The energy equivalent of CO$_2$ ($E_{eqCO_2}$) = 5.637kcal / 1 CO$_2$.

### 5.2.6 Physical Activity Level

Activity energy expenditure (AEE) was determined by the following equation (Rising, Harper et al. 1994):

$$AEE=TEE-(RMR + 0.1TEE)$$

This approach assumes that the thermic effect of feeding is 10% (Ravussin and Swinburn 1993).

The level of physical activity, often referred to as PAL was calculated as the ratio of total energy expenditure to resting metabolic rate. Another index of activity will be calculated as TEE adjusted for RMR by linear regression analysis.

### 5.2.7 Modified Continuous Scale Physical Functional Performance Test (10) (CS-PFP (10))

Subjects underwent a modified version of the CS-PFP(10) developed by Cress et al (Cress, Buchner et al. 1996). The CS-PFP (10) has been used quite extensively for the assessment of physical functionality in older people. In the original version, 15 everyday tasks
were chosen to represent activities essential to independent living. In this modified version, 10 of the 15 tasks from the original test were used to assess functionality. This test is deemed to be appropriate to a wide array of older adults, as it requires only a moderate level of effort throughout the test. That is, participants are instructed to perform the activities as quickly, but as comfortably as possible. The test includes the following: 1) weight carry test: The subject was asked to carry two 5-lb sandbags from one counter to another counter approximately 63 inches away. 2) Jacket test: The subject was asked to put on a light “windbreaker” and zip the jacket completely. 3) Scarves test: The subject was asked to pick up 4 scarves, one at a time, from the floor. 4) Reach test: The subject was asked to reach as high as possible and place a sponge on a shelf, which is an 8-ft high adjustable shelf mounted on the wall. The subject then had to remove the sponge and place their hands by their side. 5) Floor sweep test: The subject was asked to sweep up a ½ cup of kitty litter in a 4 × 3 block square. 6) Laundry test 1: Subject was asked to empty a top loading washer into a side loading dryer. The subject unloaded and loaded three 2lb. and one-3lb. bags of sand and 4 lbs. of laundry 7) Laundry test 2: Subject was asked to unload a dryer and then place only the sandbags into a laundry basket and move the basket to the cabinet, 36” high adjacent to the dryer. 8) Floor down/up test: The subject was asked to start in the standing position and then sit down on the floor and then immediately stand up finishing with their arms at their side. 9) Stair climb test: The subject was asked to climb one flight of stairs, 9-to-11 steps, 12 inches in depth, 6.5 inches high. 10) Grocery test: The subject was asked to carry a comfortable amount of groceries 16.3 yards to a set of bus steps. They ascended the bus steps, turned around, descended the steps, carried the bag to the door, opened, and closed the door. The total walking distance is 42.3 yards, excluding the steps. Subjects can make more than one trip, and the maximum weight allowed is 30lbs over two trips.
5.2.8 Accelerometers

Simultaneous to the measurement of energy expenditure by doubly labeled water, each individual was fitted with an MTI (Manufacturing Technology Inc., formerly Computer Science Applications, Fort Walton Beach, FL) activity monitor which was worn on the hip 24 hours per day including at night while sleeping, for 14 days. The only time they removed the monitor was during showering. The accelerometer was set to collect at 1-minute intervals. The actigraph uses 8-bit resolution, providing less than 0.02 G (gravity) resolution. It has a $\pm 2.15$G dynamic range with a unique filtering system, which filters out all non-human frequency components. It has been validated by numerous studies (Freedson, Melanson et al. 1998; Trost, Ward et al. 1998; Ekelund, Sjostrom et al. 2001), in one of which it was compared to doubly labeled water (Ekelund, Sjostrom et al. 2001). Data is presented as total activity counts per day expressed as total activity counts/ day and average wake counts expressed as (total count – sleep count) / (total time – sleep time) and expressed as average counts/h.

5.2.9 Statistical Analysis

Data in the text and tables are provided as means $\pm$ SEM. Statistical analysis was performed using SPSS Version 12 (SPSS Inc. Chicago, IL). Multiple regression was used to adjust total energy expenditure for FFM, FM, and sex as well as adjusting for RMR. The residuals represent the difference between predicted and measured values. A t-test was used to assess differences between the groups. Spearman correlation coefficients were run to assess relationships between assessments of physical activity.
5.3 Results

5.3.1 Body Composition

A total of 47 subjects were included in the analysis. Subject characteristics by age and sex are displayed in table 5.1. Nonagenarians weighed significantly less (p<0.001) and had significantly less fat free mass (p<0.001) and fat mass (p<0.009) than the younger subjects. As expected, males were taller and weighed more than females (both, p=0.05) (Table 5.1). Females had higher percent body fat due to less fat free mass than males (both, p<0.05). There were no differences in fat mass.

5.3.2 Total Energy Expenditure / Resting Metabolic Rate

As illustrated in the top panel of Figure 5.1 panel nonagenarians had significantly lower RMR (p=0.03) and TEE (p=0.03) than the younger group that was independent of differences in fat free mass. The bottom two panels of 5.1 illustrate the differences in adjusted TEE between the two groups. When adjusting for FFM, FM, and sex, the younger subjects have a daily TEE that was 100 kcal/d higher than predicted and the nonagenarians had TEE that was 200 kcal/d lower than predicted (5.1, bottom left). Similar results were noted when adjusted for metabolic mass (RMR) (Figure 5.1, bottom right) although the differences between groups did not reach significance. This may had to do with a low number of subjects included in the analysis. RMR in the nonagenarians was reduced by 352 kcal/d (p<0.001) and this accounted for 41% of the lower TEE in the nonagenarians (Figure 5.2, top panel).

5.3.3 Activity Energy Expenditure / PAL

Physical activity level, expressed as AEE (p<0.001, 5.2, top panel) was significantly lower in the nonagenarians. AEE was 509 kcal/d lower in the nonagenarians and this accounted for 49% of the lower TEE in the nonagenarians. Physical activity level expressed as PAL was
also significantly lower (p<0.04, 5.2, bottom panel) in the nonagenarians compared to the younger group.

Figure 5.1. Total Energy Expenditure
Table 5.1. Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Mean ±SEM</th>
<th>60-74y</th>
<th>&gt;90y</th>
<th>Age Difference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male 17</td>
<td>Female 15</td>
<td>Male 4</td>
<td>Female 11</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>71±1</td>
<td>70±1</td>
<td>95±1</td>
<td>93±1</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>90±3</td>
<td>76+5*</td>
<td>70±1</td>
<td>60+4*</td>
<td>0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>176±1</td>
<td>159±1*</td>
<td>169±1</td>
<td>158±1*</td>
<td>0.003</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>64±2</td>
<td>45±2*</td>
<td>53±2</td>
<td>38±2*</td>
<td>0.001</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>25±2</td>
<td>32±3</td>
<td>17±2</td>
<td>21±2</td>
<td>0.009</td>
</tr>
<tr>
<td>Percent Fat (%)</td>
<td>28±1</td>
<td>41±1*</td>
<td>24±2</td>
<td>35±2*</td>
<td>0.493</td>
</tr>
<tr>
<td>RMR (kcal/d)</td>
<td>1627±29</td>
<td>1297±58</td>
<td>1268±57</td>
<td>1066±45</td>
<td>0.001</td>
</tr>
<tr>
<td>TEE (kcal/d)</td>
<td>2852±112</td>
<td>2149±108*</td>
<td>2062±72</td>
<td>1608±62*</td>
<td>0.001</td>
</tr>
<tr>
<td>TEE (kcal/kg/d)**</td>
<td>+48+83</td>
<td>+24+58</td>
<td>-205.38</td>
<td>-222±75</td>
<td>0.02</td>
</tr>
<tr>
<td>AEE (kcal/d)</td>
<td>1225±91</td>
<td>997±161</td>
<td>795±42</td>
<td>542±59</td>
<td>0.001</td>
</tr>
<tr>
<td>PAL (TEE/RMR)</td>
<td>1.75±0.1</td>
<td>1.7±0.1*</td>
<td>1.6±0.1</td>
<td>1.5±0.1*</td>
<td>0.03</td>
</tr>
<tr>
<td>Accelerometer total (counts/d) T</td>
<td>264406±69634</td>
<td>197635±19800</td>
<td>101674±47146</td>
<td>224425±87217</td>
<td>0.549</td>
</tr>
<tr>
<td>Accelerometer average (counts/h) T T</td>
<td>336±144</td>
<td>177±17</td>
<td>103±54</td>
<td>263±105</td>
<td>0.657</td>
</tr>
<tr>
<td>CS-PFP(10) total score (au)</td>
<td>62.8±2.3</td>
<td>50.8±3.0*</td>
<td>26.1±1.8</td>
<td>16.2±3.9*</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Sex Differences (p<0.05)

** Expressed as residual values, i.e. measured minus predicted value or the mean of the entire group. Adjusted from Fat Free Mass, Fat Mass, and Sex.
AEE- Activity Energy Expenditure
T - Total counts per day.
T T- Average count per hour (total count – sleep count) / (total time – sleep time)
Figure 5.2. Components of Total Energy Expenditure
5.3.4 Continuous Scale Physical Functionality Test (10)

Nonagenarians scored significantly lower on the CS-PFP (10) than the younger subjects (Figure 5.3, p<0.001). In addition, the CS-PFP (10) total score was highly correlated with PAL (r=0.55, p<0.001) (Figure 5.4, panel A) and persisted when analyzing the nonagenarians only (r=0.75, p<0.003) (Figure 5.4, panel B). There was also a significant relationship between CS-PFP(10) score and TEE adjusted for FFM, FM, and sex (Figure 5.4, Panel C, r=0.40, p<0.001) however this did not remain significant when only the nonagenarians were included in the analysis (Figure 5.4, Panel D).

Figure 5.3. CS-PFP 10 Total Score

5.3.5 Accelerometers

The correlations between PAL and activity counts are illustrated in Figure 5.5. PAL was significantly correlated with total activity counts (r=0.45, p<0.008), and average wake activity counts (r=0.45, p<0.01). However, as shown in Table 5.1, the activity monitors were not able to detect differences in physical activity between the two groups.
Figure 5.4. A. Comparison between CS-PFP (10) and PAL in entire group. B. Comparison between CS-PFP (10) and PAL in nonagenarians only. C. Comparison between CS-PFP (10) and TEE adjusted for FFM, FM, and Sex. D. Comparison between CS-PFP (10) and TEE adjusted for FFM, FM, and Sex in nonagenarians only.
Figure 5.5. Activity Monitors Compared to PAL.
5.4 Discussion

To our knowledge, the Louisiana Healthy Aging Study is the first of its kind to examine the relationship between functional performance and physical activity level measured by doubly labeled water and RMR in a group of elderly individuals. Contrary to our hypothesis, the results of this study confirm previous findings that TEE is lower in nonagenarians when compared to younger individuals and that this is attributed to a decrease in both RMR and physical activity level. The present study also demonstrates that nonagenarians have impaired functional performance compared to younger individuals and this is associated with reduced physical activity. These findings in nonagenarians, known models of “successful agers” may imply that some degree of functional impairment may be a part of the natural aging process, but maintenance of physical activity may help delay some of this age-related decline. Finally, this study provides for the first time, evidence that the CS-PFP(10) is a good assessment of physical activity level in elderly individuals.

Aging is frequently characterized by a decline in total daily energy expenditure (Black 1996), attributed to a decline in RMR (Keys, Taylor et al. 1973), energy expended in physical activity (Vaughan, Zurlo et al. 1991), or both. However, whether RMR declines with age independent of changes in body composition is still controversial (Keys, Taylor et al. 1973; Tzankoff and Norris 1977). In one of the oldest populations investigated, RMR was not different between individuals ≥90y and individuals aged 73y. Furthermore, predicted RMR was not significantly different from measured RMR in nonagenarians (Rothenberg, Bosaeus et al. 2000) indicating that the decline in TEE with age is a result of a change in physical activity level. Doubly labeled water studies have provided valuable information for assessing free living physical activity level (Schoeller, Ravussin et al. 1986). When TEE and RMR are measured, it is
possible to calculate the amount of energy expended on physical activity either by dividing TEE by RMR (PAL), or by subtracting TEE from the sum of RMR and estimated TEF (AEE) (Black 1996). Using this method, Rising et al (Rising, Harper et al. 1994) observed an inverse relationship between physical activity energy expenditure and chronological age. Pannenams et al (Pannemans and Westerterp 1995) also noted that energy expended in physical activity was lower in older individuals but they observed no differences in PAL between young and old individuals. Longitudinal studies can provide more specific information on the changes in physical activity patterns over time. Binjin et al (Bijnen, Feskens et al. 1998) observed a decline in physical activity level in elderly men (65-84y) over a period of 10y. Nevertheless there are a number of factors that determine an individual’s activity level including physiological, psychological, and socio-environmental factors and it is unknown what the contributions of these factors are.

The difference in total energy expenditure in young and old individuals in the current study persisted after adjusting for body weight and composition. Contrary to our hypothesis, physical activity level was lower in nonagenarians and accounted for 49% of the decline in TEE. We also observed that nonagenarians had lower adjusted RMR and this accounted for approximately 41% of the reduction in TEE. This is in agreement with Roberts et al (Roberts, Fuss et al. 1995) who observed a similar pattern of decline in TEE, RMR, and AEE in younger individuals (20y compared to 60y). Rothenberg et al (Rothenberg 2002) also compared TEE between nonagenarians to subjects aged 70y to find that physical activity also accounted for approximately 40% of the decline in TEE in the nonagenarians. Physical activity in the elderly varies greatly with health and independence. There are a number of factors that contribute to an individual’s physical activity level including psychological and environmental factors. It is
impossible to know the contribution of each of these to an individual’s activity status.

Furthermore, it is possible that extremely old individuals make an active choice to decrease physical activity level for reasons other than inability or infirmity (Parks, Housemann et al. 2003; Greenberg and Renne 2005), therefore assessment of lifelong instead of present physical activity level may provide more information on the effect of physical activity on health and independence. This is supported by data by Hillsdon et al (Hillsdon, Brunner et al. 2005) who demonstrated that individuals (30-63y) who were physically active earlier in life were more likely to report functional independence at a 9-y follow-up.

One of the primary goals of gerontology research is the maintenance of health and independence in the elderly. Physical function tests have gained in popularity because they provide more specific information on the level of independence than typical laboratory measures of aerobic fitness. In the current study, nonagenarians were less functionally independent than younger subjects and this was associated with reduced physical activity. This is the first study to demonstrate a relationship between physical functionality and PAL. In the past, the CS-PFP(10) has proven reliable when compared to VO2peak in elderly individuals (Cress, Buchner et al. 1996). Our results indicate that when compared to the gold standard assessment of physical activity, energy expenditure by doubly labeled water and indirect calorimetry, the CS-PFP (10) is directly related to physical activity level. It is not possible to determine from the current study whether physical activity level determines physical functionality or whether functionally independent individuals are more physically active. However, previous data demonstrates that six months of structured exercise resulted in significant improvements in performance on the CS-PFP(10) test (Cress, Buchner et al. 1999). In fact, exercise intervention in disabled women or women diagnosed with CHD resulted in improvements in physical capacity over a wide range of
household activities (Brochu, Savage et al. 2002; Ades, Savage et al. 2003). Finally, Toraman et al (Toraman and Ayceman 2005) demonstrated that six weeks of detraining resulted in declines in components of physical function indicating that maintenance of physical activity results in improved physical function.

The benefit of physical activity in the prevention of disease makes it imperative to accurately assess physical activity level on the population level. While doubly labeled water remains the gold standard for assessment of energy expenditure and physical activity level, the expense makes it inappropriate for use in large populations. Therefore, it is important to determine the accuracy of less expensive methods to assess physical activity level on a large scale. To our knowledge, this study is the first of its kind to assess the accuracy of the MTI accelerometer to assess physical activity level in nonagenarians. The results of this study demonstrate that the MTI accelerometer can accurately predict physical activity level in an elderly population including individuals ≥90y. This is agreement with Ekelund et al (Ekelund, Sjostrom et al. 2001) who also found that activity counts measured by the MTI accelerometer correlated well with activity energy expenditure measured by doubly labeled water in younger subjects. The fact that the accelerometer was able to accurately predict PAL in this population is important because it indicates that the accelerometer is sensitive to the lower range of activity intensity which is more common in the elderly. However, the fact that it was not able to detect differences between the groups may be of concern. The monitors were placed on the hip in the current study. Placement of the monitor on the wrist or the use of two monitors, one on the wrist and one on the hip may be more useful in assessment of physical activity in elderly individuals (Swartz, Strath et al. 2000).
Aging is associated with increased risk of dependency and disability characterized by
generalized weakness, impaired mobility, and poor endurance (Lamberts, van den Beld et al.
1997; Fried, Tangen et al. 2001). This dependent state is known as frailty and is defined as “a
state of reduced physiological reserves associated with increased susceptibility to disability”
(Buchner 1992). It results in fractures, impairment in activities of daily living, and loss of
functional independence (Tinetti, Speechley et al. 1988). While most elderly individuals die
from age-related illness such as cardiovascular disease and cancer, many of the oldest old,
having escaped these age-related diseases will suffer from frailty thereby impairing physical
function and their ability to live independent lives (Lamberts, van den Beld et al. 1997). The
maintenance of strength and endurance helps to delay the age-related increase in frailty and
dependence (Frontera, Hughes et al. 1991). Physical activity and strength training helps to
maintain strength and endurance (Seals, Hagberg et al. 1984; Meredith, Frontera et al. 1989;
Frontera, Hughes et al. 1991) and may be beneficial in the maintenance of functional
independence.

5.5 Conclusion

In conclusion, TEE is lower in nonagenarians independent of differences in body weight
and body composition, and this is attributed to a lower RMR and reduced physical activity.
Furthermore, nonagenarians were less functionally independent and this may be due to reduced
physical activity. Finally, the MTI accelerometer was able to predict physical activity level in an
elderly population.
Individuals living into their nineties and beyond are often referred to as “successful agers”. The term successful aging refers to the fact that these individuals have made it to extreme old age, while still maintaining physical and cognitive function. Therefore, successful agers have often been characterized as human models of longevity. Data obtained from these individuals can provide valuable information into the aging process.

It has been hypothesized that successful agers may possess genetic and physiological traits that protect them from age associated disease and promote healthy aging. There are a number of mechanisms that have been proposed to play a role in this including genetics, energy metabolism, and oxidative stress. The overall hypothesis of this study was that characteristics of an individual’s metabolism predispose to long life (or not so long life) and retention of physical and cognitive capacity, promoting the sense of well being that is associated with healthy aging. The question is not only who lives the longest, but rather who lives the longest, the healthiest.

There are a number of proposed biomarkers of aging. These are characteristics (i.e. insulin) known to either increase or decrease with chronological age. However, it is not a strict linear relationship and inevitably, individuals will either be higher or lower than their chronological age and thus either “biologically” older or younger than expected. The overall result of this study, as displayed in figure 6.1 is that nonagenarians have health profiles comparable to middle age individuals (60-74y). Nonagenarians in the current study had a number of physiological traits, including waist circumference, total serum cholesterol and triglyceride concentrations, systolic and diastolic blood pressure, and DNA damage comparable to middle aged individuals and healthier than expected based on their chronological age.
While this may be related to reduced RMR and oxidative damage to DNA, it is not related to increased physical activity.

Figure 6.1 Hypothesis revisited.

The results of this study indicate that nonagenarians have a metabolic rate that is lower than expected based on body weight and body composition. However, although nonagenarians also have less accumulation of oxidative damage to DNA, it does not appear to be due to a lower metabolic rate. The accumulation of oxidative stress may not be the result of a direct relationship between oxygen consumption and free radical production. Rather, free radical production may be more dependent on the health and efficiency of the mitochondria rather than total oxygen consumption per se. It is interesting to note that although nonagenarians had comparable levels of protein carbonyl and urinary isoprostane concentrations, there were also no differences in oxidative stress between the two younger groups. This would lead to the conclusion that oxidative stress does not accumulate with age, however, the cross sectional study design lends itself to limitations in that it is impossible to determine those individuals in the younger groups who will live to extreme old age emphasizing the importance of longitudinal
studies. Furthermore, the accumulation of oxidative stress is dependent on a number of factors including free radical production, tissue susceptibility, and the strength of the defense and repair systems. The examination of each of these factors is important to truly characterize an individual’s oxidative status. Finally, oxidative damage does not accumulate in tissues uniformly; therefore larger studies with multiple markers of oxidative damage from multiple tissues are needed to fully answer this question.

Nonagenarians in the current study had a cardiovascular risk profile that was comparable to middle aged individuals. The current findings support the hypothesis that nonagenarians, like centenarians, may be protected from age-related disease, a factor that may contribute to their longevity. Although results from the present study do not demonstrate direct relationships between markers of oxidative stress and components of the metabolic syndrome and risk factors for cardiovascular disease, the fact that nonagenarians have comparable levels of oxidative stress and risk factors for CVD, one can only speculate that the two are related. The fact that the current study included individuals who were taking medications to treat hyperlipidemia and hypercholesterolemia as well as excluding individuals with elevated fasting glucose is a limitation because this could mask potential differences that could have been observed between groups. Furthermore, the current study was limited by the fact that very old individuals had to be healthy enough to come to the Pennington Center for testing. This excludes individuals of extreme old age who may be more characteristic of “normal” aging.

Finally, nonagenarians expended less energy than middle aged individuals independent of their lower body weight. The reduced TEE appears to be related to a reduction in RMR and physical activity level. Contrary to our hypothesis, nonagenarians were less physically active than younger individuals and this was related to increased functional impairment, although it is
not possible to determine cause and effect from the current study. However, if physical activity does determine functional capacity, the maintenance of regular physical activity may help to promote functional independence that is associated with healthy aging. Again, prospective studies are needed to address this issue.

Taken together, these results indicate that nonagenarians may be biologically younger than their chronological age. Future studies should prospectively examine, in younger individuals (70-90y), which factors, such as energy metabolism and mitochondrial function, physical activity, and oxidative stress significantly impact the aging process.
Literature Cited


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Appendix A: Proposal

SPECIFIC AIMS

Aging can be defined as a progressive decline in the ability of an organism to resist stress, damage, and disease. It is characterized by an increase in the incidence of degenerative disorders including cancer, cardiovascular disease, decreased immune function, and diabetes. The aging process proceeds at a varied rate in different individuals. Consequently, some individuals age slower than others predisposing them to long life and retention of physical and cognitive capacity promoting the sense of well being associated with healthy aging. Caloric restriction (CR), which is the only intervention to consistently extend average and maximum lifespan, is associated with a number of changes including a reduction in energy metabolism and a decrease in oxidative stress. One intriguing hypothesis is that CR lessens oxidative damage and repair by reducing energy flux and metabolism. It is not yet known if individuals who successfully reach “old age” have a reduced metabolic rate adjusted for respiring body mass and whether this is associated with lower levels of oxidative stress.

The purpose of this study and the major hypothesis tested in this project is that the relatively low metabolic rate (adjusted for body size and composition) is associated with “reduced” oxidative stress. We will test whether “healthy nonagenarians” have a relatively low metabolic rate when compared to middle age and young individuals and whether the low metabolic rate is associated with a low level of oxidative stress when compared to that of middle-aged individuals. Regular physical activity has been proven to induce weight loss, improve glucose and lipid profiles, as well as maintain bone density and muscle mass, improving the aging process. Although evidence does not support the idea that exercise increases lifespan, it may decrease morbidity and improve quality of life. Very little is known about physical activity in very old people. Therefore, we will measure levels of physical activity in nonagenarians using state of the art methods (doubly labeled water). Finally, we will measure predictors or biomarkers of disease and longevity.

Aim 1: Measure and compare resting metabolic rate (RMR) and oxidative stress in three groups of non-diabetic subjects i.e. 20-34, 60-74, ≥ 90 years.
We hypothesize that those subjects ≥ 90 years old have lower RMR (adjusted for fat free mass, fat mass, and sex) which will protect against aging by reducing oxidative stress. The low metabolic rate is associated with lower body temperature, and evidence of lower tissue oxidative stress when compared to middle age subjects (60-74y).

Aim 2: Measure and compare the level of physical activity by doubly labeled water in three groups of non-diabetic subjects i.e. 20-34, 60-74, ≥ 90 years.
We hypothesize that those subjects ≥ 90 years old still have relatively high levels of physical activity which confer them to a high level of physical and cognitive functionality which will be measured in other projects in the study.

Aim 3: Measure and compare markers metabolic syndrome (BP, fasting glucose and insulin, lipid profile, hemostasis factors, homocysteine) and markers of inflammation in three groups of non-diabetic subjects i.e. 20-34, 60-74, ≥ 90 years.
We hypothesize that because those subjects ≥ 90 years old have lower levels of oxidative stress, they will have lower concentrations of markers of metabolic syndrome than middle age subjects (60-74y).

BACKGROUND AND SIGNIFICANCE

Aging is characterized by a number of physiological changes, all of which contribute to a decreased ability to defend against disease ultimately resulting in death. However, in order to make it to extreme old age, “successful agers,” (individuals who have made it to extreme old age while still maintaining the ability to perform normal activities of daily life) markedly delay or even escape diseases that would otherwise be lethal at younger ages. This leads to questions concerning what factors have played a role in the apparent resistance to aging in these individuals. Energy metabolism is indirectly related to lifespan. The Rate of Living Theory hypothesizes a direct relationship between metabolic rate and the rate of aging by demonstrating that larger animals live longer and have lower metabolic rate per unit of muscle mass (Greenberg 1999). The link between energy metabolism and aging may be oxidative stress. Adelman et al reported a significant relationship between species- specific metabolic rate and DNA damage (Adelman, Saul et al. 1988). In addition, Sohal and Weindruch demonstrated an inverse relationship between maximum lifespan potential and species specific metabolic rate as well as free radical production. Oxidative stress is one of the prevailing theories of aging. The theory states “aging and degenerative diseases associated with it are attributed basically to side attacks of free radicals on cell constituents and on connective tissue.” This hypothesis is supported by numerous observations. For example, maximal lifespan is related to the amount of free radical production (Sohal and Allen 1985). Furthermore, overexpression of anti-oxidative enzymes or activation of defense mechanisms against oxidative stress retards aging and extends lifespan (Orr and Sohal 1994). Thus, the amount of oxidative damage increases as an organism ages and is postulated to be one of the major causal factors of aging and therefore lifespan.

B1. Aging and Energy Metabolism

In this study we are hypothesizing that successful aging people will have a lower metabolic rate that is out of proportion of respiring mass. Total energy expenditure can be divided into three main components, resting metabolic rate (RMR), thermogenesis, and energy cost of physical activity. The decline in total energy expenditure with age can result from a decline in any or all of these three components. Total daily energy expenditure (TEE) measured by doubly labeled water and adjusted for fat free mass declines with age (Pannemans and Westerterp 1995). However, whether RMR declines with age is still a matter of debate. Early cross sectional studies have revealed that absolute metabolic rate declines as a function of age. McGandy et al (McGandy, Barrows et al. 1966) measured basal oxygen consumption in 252 men aged 20-99 years. Their results indicate a decline in RMR per decade of approximately 52 cal per day. However, studies since then have yielded mixed results (Cunningham 1980; Fukagawa, Bandini et al. 1990; Hunter, Weinsier et al. 2001). Many have attributed the changes in RMR to age related changes in body composition i.e. decline in fat free mass and accumulation of fat mass. Calloway and Zanni (Calloway and Zanni 1980) investigated RMR in a group of healthy men aged 63-77 years. BMR of the older group was 13% below the rate of a younger group. However once adjusting for whole body potassium content this difference disappeared. They concluded that the decline in RMR with age was attributed to changes in body composition. Tzankoff and Norris (Tzankoff and Norris 1977) reported that after 45 years of age there was a
decline in RMR, which was accompanied by a decline in muscle mass measured by creatinine excretion. They concluded that the decline in RMR with age was solely due to the decline in muscle mass also seen with age. Roubenoff et al (Roubenoff, Hughes et al. 2000) also concluded that it is the decline in body cell mass not detectable by many measures of body composition that is responsible for differences in RMR. However, studies of the very old (>90y) compared to those aged 73y, detected no differences in RMR between the groups. In addition, predicted RMR from fat- free mass is not different from measured RMR in nonagenarians suggesting differences in TEE are due to activity level (Rothenberg, Bosaeus et al. 2000). Even still, it is still unclear whether resting energy expenditure declines with age. In studies by our own group (Ravussin) it was shown that RMR after adjusting for both FFM and FM is lower in older adults (Vaughan, Zurlo et al. 1991). Moreover, this study examining components of energy expenditure (RMR, sleeping metabolic rate, TEF, physical activity) using a respiratory chamber indicated that RMR was the only significantly lower measure old as compared to young adults once adjusting for FFM. In addition very few studies have examined energy expenditure in very old populations i.e. nonagenarians. Therefore, we intend to measure RMR using indirect calorimetry in three groups of individuals i.e. >90y, 20-34, and 60-74y).

B2. Energy Metabolism and Oxidative Stress
Energy metabolism has been associated with production of deleterious byproducts known as reactive oxygen species (ROS). Approximately 2% of the oxygen consumed results in the production of ROS such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH$^\cdot$) (Jenkins 1988). Earlier studies demonstrated under normal conditions, the electron transport is the primary producer of the superoxide anion, precursor to other dangerous free radicals. Early studies indicated that under normal conditions, electrons leak during their transfer from Complex I through ubiquinone to Complex III or within Complex III at the point of cytochrome b. These electrons are then able to react with oxygen to produce superoxide. This is the starting point of a chain reaction that leads to the production of other harmful radicals that result in damage to cells and tissues (Turrens and Boveris 1980). Oxidative stress has been implicated in a number of disease states. Free radicals contribute to endothelial dysfunction and are involved in the pathogenesis and development of cardiovascular diseases, such as atherosclerosis (Vassalle, Petrozzi et al. 2004). Oxidative stress is elevated in individuals with hyperlipidemia, and hypercholesterolemia (Araujo, Barbosa et al. 1995; Reilly, Pratico et al. 1998; Stojiljkovic, Lopes et al. 2002). It has also been implicated in a number of neurological disorders including Alzheimer’s, Parkinson’s, and Schizophrenia (Mecocci, MacGarvey et al. 1994; Milton 2004; Nishioka and Arnold 2004). In addition, oxidative stress has been implicated in the development of insulin resistance and diabetes (Pennathur and Heinecke 2004). Related to this, oxidative stress may be the cause of mitochondrial dysfunction in obesity. Accumulation of free fatty acids in the mitochondrial matrix, where oxidative processes takes place makes them prone to lipid peroxidation, which can lead to damage of the mitochondria. Mitochondrial dysfunction is thought to be responsible for insulin resistance. Uncoupling proteins (particularly UCP3), originally thought to be responsible for uncoupling in times of low energy demand are now thought to be primarily responsible for removing fatty acid anions from the mitochondria (Schrauwen and Hesselink 2004). There is a delicate balance between the production of free radicals and the antioxidative defense mechanisms that aerobic organisms have evolved to protect themselves. These defense mechanisms include enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase that catalytically remove free radicals and other
reactive species (Mills 1960; Kwong, Mockett et al. 2000; Ookawara, Haga et al. 2003). Overexpression of SOD in flies results in the extension of lifespan (Orr and Sohal 1994). In addition, dietary antioxidants such as tocopherols carotenoids, bilirubin, uric acid, ascorbic acid, coenzyme Q10, and selenium are responsible for scavenging free radicals as well as minimizing the availability of other reactive molecules such as heme (Halliwell 1999). Finally, another line of defense consists of DNA repair by the cells in which it was damaged (Breimer 1991; Demple and Harrison 1994). Oxidative stress results when the balance tips in favor of the prooxidants.

Figure 1

Steady state measures of oxidative stress represent the equilibrium among the rate of ROS generation, the rate of oxygen scavenging, and the rate of repair. Figure 1, adapted from Beckman and Ames (Beckman and Ames 1998) illustrates the delicate balance that exists between these 3 systems. Aging may be due to an increase in free radical production, but also by a decrease in ROS removal. For example, overexpression of antioxidant genes, such as SOD and catalase in houseflies results in the extension of lifespan as well as decrease in protein oxidation and improved physical function (Orr and Sohal 1994). Studies in centenarians reveal that this population has elevated levels of antioxidants when compared to younger individuals (Klapcinska, Derejczyk et al. 2000). Finally, calorie restriction studies reveal that reduced energy intake results in both a decline in metabolic rate but also a decrease in oxidative stress, as reviewed in (Heilbronn and Ravussin 2003).

Lipid molecules are the molecules most susceptible to free radical attack. Early studies have demonstrated that free radicals can react with unsaturated fatty acids in the lipid membrane leading to damage of the membrane (Bielski, Arudi et al. 1983). In addition, products of lipid peroxidation can react with DNA bases and proteins (Uchida and Stadtman 1992; Cai, Tian et al. 1996). Lipid peroxidation results in the formation of isoprostanes. Isoprostanes are prostaglandin like products resulting from the peroxidation of arachidonic acid. They are released in plasma and excreted in the urine and are therefore a useful index of current oxidative
status (Morrow, Hill et al. 1990). Isoprostane levels are higher in smokers, in individuals with disease such as hypercholesterolemia, cardiovascular disease, and diabetes (Morrow, Awad et al. 1992; Morrow, Frei et al. 1995). In addition, the administration of antioxidants in disease models results in a decrease in isoprostanes (Morrow, Awad et al. 1992).

Reactions of free radicals with proteins results in the formation of abnormal protein modification and cross links. Bonnes-Taurel demonstrated that carbonyl groups accumulate with age in mice (Bonnes-Taurel, Guerin et al. 1993). Yan et al observed in houseflies the accumulation of carbonyl groups with age and the corresponding decline in enzyme and physical function (Yan, Levine et al. 1997).

While DNA is generally very stable it can undergo spontaneous decomposition over time. This is exacerbated by the presence of ROS. Reactions between DNA and free radicals result in adducts which are implicated in carcinogenesis and neurological disorders. The hydroxyl radical can react with all four DNA bases as well as the sugar backbones (Halliwell 1999). Studies in animals indicate that exposure of cells to H2O2 resulted in the formation of mutations (Heaton, Ransley et al. 2002). One particular DNA mutation that is common to oxidative stress is to guanine resulting in the formation 8-oxoguanine (8-oxodG). Its formation can lead to G→T transversion mutation since adenine is misincorporated opposite 8-oxodG during DNA replication (Cabrera, Nghiem et al. 1988). Free radical attack can also result in the formation of baseless or apurinic/apyrimidinic (AP) sites (Sagher and Strauss 1983; Loeb and Preston 1986). The most likely mechanism for this observation is that DNA damage produced by oxidative stress results in the destabilization of the N-glycosylic bond and the formation of an AP site. In addition, repair of base mutations by glycosylases also results in the formation of baseless sites. The presence of damaged DNA can cause mutations and can possibly be lethal to the cell. Recent research indicates that DNA mutations increase with age in animals (Hamilton, Van Remmen et al. 2001). Furthermore, the ability of cells to repair damaged DNA declines with age. DNA damage from oxidative stress produced by energy metabolism is a potential cause of natural aging.

In the proposed studies, we will measure protein carbonyls, isoprostanes as a marker of lipid peroxidation, DNA fragmentation by the comet assay, and the ARP assay to measure baseless sites in DNA. We hypothesize that nonagenarians have relatively low levels of damage to protein, lipids, and DNA when compared to individuals in there sixties. Whether the lower oxidative damage is associated with a low metabolic rate remains to be proven.

**B3. Aging and Metabolic Syndrome**

Aging is associated with increase risk of disease, particularly cardiovascular disease and type II diabetes. The factors known to be associated with these conditions, collectively known as metabolic syndrome (dyslipidemia, hypertension, impaired glucose tolerance, insulin resistance, and obesity) become altered with age ultimately resulting in the progression of disease. Metabolic syndrome is associated with the occurrence of endothelial dysfunction, intimal-medial thickening, and elevations of plasma C-reactive protein (CRP). These factors are also associated with obesity, dyslipidemia, hypertension, insulin resistance and type 2 diabetes (Sonnenberg, Krakower et al. 2004). Endothelial dysfunction is characterized by a reduction of the bioavailability of vasodilators and in increase in endothelium-derived contracting factors. It
promotes lipid and cell permeability, lipoprotein oxidation, inflammation, platelet activation, and thrombus formation. A dysfunctional endothelium is involved in the recruitment of inflammatory cells into the vessel walls (Viles-Gonzalez, Anand et al. 2004). Endothelial cells produce cytokines and express adhesion molecules. Monocytes migrate into the subendothelium, where they transform macrophages and modulate inflammatory reactions and the secretion of chemoattractants and migration and proliferation of underlying smooth muscle cells (Ridker, Buring et al. 1998; Ridker, Glynn et al. 1998; Koenig, Sund et al. 1999). In addition, CRP, which is primarily regulated by IL-6, enhances the uptake of LDL and also stimulates macrophages to express cytokines further promoting endothelial dysfunction and worsening intimal medial thickening (Sonnenberg, Krakower et al. 2004). Oxidative stress is independently associated with hypertriglyceridemia, hypertension, hyperglycemia, and (Bae, Bassenge et al. 2001; Keaney, Larson et al. 2003; Redon, Oliva et al. 2003). Hansel et al demonstrated that in individuals with metabolic syndrome, isoprostane levels were elevated and they had attenuated antioxidative activity of small, dense, HDL subfractions, known to be protective against systemic oxidative stress (Hansel, Giral et al. 2004). Furthermore, Furukawa et al (Furukawa, Fujita et al. 2004) demonstrated that in cultured adipocytes, elevated levels of fatty acids increased oxidative stress via NADPH oxidase activation, and oxidative stress caused dysregulated production of adipocytokines (fat-derived hormones), including reduced adiponectin, and elevated plasminogen activator inhibitor-1, and IL-6. They concluded that accumulated oxidative stress in obesity, through adipocytokines dysregulation, could ultimately lead to metabolic syndrome. Inhibition of ROS resulted in improvement in diabetes, hyperlipidemia, and hepatic steatosis. We intend to measure blood pressure, fasting lipids, hemostasis factors (factor VII, fibrinogen, plasminogen activator inhibitor-1), C-reactive protein, and IL-6 (Ross 1999). One would expect that aged individuals with a resistance to oxidative stress would see levels of markers of metabolic syndrome similar to younger individuals (20-34y) and lower than middle age subjects (60-74y).

B4. Aging and physical activity

Regular physical activity is known to improve health and reduce morbidity and mortality. The beneficial effects of physical activity include maintaining a healthy weight, favorable blood glucose and lipid profiles, all of which have beneficial effects on disease. It is assumed that physical activity declines with age, which may play a role in the increase risk of disease that occurs with age. Furthermore, the decline in physical activity with age may be partly responsible for the decline in total energy expenditure measured by doubly labeled water. Earlier studies by
McGandy et al (McGandy, Barrows et al. 1966) who examined dietary records of 252 men aged 20-99 years of age indicated that total energy expenditure declined with age and was caused by a decline in resting metabolism and physical activity. This is supported by Rising et al (Rising, Harper et al. 1994) who noted an inverse correlation between physical activity level and age. This is also supported by Roberts et al (Roberts, Fuss et al. 1995) who reported an average 12% decrease in the ratio of daily to resting energy expenditure from age 24 to 70 y using indirect calorimetry and doubly labeled water. Furthermore, there are no differences in sedentary energy expenditure measured in a respiratory chamber between young and old (Vaughan, Zurlo et al. 1991). In addition, Black et al analyzed 574 measurements of TEE, RMR, activity energy expenditure, and physical activity level (PAL). As illustrated in 2, there is a general decline in physical activity across age (Black, Coward et al. 1996). Furthermore, multiple regression analysis revealed that age is a significant predictor of PAL (p<0.0001) even when including weight, height, and sex in the equation. Comparison of nonagenarians and 73 year olds demonstrated that all of the reduction in total energy expenditure was due to decrease physical activity because RMR was not different between groups (Rothenberg 2002). However, other studies have not been able to report a difference in physical activity levels between young and old (Vaughan, Zurlo et al. 1991; Pannemans and Westerterp 1995). The decline in TEE and RMR with age may be due to an increase in body fat with a decrease in fat free mass. Cross-sectional studies show that FFM is approximately 15% lower in subjects aged > 85y as compared to 35-55 year olds (Kyle, Genton et al. 2001). However, it has been demonstrated that regular physical activity helps to prevent the decline in fat free mass and RMR that occurs with age (Starling, Ades et al. 1999; Roth, Ivey et al. 2001; Van Pelt, Dinneno et al. 2001). Furthermore, exercise training has been shown to increase antioxidant levels in previously inactive individuals (Elosua, Molina et al. 2003). It is possible that older individuals, who maintain their physical activity also maintain muscle mass, prevent the accumulation of body fat, and have a better defense against oxidative stress. In this study we intend to investigate whether successful “agers” (nonagenarians) have a level of physical activity not as low as expected when compared to middle aged volunteers. We will measure physical activity level by combining the doubly labeled water method for total energy expenditure and indirect calorimetry for RMR. In addition, we will measure physical activity using 2 separate physical activity monitors. This information will be compare to PAL calculated from TEE and RMR.

C. PRELIMINARY STUDIES
C1. Description of the Pennington Center Professional Staff who will participate in these studies.

The PBRC faculty who will participate in these studies has had extensive experience in studies of energy metabolism (Ravussin and DeLany), body composition (Ravussin and DeLany), and oxidative stress (Deutsch).

Eric Ravussin, Ph.D., the PI for this Project has been working in the field of energy balance and obesity for more than 20 years. Relevant to this proposal, he conducted a study on the effect of age and caloric restriction on energy metabolism in 8 biospherians confined for 2 years in Biosphere II in Arizona (Vaughan, Zurlo et al. 1991; Rising, Harper et al. 1994).

James DeLany, Ph.D., is an associate Professor who was trained in the use of stable isotopes to measure energy expenditure by Dr. Dale Schoeller at the University of Chicago. He is Director
of the Stable Isotope Core Facility and will supervise all the procedures related to the use of doubly labeled water to measure total energy expenditure and physical activity level.

Andrew Deutsch, Ph.D., is a Professor at the Pennington and is head of the DNA Damage and Repair Laboratory. He is routinely using the comet assay for assessing DNA damage. Madlyn Frisard, MS., is a doctoral graduate student under the direction of Eric Ravussin. She has been an employee of Pennington Center since August of 2000. She has worked closely with both Dr Ravussin and Dr DeLany learning techniques related to measuring energy metabolism including indirect calorimetry and doubly labeled water. In addition, she has learned the techniques for measuring oxidative stress. She is currently measuring and analyzing all of the data related to oxidative stress in another study examining the effect of acute high intensity exercise on oxidative stress in endurance athletes.

C2. Preliminary Results  
 a) Energy Expenditure

Dr Ravussin has vast experience with the measurement of energy expenditure in humans over the entire lifespan. Dr DeLany is one of the national experts on the use of doubly labeled water to measure total energy expenditure. In addition, Madlyn Frisard under their direction has become proficient in the methods to measure energy expenditure including indirect calorimetry to measure resting metabolic rate and doubly labeled water to measure total energy expenditure. Data has been collected on 95 non-diabetic individuals: 20-34y, n=28; 60-74y, n=26; and ≥90y, n=40. Test retest reliability has been performed in another study that performed DLW on two separate occasions of weight maintenance. The r-square between the two measurements is 0.86. Furthermore, The percent difference between the two measures was 3.2± 6.7%. Figure 3 illustrates the lower resting metabolic rate plotted against FFM in the three different age groups. RMR is significantly lower in the 90y (p<0.000) and 60-74y (p<0.02) when compared to the 20-34y. In addition, there are significant differences between groups with regards to body temperature. Both the 60-74 years olds (p<0.008) and the nonagenarians (p<0.02) have significantly lower body temperature than the 20-34y. The current observed power for detecting these differences is 0.86.

b) Oxidative Stress

![Figure 3](image-url)

20-34 y  
60-74 y  
> 89 y

FFM
Dr DeLany is performing the isoprostane analysis. Madlyn has spent time in Dr DeLany’s lab learning the HPLC-MSMS technique to measure urinary isoprostanes. She currently performs the extraction technique, which prepares the urine samples for analysis by HPLC. Figure 4 illustrates preliminary data from our lab comparing urinary isoprostanes in a group of smokers to a group of nonsmokers. In addition, we are collaborating with Dr Jackson Roberts from the University of Vanderbilt. Dr Roberts is a pioneer in the study of lipid peroxidation and has extensive experience in measuring isoprostanes under a number of conditions including aging and disease.

Dr Deutsch will be performing a number of tests to determine the amount of endogenous DNA damage that exists in each of the three test groups. The assumption here is that a higher metabolic rate translates into increased oxidative stress and therefore increased levels of DNA lesions. One assay that will be employed is the single-cell gel electrophoresis, or Comet assay, that reveals single- and double-strand breaks and baseless sites in DNA. It can also be used to detect the presence of modified bases in DNA through the use of specific DNA repair enzymes. An example of this would be the use of 8-oxoguanine DNA glycosylase, which forms and alkali-labile site as an intermediate in the repair of 8-oxoguanine. Because the rapidly changing intensities of the individual cells are difficult to control, a large variance within each experiment is an unavoidable consequence of using this method. Therefore, a normalizing and variance stabilizing logarithmic transformation is applied to the calculate tail moments. After the data are transformed, an analysis of variance (ANOVA) with multiple comparisons is applied with respect to the different treatment groups. In order to maintain an overall significance level of 0.05, the Tukey-Kramer adjustment is used.

Madlyn Frisard has spent a number of hours in Dr Deutsch’s lab learning the comet assay. Figure 5 shows the results of a typical experiment using the Comet Assay. The slide on the left illustrates cells from a young individual and the slide on the right illustrates cells from an older individual. The cell from the younger individual is brighter and denser than the cell from the older individuals. The Intra-class correlation (ICC) point estimate for the comet assay is 0.9492 with a 95% confidence interval (CI) of 0.8784-0.9794. This indicates that variation is due to
subject and not measurement error.

Finally, analysis of protein carbonyls will measured in the clinical chemistry lab under the direction of Dr Jennifer Rood. They have vast experience in Elisa Assays which is the method used to measure protein carbonyls in plasma.

D. RESEARCH DESIGN AND METHODS
D1. Overall description of the Experiments
The description of the study design is shown in Figure 6 below. This project is part of a larger project; therefore the describes the testing that a subject will go through if they are involved in all the projects including this one.

As presented in the specific aims, my primary hypothesis is that subjects ≥ 90 years old have lower RMR (adjusted for fat-free mass, fat mass, and sex) than younger people. The low metabolic rate will be associated with low body temperature, and evidence of relatively low oxidative stress when compared to younger subjects and especially to group of middle age volunteers (60-74 years old). The other aims will assess in this unique population of nonagenarians the level of physical activity, markers of cardiovascular disease, and insulin resistance.

D2. Study Population

All subjects will be randomly selected in a radius of 40 miles around Baton Rouge and list of names to be contacted will be provided by Core B of the program project. Subjects will then be
contacted and asked their willingness to participate in the study. We are seeking to enroll the following numbers of subjects in the following groups:

1) 100 subjects, age ≥ 90 y
2) 50 subjects, 20 > age < 35
3) 50 subjects, 60 ≥ age < 75

The data for my dissertation will be a subset taken from this larger dataset.

**Inclusion**
All above persons ages 20 to 34, 60 to 74, and ≥ 90, with a normal hematocrit (> 33%).

**Exclusion**
Subjects are not eligible if they:

- Have diabetes
- Have unstable cardiovascular disease
- Have mental health problems requiring drug treatment.
- Have had a heart attack or stroke in the last three months
- Have severe high blood pressure
- Have blood vessel aneurysm
- Are taking certain medications used for myasthenia gravis
- Have uncontrolled asthma, an asthma-like condition or emphysema/Chronic Obstructive Pulmonary Disease (COPD)

**D3. Outcome Measures**

*a) Primary outcome.* The primary endpoint will be the rate of energy expenditure under resting condition, i.e. resting metabolic rate (RMR). RMR will be measured by a ventilated hood system.

*b) Secondary outcomes.* We will also be able to utilize this unique population to uncover suspected differences for a number of secondary outcomes.

1. **Oxidative stress.** Oxidative stress will be measured indirectly by DNA damage, lipid peroxidation, and protein carbonylation. Results will be directly correlated to resting metabolic rate adjusted for fat free mass, fat mass, and sex. This will be one of the first attempts to correlate directly measures of oxidative stress to measures of oxidative metabolism. Since 2-5% of oxygen consumption is not used in oxidative metabolism of fuels but is associated with the production of highly reactive oxygen molecules, we expect to detect a positive correlation between oxidative stress (DNA damage, lipid peroxidation, and protein carbonylation) and metabolic rate. We intend to derive an overall oxidative stress index based on the information from the oxidative stress measures.

2. **Body composition.** Differences in body composition will be measured by DEXA, the new gold standard. Because of the precision and reliability of the method, small differences between groups can be detected. This will be compared to body composition obtained by total body water from DLW.
3. **Total daily energy expenditure.** Total daily energy expenditure in free-living conditions will be measured by doubly labeled water over a two week period.

4. **Physical activity.** The level of physical activity will be assessed by the ratio of total daily energy expenditure measured by doubly labeled water and RMR measure by indirect calorimetry. Data will also be obtained by accelerometers worn during the 2-week period of the doubly labeled water.

5. **Cardiovascular disease and insulin resistance markers.** Fasting insulin concentration along with glucose will be used as an index of insulin sensitivity (HOMA) and fasting levels of C-reactive protein and interleukin 6 will be used as markers of inflammation.

### D4. Methods

#### D4a. Body Composition and Bone Mineral Density.

**DEXA** scans will be performed using a new Hologic QDR 4500A whole-body scanner. The protocol requires that subjects lie on a table wearing a hospital gown and no metal containing objects, while the scanner emitting low energy X-rays, and a detector passes along the body. The scan takes less than 4 min. and the radiation dose is less than 1 mrem, equal to about 12-h of background radiation. The scans are analyzed with the latest software QDR for Windows V11.1. Using our old instrument, (QDR 2000), coefficients of variation (CV) for body composition measurements of lean mass, fat mass, and percentage of body fat were 0.8%, 1.6%, 1.7%, respectively. Using our new machine, (QDR 4500), the CV for body composition measurements of lean mass, fat mass, and percent fat were 0.6%, 1.1%, and 1.1% respectively. We will also use the DEXA to estimate muscle mass using a method developed by Dr Heymsfield’s group (Heymsfield, Gallagher et al. 2002).

The bone mineral density of the hip and proximal femur will be measured on the same scanner in a matter of minutes. Subjects lie supine and motionless while the DEXA scanner moves along the length of the subject’s left femur and lasts approximately 2 min, for a 6-inch length. The hip regions of interest are the femoral neck, ward’s triangle, greater trochanter, and inter-trochanter region. Area, BMC, and BMD are all measured. For the most part, analysis is automated, with Ward’s triangle being automatically located by the system, because this is the area of initial bone loss in the femoral neck. There is a references database for comparing BMD vs. age. Potential changes with intervention will be assessed.

#### D4b. Energy Expenditure

**RMR.** Indirect calorimetry will be performed using a Deltratrac II Metabolic cart (Datex-Ohmeda, Helsinki, Finland) over a 30-min period. The analyzer will be calibrated before each study with standardized gases containing 5%CO2 and 95% O2. After quietly resting for 10 min., a transparent plastic hood connected to the device will be placed over the head of the subjects. Calculations of O2 consumption and CO2 production will be made from continuous CO2 and O2 concentrations in inspired and expired air diluted with a constant air flow (40L/min) generated by the analyzer and energy expenditure calculated. Subjects will be asked to calculate RMR. The rate of protein oxidation will be estimated and the rates of carbohydrate and lipid oxidation calculated according to Elia and Livesay (Livesey and Elia 1988).

**Doubly-labeled Water.** On the morning of the visit, a baseline urine sample will be collected. Soon after, subjects will drink from a stock solution with 0.2g/kg total body water (TBW) of
H$_2^{18}$O (Cambridge Isotopes, Cambridge, MA) and 0.16 g/kg TBW of $^2$H$_2$O (Isotec Inc, Miamisburg OH, or Cambridge Isotopes, Cambridge, MA), followed by 100-ml of tap water used to rinse the dose container. After one void, 2 more urine samples will be collected the same day. On the morning of days 13 and 14 after dosing, 2 more urine samples will be collected either at the testing site or at the home of the subjects depending on the age of the subjects. The isotope abundances will be measured on a Finnegan MAT DeltaS dual inlet Gas Isotope Ratio Mass Spectrometer with a CO$_2$-water equilibration device. The hydrogen isotope abundances are measured on a Finnigan MAT 252 dual-inlet Gas Isotope Ratio Mass Spectrometer using zinc reduction at 500°C, as previously described. The $^2$H and $^{18}$O isotope elimination rates ($k_H$ and $k_O$) will be calculated using linear regression using the isotopic enrichment relative to predose enrichment of the urine samples from the beginning (2 postdose samples on day of dosing) and end of the metabolic study days (13 and 14). Total body water will be determined from the enrichment at time zero, obtained from the regression line. The rate of CO$_2$ production is calculated using the equations of Schoeller et al (Schoeller 1988) and later modified (Racette, Schoeller et al. 1994) as follows: $r_{CO_2} = \left( N/2.078 \right) \left( 1.007 k_O - 1.041 k_H \right) - 0.0246r_{H_2}O_f$, where $N$ is the total body water and $r_{H_2}O_f$ is the rate of fractionated evaporative water loss which is estimated to be 1.05N(1.007$k_O$ – 1.041$k_H$). Total energy expenditure (TEE) is calculated by multiplying $r_{CO_2}$ by the energy equivalent of CO$_2$ for an assumed RQ of 0.882 or an estimated RQ on the basis of the Food Frequency Questionnaire administered prior to the test day.

The level of physical activity often referred to as PAL will be calculated as the ratio of total energy expenditure to resting metabolic rate. Another index of activity will be calculated as TEE adjusted for RMR by linear regression analysis. Finally, the energy cost of activity will be calculated as TEE –((TEE × 0.1) + RMR). TEE × 0.1 is an accepted estimate of the thermic effect of food.

Simultaneously to the 2-week measurement of energy expenditure by doubled water, we will assess activity in all subjects using an accelerometer. The MTI/SCA activity monitor was chosen to monitor activity levels and step counts simultaneously for periods up to 11 days. The actigraph uses 8-bit resolution, providing less than 0.02 G (Gravity) resolution. It has a ± 2.15 G dynamic range with a unique filtering system, which filters out all non-human frequency components. It was validated by numerous studies, one of which was compared to doubly labeled water. Total energy expenditure was significantly related to the doubly labeled water data ($r$=0.39; $p<0.005$). It is also the only actigraph that has been validated for the measurement of kcals expenditure and metabolic equivalent (METs). Results from the combination of doubly labeled water and indirect calorimetry will be compared to those obtained by accelerometers. Recent studies have indicated that spontaneous physical activity is highly variable among individuals and accounted for a major portion of individual differences in 24-hour energy expenditure (Ravussin, Lillioja et al. 1986). Studies in families (Zurlo, Ferraro et al. 1992) show that although the degree of spontaneous physical activity is highly variable, it is more similar among siblings that among unrelated individuals. In the current issue of Science, Levine et al (Levine 2005) use a combination of physical activity monitors and inclinometers to demonstrate that overweight individuals sit an average of 2 hours longer than lean individuals. In addition, they demonstrated that this accounted for approximately 250 calories per day (Levine 2005). Recently, a new portable physical activity device, Intelligent Device for Energy Expenditure and
Activity (IDEEA) (Minisun, Fresno, CA), has been designed to accurately identifying type
duration, frequency, and intensity of daily physical activity. In addition, this new device is able
to collect postural information such as time spent sitting, lying, and standing as well as
information on gait analysis (Zhang, Werner et al. 2003). We propose to use this device to
compare physical activity and posture in three groups of individuals and compare this
information to the results from doubly labeled water.

D4c. Oxidative Stress

Isoprostanes. The isoprostane that has been most often studied (shown to be a potential marker
for oxidative stress) is 8-epi PGF2 alpha, although there are several names for this compound.
We propose to measure 8-epi alpha as a marker of oxidative stress using HPLC/MS-MS.
Briefly, an internal standard (deuterated 8-epi PGF2 alphah, Caymen Chemical) will be added to
urine, followed by the addition of 15% KOH and allowed to stand for 30 min. The sample is
then subjected to solid phase extraction after adjustment of pH. The samples is dried and
resuspended in 20% acetonitrile in water and subjected to HPLC/MS-MS.

Protein Carbonyl content. Serum samples will be analyzed for carbonyl content, an indicator
of oxidative damage to proteins. Briefly, serum samples are combined with 2,4
dinitrophenylhydrazine. The carbonyls in the samples react with the 2,4 DNP to form protein
hydrazones. The product is measured spectrophotometrically at 255-290. Each sample is
scanned against a sampled treated with 2.5M HCL. Carbonyl content is calculated from the
absorption spectra using an absorption coefficient of 22,000M⁻¹ cm⁻¹.

DNA Damage

Comet Assay for DNA Damage – Single-cell gel electrophoresis (SCGE, comet assay) provides
a very sensitive method for detecting strand breaks and measuring repair kinetics at the level of
single cells. A variety of possible modifications to the assay facilitate the detection of single-
strand breaks, incomplete excision repair sites, and interstrand cross links. In addition to the
above, DNA fragmentation associated with cell death or related to apoptosis can be evaluated
with the comet assay. After initial treatment, cells are embedded in agarose layers, lysed, and
electrophoresed. After staining with a fluorescent DNA binding dye, cells with increased DNA
damage display increased DNA migration from the nucleas toward the anode, thus forming the
shape of a “comet”. The comet assay takes 2 days and utilizes a small number of cells. Briefly,
lymphocytes are suspended in low melting point agarose and immobilized on slides. The slides
are then treated with a gentle lysing solution, followed by a highly alkaline solution (>pH 12.6)
to expose AP and other alkali labile sites. Alternatively, lysed cells can be treated with DNA
glycosylases to reveal damaged DNA bases. The slides are then placed into an electrophoresis
chamber. Following electrophoresis, the slides are fixed and stained. For quantification, digital
imaging will be used. There is a commercially available kit from Trevigen for conducting the
comet assay.

Determination of baseless sites in DNA – The action of ROS destabilize bases that result in the
formation of baseless DNA (apurinic/apyrimidinic= AP). These baseless sites also arise as an
intermediate in the repair of ROS damages DNA bases such as 8-oxoguanine. A specific method
for the detection of AP sites has been developed. This method utilized the reagent O-
(carboxymethyl) hydroxylamine that has been reacted with biotin hydrazide in the presence of
carbodiimide. Aldehyde reactive probe (ARP) specifically reacts with AP sites in DNA,
identifying them with biotin residues. The biotin-marked AP sites are determined using color detection system in a slotblot or ELISA type analysis and avidin/biotin complex conjugated to horseradish peroxidase. This system has been modified to use the more sensitive chemiluminescence detection kits (Boehringer-Mannheim) in a slot-blot apparatus. One AP site for every 10,000 bases of DNA (approximately 10fmol of AP sites) can be detected and requires as little as 300ng of DNA per reaction. Band densities are normalized with GAPDH expression.

**D4d. CVD markers and vascular function.** Blood pressure will be measured on the morning of the testing visit. Serum lipids will be analyzed using a Beckman-Coulter Synchron CX7 (Brea, CA). Serum cholesterol will be assayed by the cholesterol esterase/oxidase/peroxidase method. Triacylglycerols will be measured using the GPO-Trinder method. HDL-C will be measured following of apoB containing lipoproteins with 50,000 mol wt. dextran sulfate (Sigma Chemical Co.). LDL cholesterol is calculated using the Friedewald equation. The coefficient of variation for these assays is less than 2.0%. Our clinical laboratory participates in CDC’s ongoing lipid standardization program.

**Plasma hemostatic factors, inflammatory markers and homocysteine.** Factor VII and fibrinogen will be assayed on an Instrumentation Laboratory ACL 3000+ (Lexington, MA). Factor VII activity is assayed by determining the ability of test plasma to correct the clotting time of factor VII deficient plasma. Factor VII activity is expressed relative to serial dilution of pooled plasma. Fibrinogen will be measured by automated immunoassay with chemiluminescent detection on a DPC-Immulite instrument with reagents supplied by the instrument manufacturer.

**D4e. Power calculation and Data Analysis**

The power calculations and sample size determination were done for the primary endpoint, RMR measured by Deltatrac. As specified in Aim A1, in the final analysis, it is assumed that there will be a comparison between the three age groups:

1. Older than or equal to age 90
2. Ages between 60 –74
3. Ages between 20 –34

We hypothesize that in the oldest age group RMR is lower by 5, 10, or 15% than in the younger groups. The power calculations were carried out for the reference RMR of 1250, 1500, and 1750 Kcal/day. For the estimate of between subject population variance, combined baseline data from two Pennington studies were used (Fat Challenge II study and the Chamber adaptation study). Altogether there were 95 subjects (47 females and 48 males) included in the estimation. The smallest population variance was achieved after adjusting RMR for gender, age, fat free mass, and fat mass, with no interaction terms in the model. We intend to consider an unbalanced design for this study with twice as many subjects in the oldest age category than in each of the two younger groups. The significance level is set to 0.05 and the desired power is at least 80%. Table 1 below shows the results of power calculations. The minimum differences in Kcal between the age groups that is detectable with a given power is denoted by delta and results from 5,10, or 15% of the reference level of RMR in Kcal is shown. The population standard deviation is estimated by 137.2 Kcal. With our design including 160 subjects, we will easily detect a 10% reduction in RMR in the older group.
In the final analysis, we will investigate the difference in metabolic rate between groups and attempt to correlate these differences to group differences in indices of oxidative stress. RMR data will be analyzed by an analysis of covariance in which fat-free mass, fat mass, and sex are used as independent covariates.

**D5. Strengths and Weaknesses**
The techniques described in this application for measuring energy metabolism, physical activity, body composition, markers of cardiovascular disease and insulin resistance, and markers of oxidative stress are routinely used at the Pennington Center. Madlyn has spent a number of hours in the labs of Jim DeLany and Andy Deutsch to become proficient in the techniques. She oversees all measures of energy metabolism including indirect calorimetry to measure RMR and doubly labeled water to measure total energy expenditure. She has also learned and become proficient in each of the measurements of oxidative stress. She has performed measurements of both lipid peroxidation and DNA damage for other studies performed at the Pennington Center including one study performed in athletes.

**D6. Timeline**
Currently, we have enrolled 110 subjects into the study. The numbers of subjects are broken down into the following groups:

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>46 subjects, age ≥ 90 y (23 males and 23 females)</td>
</tr>
<tr>
<td>2)</td>
<td>30 subjects, 20 ≥ age &lt; 35 (9 males and 21 females)</td>
</tr>
<tr>
<td>3)</td>
<td>34 subjects, 60 ≥ age &lt; 75 (17 males and 17 females)</td>
</tr>
</tbody>
</table>

If we assume that we collect 2 subjects per month, there will be an additional 16 subjects included in the dataset. However, we have recently increased our recruiting staff and hope to collect more subjects than the minimum 2 per month. I am performing all of the testing on our subjects could ultimately test four subjects per week, which is the maximum allowed by the blood requirements. As stated previously, our current power to detect differences in RMR is Figure 7 below illustrates my proposed timeline for the duration of my doctoral program. By the end of the spring semester, May 2005, my residency requirements (enrolling in two back to back
full time semesters) will be fulfilled. In addition, I plan to submit my literature review manuscript by March 15, 2005. I intend on having my first draft out to the committee members by February 11, 2005. I have been analyzing data over the duration of the study. All of the measurements including blood work are being directly entered into the central database. Therefore, other than some blood parameters including inflammatory markers that still need to be analyzed, all of the data for tested subjects is available for statistical analysis. I am planning on having my data analyzed by the end of July, allowing the month of August for the final write-up of the results and discussion section of the dissertation. I have tentatively set my defense date for September 9, 2005, which leaves 2.5 months for revision and submission to the graduate school, which is due mid November.

**Figure 7.**
Appendix B: Consent Forms

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Title of Study:
Determinants of Human Longevity and Healthy Aging

Study 1: Genetic contributions to longevity and healthy aging in the LA population

What you should know about a research study

- We give you this consent form so that you may read about the purpose, risks and benefits of this research study.
- The main goal of research studies is to gain knowledge that may help future patients.
- You have the right to refuse to take part, or agree to take part now and change your mind later on.
- Please review this consent form carefully and ask any questions before you make a decision.
- Your participation is voluntary.
- By signing this consent form, you agree to participate in the study as it is described.

1- Who is doing the study?
Principal Investigators: Eric Ravussin, Ph.D. Tel: 225-763-3186
S. Michal Jazwinski, Ph.D. Tel: 504-568-4725

Medical Investigators: Alok Gupta, M.D.,
Day Phone: 225-763-2656
24-hr. Emergency Phone Nos.:
225-763-2640 (Weekdays 8:00 a.m.-5:00 p.m.)
225-765-4644 (After 5:00 p.m. and Weekends)

Steven Smith, M.D.
Day Phone: 225-763-3028

Henry Rothschild, M.D. Tel: 504-568-5842
L.S.U. Health Sciences Center

Co-Investigators: Mark Batzer, Ph.D.
Katie Cherry, Ph.D.
James P. DeLany, Ph.D.
Lauri Byerley, PhD.
Elizabeth T.H. Fontham, Dr.P.H., M.P.H.
W. Andrew Deutsch, Ph.D.
Paula J. Geiselman, Ph.D.
John Mountz, M.D., Ph.D.
Drs. Eric Ravussin and Michal Jazwinski direct this study, which is under the medical supervision of Drs. Steven Smith and Henry Rothschild. We expect to enroll about 800 people in this study, but with different time commitments. The study will target volunteers from age 20-100+. The study will take place over a period of five years. For this study, all volunteers less than 70 will spend between 1 and 8 hours at the Pennington Biomedical Research Center, depending upon eligibility in the different projects. All volunteers age 70 or greater, will have the option of either coming to the Pennington Biomedical Research Center for 2 hours or being visited in their home for the same amount of time. For those eligible, additional tests will be run at the Pennington Center. This study is part of a state wide study being done by researchers at the Pennington Biomedical Research Center in Baton Rouge (Drs Ravussin, DeLany, Deutsch, Smith, and Geiselman), at the Louisiana State University Health Sciences Center in New Orleans (Drs Jazwinski, Rothschild, Su, Schmidt, Fontham, Welsh and Scott), at Louisiana State University and A&M College in Baton Rouge (Drs. Batzer, Cherry, Welsch, and Wood), and at the University of Alabama, Birmingham (Dr. Mountz).

2- Where is the study being conducted?

This study takes place at your home if you are over 90 years old, or if you are between 70 and 90 without transportation, and at the Pennington Biomedical Research Center’s inpatient and outpatient clinics.

3- What is the purpose of this study?

It is now well accepted that a long duration of life and good quality aging are influenced by genetic factors. Such genes may influence an individual’s metabolism, which in turn may make a long life with good physical and mental capabilities more likely. This may promote the sense of well being associated with healthy aging. It is thought that a low metabolic rate is associated with reduced levels of cell damage, a factor known to contribute to successful aging. The study will target approximately 800 volunteers from age 20-100+. For the volunteers over age 90, we will emphasize those persons who require minimal to moderate assistance with activities of daily living. In all 800 volunteers, we will look at the genetic contributions to a long duration of life and healthy aging in the LA population.

4- Who is eligible to participate in the study? Who is ineligible?

Inclusion Criteria:  

Study 1:
All persons ages 20 to 100 plus.

**Exclusion Criteria:**
- Women who are pregnant or nursing.

5- What will happen to you if you take part in the study?

**A. If you are 60 years old or older you will fall into one of the following categories:**

I. **a) Home Visit:** For those 90 or over or those 70-90 without transportation to the Pennington, a nurse practitioner and recruiter will visit your home to check for your eligibility in the study. The nurse will draw a blood sample of about 60 ml (just about 5 tablespoons) for genetic testing and blood chemistry. Some of the blood drawn (35 ml) will be used to obtain genetic material, called DNA. The DNA will be taken from your white blood cells. We will grow your white blood cells in the laboratory, and then freeze them so that we will always have a supply of the DNA. The DNA may be sent to other scientists who work with us on studies of aging and related medical problems. If we did this, the DNA would be coded and you would not be personally identified. The nurse will also obtain a urine, a weight, medical history information, administer questionnaires, and assess overall physical ability. Cognitive function will be evaluated using a variety of questionnaires administered by the visiting team. If you are further eligible, you will come to the Pennington Clinic via transportation provided by the Pennington Center. You will also be asked information about your spouse, siblings, and/or children. We may contact your spouse, siblings and/or children to ask them if they would be interested in volunteering in the study or some part of the study. You have the option of contacting your family about the study or our staff can contact them if you prefer.

b) **Screening Visit:** For those 60 and over that do not require transportation to the Pennington, you will come for two separate visits. The screening visit will consist of explaining the study and study consent forms, obtaining a height, weight, a hip/waist ratio, a medical history, completing questionnaires, and getting instructions for the next visit. Approximately 60 ml (about 5 tablespoons) of blood will be drawn at the screening visit for those who qualify for Study 2. Some of the blood drawn (35 ml) will be used to obtain genetic material, called DNA. The DNA will be taken from your white blood cells. We will grow your white blood cells in the laboratory, and then freeze them so that we will always have a supply of the DNA. The DNA may be sent to other scientists who work on studies of aging and related medical problems. If we did this, the DNA would be coded and you would not be personally identified.

II. **Pennington Visit:** Once you have qualified and agree to continue in the study, you will arrive early at PBRC either on your own or by transportation provided for additional tests on different biomarkers of aging.
1) For those who have not had a home visit, 60 ml of blood will be drawn upon arrival. This amount may be increased to 125 ml (a little less than 9 tablespoons) if you qualify for study 2. Some of the blood drawn (35 ml) will be used to obtain genetic material, called DNA. The DNA will be taken from your white blood cells. We will grow your white blood cells in the laboratory, and then freeze them so that we will always have a supply of the DNA. The DNA may be sent to other scientists who work with us on studies of aging and related medical problems. If we did this, the DNA would be coded and you would not be personally identified.

2) Vision – will be measured by reading letters on an eye chart

3) Vital signs - blood pressure, heart rate, respirations, and temperature will be taken

4) Height and weight –will be measured on calibrated equipment

5) Physical Exam will be performed by a physician or a nurse practitioner

6) ECG – electrodes placed on the arms, legs and chest to determine activity of the heart

7) Body composition and bone mineral density by DEXA: This test involves changing into a hospital gown and lying on a table called a DEXA scanner. The scanner uses low-dose X-rays to determine the amount of fat, bone and muscle in your body.

8) Echocardiogram, Brachial, and Carotid Artery Tests – The echocardiogram monitors the heart with an ultrasound machine. The brachial artery test measure how elastic your veins are, and the thickness of the lining of your brachial arteries. After resting on your back for 10 minutes an ultrasound probe is placed over your brachial artery (elbow area) on your non-dominant arm (the side you do NOT write with). After the baseline measurement, a blood pressure cuff is placed around your forearm and inflated to reduce blood flow for 5 minutes. When the cuff is removed the ultrasound will be continued for 6 more minutes. You may feel a warm tingling on your elbow from the ultrasound. The carotid ultrasound test measures how elastic your veins are, and the thickness of the lining of your carotid arteries. A measurement of your carotid artery will be taken non-invasively by using an ultrasound probe that will be applied to the side of your neck.

9) Pulmonary Function Testing (lung function) - measured by spirometry and body plethysmography (breathing into a plastic tube before and after inhaling a medication, albuterol, to dilate your bronchial tubes).

10) Hearing – checked by response to sounds

11) Additional questionnaires

The nurse will then make an assessment as to which other studies (2, 3, and 4) you may be eligible for. If you are eligible for studies 2, 3, and 4 and want to participate, you will sign other consent forms explaining the requirements of the study. The time commitment for this part of the study will range from 3-8 hours.

B. If you are 59 years old or younger:

Anyone less than 59 years old will be asked to come to the Pennington clinic on two separate occasions.

- The first visit will consist of a screening visit to determine study eligibility for Studies 1 and 2. The screening visit will consist of explaining the study and study consent forms,
obtaining a height, weight, a hip/waist ratio, a medical history, completing questionnaires, and getting instructions for the next visit

- During the second visit, you will participate in Study 1 (Genetic Testing) and/or Study 2-4. You will have a 53ml blood sample drawn (about 4 tablespoons) and urine collected if you participate only in Study 1. This amount of blood will be increased to about 125ml (a little less than 9 tablespoons) if you are eligible and consent to participate in both Study 1 and 2 (see separate consent form). You will fill out additional questionnaires, have your vital signs taken and your weight measured once more. There will be a brief physical exam, and a measure of your body composition and bone mineral density by DEXA.

6- **What are the possible risks and discomforts?**

**Blood drawing** – possible bruising at the puncture site of the vein; possible fainting; pain.

**Pulmonary function (lung) testing** - this test is commonly used and is considered safe. However, there is a possibility that you could have a reaction to the inhaled medicine given. A reaction could cause you to have breathing problems such as shortness of breath and wheezing. This is unlikely since the medicine is opening your airways and not closing them.

**Echocardiogram, Brachial, and Carotid Artery Test** – There is no risk associated with a low level ultrasound. Occlusion of the forearm also carries no risk but you may experience temporary discomfort when the blood pressure cuff is inflated. The cuff can be deflated immediately upon your request. With the carotid artery test there is a possibility of fainting and/or temporary slowing of the heart rate and the remote possibility of carotid plaque destabilization with resulting stroke.

**DEXA**- the test involves exposure to low doses of x-ray approximately equal to 12 hours of background radiation from the sun.

None of the other testing procedures have any known risks.

7- **What are the possible benefits?**

We cannot promise any benefits from your being in the study. However, you will be given the results of applicable tests such as blood chemistry, an EKG for those ages 40 and over, an echocardiogram for those ages 60 and over, and body composition. This information may be given to your doctor if you agree.

8- **If you do not want to take part in the study, are there other choices?**

You have the right to withdraw from this research study at any time without penalty.
If you choose not to participate in this study this will not affect any rights or benefits to which you are otherwise entitled.

9- If you have any questions or problems, whom can you call?

If you have any questions about your rights as a research volunteer, you should call the Institutional Review Board Office at 225-763-2693 or Dr. Claude Bouchard, Executive Director of PBRC at 225-763-2513. If you have any questions about the research study, contact Dr. Eric Ravussin at 225-763-3186 or Dr. Michal Jazwinski at 504-568-4725. If you think you have a research-related injury or medical illness, you should call Dr. Steven Smith at 225-763-3028 during regular working hours. After working hours and on weekends you should call the answering service at 225-765-4644. The on-call physician will respond to your call.

10- What information will be kept private?

Every effort will be made to maintain the confidentiality of your study records. However, someone from the Pennington Biomedical Research Center, LSUHSC, LSU A&M and University of Alabama may inspect and/or copy the medical records related to the study. Results of the study may be published and made available to other researchers; however, we will keep your name and other identifying information private. Other than as set forth above, your identity will remain confidential unless law requires disclosure.

In the future, your biomaterial (blood and/or urine samples) may be used by other investigators at Pennington or at other sites. There is a possibility that the samples that you are donating and the information you provide in this study may be used in research leading to commercial value. Should they lead to the development of a commercial product, the Pennington Biomedical Research Center, LSUHSC, LSU A&M and University of Alabama will own it and it is possible that it will be patented and licensed by these institutions.

11- Can your taking part in the study end early?

Dr. Eric Ravussin, Dr. Steven Smith, or Dr. Alok Gupta can withdraw you from the study for any reason or for no reason. You may withdraw from the study at any time without penalty. Possible reasons for withdrawal include inability or unwillingness to complete the required testing.

12- What if information becomes available that might affect your decision to stay in the study?

During the course of this study there may be new findings from this or other research, which may affect your willingness to continue participation. Information concerning any such new findings will be provided to you.

13- What charges will you have to pay?

None.
14- What payment will you receive?

Subjects participating in this study will be compensated $100.00 for completion of the study. If you are or have been an employee of LSU within the last two years, the normal employee payroll deductions will be withheld. For those who have Home Visits, you will be compensated $50.00 if you decide to stop participating after the Home Visit.

15- Will you be compensated for a study-related injury or medical illness?

No form of compensation for medical treatment or for other damages (i.e., lost wages, time lost from work, etc.) is available from the Pennington Biomedical Research Center, LSU Health Sciences Center, LSU A&M College or UAB. In the event of injury or medical illness resulting from the research procedures in which you participate, you will be referred to a treatment facility. Medical treatment may be provided at your expense or at the expense of your health care insurer (e.g., Medicare, Medicaid, Blue Cross-Blue Shield, Dental Insurer, etc.), which may or may not provide coverage. The Pennington Biomedical Research Center is a research facility and provides medical treatment only as part of research protocols. Should you require ongoing medical treatments, they must be provided by community physicians and hospitals.

16- Healthy Insurance Portability and Accountability Act (HIPAA)

Records that you give us permission to keep, and that identify you, will be kept confidential as required by law. Federal Privacy Regulations provide safeguards for privacy, security, and authorized access. Except when required by law, you will not be identified by name, social security number, address, telephone number or any other direct personal identifier in screening records disclosed outside of Pennington Biomedical Research Center (PBRC) and kept in the study archives which are at LSUHSC in New Orleans. For records disclosed outside of PBRC, you will be assigned a unique code number.
17- Signatures

The study has been discussed with me and all my questions have been answered. I understand that additional questions regarding the study should be directed to the study investigators. I agree with the terms above and acknowledge that I have been given a copy of the consent form.

With my signature, I also acknowledge that I have been given either today or in the past a copy of the Notice of Privacy Practices for Protected Health Information

__________________________________                              _____________
Signature of Volunteer         Date

__________________________________
Date of Birth of Volunteer

I consent to have my blood stored for future DNA analysis. ☐ Yes ☐ No

Volunteer’s Initials

__________________________________________             _____________
Signature of Person Administering Informed Consent             Date

The study volunteer has indicated to me that the volunteer is unable to read. I certify that I have read this consent form to the volunteer and explained that by completing the signature line above the volunteer has agreed to participate.

__________________________________________             _____________
Signature of Reader             Date
CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Title of Study:
Determinants of Human Longevity and Healthy Aging
Study 2: Physiology of successful aging: emphasis on RMR, oxidative stress and gene expression

What you should know about a research study
• We give you this consent form so that you may read about the purpose, risks and benefits of this research study.
• The main goal of research studies is to gain knowledge that may help future patients.
• You have the right to refuse to take part, or agree to take part now and change your mind later on.
• Please review this consent form carefully and ask any questions before you make a decision.
• Your participation is voluntary.
• By signing this consent form, you agree to participate in the study as it is described.

1- Who is doing the study?
Principal Investigators:  Eric Ravussin, Ph.D.     Tel: 225-763-3186
S. Michal Jazwinski, Ph.D. Tel: 504-568-4725

Medical Investigators:  Alok Gupta, M.D.,
Day Phone: 225-763-2656
24-hr. Emergency Phone Nos.:
225-763-2640 (Weekdays 7:00a.m.-4:30 p.m.)
225-765-4644 (After 4:30 p.m. and Weekends).

Steven Smith, M.D.
Day Phone: 225-763-3028

Henry Rothschild, M.D.  Tel: 504-568-5842
L.S.U. Health Sciences Center

Co-Investigators:  Mark Batzer, Ph.D.
Katie Cherry, Ph.D.
James P. DeLany, Ph.D.
Lauri Byerley, PhD.
W. Andrew Deutsch, Ph.D.
Elizabeth T.H. Fontham, Dr.Ph., M.P.H.
Paula J. Geiselman, Ph.D.
John Mountz, M.D., Ph.D.
Beth A. Schmidt, M.S.P.H.
Donald Scott, Ph.D.
Drs. Eric Ravussin and Michal Jazwinski direct this study, which is under the medical supervision of Drs. Steven Smith and Henry Rothschild. We expect to enroll about 200 people in this study. 100 elderly volunteers, physically functional (age > 90) will be compared to 100 younger volunteers; 50 (age 20-34) and 50 (age 60-74). The study will take place over a period of five years. Your expected time in this study will be a maximum of three weeks with a maximum of one full day at the clinic of the Pennington Center.

This study is part of a state wide study being done by researchers at the Pennington Biomedical Research Center in Baton Rouge (Drs Ravussin, DeLany, Deutsch, Smith, and Geiselman), at the Louisiana State University Health Sciences Center in New Orleans (Drs Jazwinski, Rothschild, Su, Schmidt, Fontham, Welsh, and Scott), at Louisiana State University and A&M College in Baton Rouge (Drs. Batzer, Cherry, Welsch, and Wood), and at the University of Alabama, Birmingham (Dr. Mountz).

2- Where is the study being conducted?

This study takes place at the Pennington Biomedical Research Center’s inpatient and outpatient clinics.

3- What is the purpose of this study?

It is now well accepted that a long duration of life and good quality aging are influenced by genetic factors. Such genes may influence an individual’s metabolism, which in turn may make a long life with good physical and mental capabilities more likely. This may promote the sense of well being associated with healthy aging. It is thought that a low metabolic rate is associated with reduced levels of cell damage, a factor known to contribute to successful aging. The study will target approximately 200 volunteers from age 20-100+. We will compare metabolic rate and cell damage in physically functional elderly volunteers over the age of 90 and in two groups of younger volunteers, i.e. ages 60-74 and 20-34. We will also study the genes involved in aging and glucose metabolism in T lymphocytes (cells in blood helping the immune system).

4- Who is eligible to participate in the study? Who is ineligible?

Inclusion Criteria:
- Having participated in Study 1 of this protocol.

Exclusion Criteria:
- Women who are pregnant or nursing.
- Persons who have alcohol or drug abuse problems.
- Abnormally low hematocrit (blood cell volume evaluated in the home visit for those
between the ages of 70-100+
- Diabetes
- Unstable heart disease
- Thyroid Disease
- Mental health problems requiring medication
- Those subjects with a recent (past 3 months) cancer diagnosis, undergoing immunotherapy, taking immune suppressants, and those with allergies or infections requiring antibiotics
- For volunteers who are 70-90 years old:
  - Heart attack or stroke in the last 3 months
  - Uncontrolled high blood pressure
  - Blood vessel aneurysm (weakness or enlargement)
  - Uncontrolled asthma or emphysema
  - Myasthenia gravis (neuromuscular disorder characterized by fatigue)

5- What will happen to you if you take part in the study?

You will come to the Pennington Center early in the morning after a 10 hour fast.

a) Blood drawing. Screening blood work (approximately 53ml – about 4 tablespoons) will determine eligibility for those age 60 and above. If you are age 60 and over and eligible for Study 2 an extra 72ml (a little less than 5 tbsp) blood sample will be drawn for glucose regulated gene activity testing and measures of blood proteins during your Pennington visit. If you are age 20-34 all of your blood work (125ml – about 9 tbsp) will be drawn during your Pennington visit.

b) Total Daily Energy Expenditure by Doubly-Labeled Water (D2O): 30 minutes
This is a test to measure your energy expenditure over a 2 week period. After providing a fasting urine sample, you will drink a glass of water that contains 2 atoms found in normal water. However, for the purpose of our measurements, this water has been enriched with these 2 atoms, which are called stable isotopes (non-radioactive). During the rest of the day, you will be asked to provide us with 2 more urine samples. Two weeks later while at home, you will collect another 2 urine samples. The urine samples collected will be either returned by you, or picked up at your home by a Pennington employee for analysis. Measures of the 2 atoms in your urine will tell us how many calories you will burn during these 2 weeks and about your level of physical activity.

c) Resting metabolic rate (RMR): 45 minutes
After drinking the water for the doubly labeled water test, we will measure your metabolic rate while resting quietly in a bed at the Pennington Center. While you are lying comfortably and awake, we will put a clear plastic bubble over your head and seal it around your pillow. The hood is comfortably ventilated with fresh room air. This will permit the measurement of how much oxygen you breathe in and how much carbon dioxide (CO2) you breathe out. From these
measures, we can calculate how many calories you burn at any moment during the 45-minute test.

d) Macronutrient Self-Selection and Fat Preference  30 minutes
For your lunch, you will have to select pre-prepared food with different contents of carbohydrate, fat and protein. This macronutrient self-selection paradigm along with a food preference questionnaire is designed to tell us about the kind of food you prefer.

e) Physical Activity Level
We will measure your physical activity level using a portable activity monitor. This monitor is attached to a strap that is worn around the waist. You will wear the monitor continuously only taking it off to shower. We will collect the monitor two weeks later either at your home or at the Pennington Center.

If you are 90 years of age or older we will measure your total energy expenditure and posture using a motion-sensing device (IDEEA). We will set up 5 motion sensors on your body, 1 on each foot, 1 on the front of each thigh and one on the chest (20 minutes). We will then hook these sensors up to a little recording box that you will wear on a belt for two consecutive days. You will not be able to take it off while sleeping. You will then come back two days later early in the morning to the Pennington clinic to return the sensors and the recording box (30 minutes).

6- What are the possible risks and discomforts?

The risks associated with the procedures required for these studies include, but are not limited to:

<table>
<thead>
<tr>
<th>Procedure:</th>
<th>Risk:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venipuncture (blood draw)</td>
<td>Bruising, bleeding, pain</td>
</tr>
<tr>
<td>Doubly labeled water</td>
<td>The measure of your energy expenditure by doubly labeled water carries no risk. The two natural atoms given in the water are not dangerous at all and are often given to small children or pregnant women.</td>
</tr>
<tr>
<td>Resting Metabolic Rate</td>
<td>The measure of your resting metabolic rate using a ventilated hood carries no risk. The only adverse factor in this 45-minute testing may be a feeling of claustrophobia. A person will be at the bedside at all times and will check to see that you are comfortable. You may easily and rapidly remove the transparent hood, if necessary.</td>
</tr>
</tbody>
</table>
Physical activity monitors

The little box and the wires on your chest and thighs may be an inconvenience but they will not hurt. You will also not be able to shower, swim or bathe while wearing this device.

7- What are the possible benefits?

We cannot promise any benefits from your being in the study. However, possible benefits include receiving information about your blood chemistry, metabolism, and body composition. This information may be given to your doctor if you agree.

8- If you do not want to take part in the study, are there other choices?

You have the right to withdraw from this research study at any time without penalty.

If you choose not to participate in this study this will not affect any rights or benefits to which you are otherwise entitled.

9- If you have any questions or problems, whom can you call?

If you have any questions about your rights as a research volunteer, you should call the Institutional Review Board Office at 225-763-2693 or Dr. Claude Bouchard, Executive Director of PBRC at 225-763-2513. If you have any questions about the research study, contact Dr. Eric Ravussin at 225-763-3186 or Dr. Michal Jazwinski at 504-568-4725. If you think you have a research-related injury or medical illness, you should call Dr. Steven Smith at 225-763-3028 during regular working hours. After working hours and on weekends you should call the answering service at 225-765-4644. The on-call physician will respond to your call.

10- What information will be kept private?

Every effort will be made to maintain the confidentiality of your study records. However, someone from the Pennington Biomedical Research Center, LSUHSC, LSU A&M and University of Alabama may inspect and/or copy the medical records related to the study. Results of the study may be published and made available to other researchers; however, we will keep your name and other identifying information private. Other than as set forth above, your identity will remain confidential unless law requires disclosure.

In the future, your biomaterial (blood and/or urine samples) may be used by other investigators at Pennington or at other sites. There is a possibility that the samples that you are donating and the information you provide in this study may be used in research leading to commercial value. Should they lead to the development of a commercial product, the Pennington Biomedical
Research Center, LSUHSC, LSU A&M and University of Alabama will own it and it is possible that it will be patented and licensed by these institutions.

11- Can your taking part in the study end early?

Dr. Eric Ravussin, Dr. Steven Smith, or Dr. Alok Gupta can withdraw you from the study for any reason or for no reason. You may withdraw from the study at any time without penalty. Possible reasons for withdrawal include inability or unwillingness to complete the required testing.

12- What if information becomes available that might affect your decision to stay in the study?

During the course of this study there may be new findings from this or other research, which may affect your willingness to continue participation. Information concerning any such new findings will be provided to you.

13- What charges will you have to pay?

None.

14- What payment will you receive?

You will receive an additional $200 for your participation in this part of the study whether or not you participate in study 3 or 4. If you are or have been an employee of LSU within the last two years, the normal employee payroll deductions will be withheld.

15- Will you be compensated for a study-related injury or medical illness?

No form of compensation for medical treatment or for other damages (i.e., lost wages, time lost from work, etc.) is available from the Pennington Biomedical Research Center, LSU Health Sciences Center, LSU A&M College or UAB. In the event of injury or medical illness resulting from the research procedures in which you participate, you will be referred to a treatment facility. Medical treatment may be provided at your expense or at the expense of your health care insurer (e.g., Medicare, Medicaid, Blue Cross-Blue Shield, Dental Insurer, etc.), which may or may not provide coverage. The Pennington Biomedical Research Center is a research facility and provides medical treatment only as part of research protocols. Should you require ongoing medical treatments, they must be provided by community physicians and hospitals.
16- Healthy Insurance Portability and Accountability Act (HIPAA)

Records that you give us permission to keep, and that identify you, will be kept confidential as required by law. Federal Privacy Regulations provide safeguards for privacy, security, and authorized access. Except when required by law, you will not be identified by name, social security number, address, telephone number or any other direct personal identifier in screening records disclosed outside of Pennington Biomedical Research Center (PBRC) and kept in the study archives which are at LSUHSC in New Orleans. For records disclosed outside of PBRC, you will be assigned a unique code number.
17- Signatures

The study has been discussed with me and all my questions have been answered. I understand that additional questions regarding the study should be directed to the study investigators. I agree with the terms above and acknowledge that I have been given a copy of the consent form.

__________________________________                              _____________
Signature of Volunteer         Date

__________________________________
Date of Birth of Volunteer

I consent to have my blood stored for future DNA analysis. □ Yes    □ No
Volunteer’s Initials

__________________________________________             _____________
Signature of Person Administering Informed Consent             Date

The study volunteer has indicated to me that the volunteer is unable to read. I certify that I have read this consent form to the volunteer and explained that by completing the signature line above the volunteer has agreed to participate.

__________________________________________             _____________
Signature of Reader                                                                              Date
VITA

Madlyn Frisard was born in New Orleans, Louisiana, on December 1, 1974. She received her bachelor’s degree from Louisiana State University in May of 1997 and her master’s degree in August of 1999. She currently resides in Baton Rouge and is working at the Pennington Biomedical Research Center, where she has worked for the past five years. At the December 2005 commencement ceremony, Madlyn Frisard will be awarded the Doctor of Philosophy in kinesiology from the graduate School at Louisiana State University.