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MARKERS AND MECHANISMS OF METABOLIC AGING

By

Peter Dziewornu Ahiawodzi

BSc., Kwame Nkrumah University of Science and Technology, 2001

M.P.H., University of Louisville, 2010

A Dissertation

Submitted to the Faculty of the

School of Public Health and Information Sciences of the University of Louisville

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

Department of Epidemiology and Population Health

University of Louisville

Louisville, Kentucky

May 2014

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ABSTRACT

MARKERS AND MECHANISMS OF METABOLIC AGING

Peter D. Ahiawodzi

April 17, 2014

Oxidative stress has been implicated in aging and age-related diseases. Using three distinct studies, this dissertation examines associations between lifestyle factors and oxidative stress, and their effects on advanced glycation end products, telomere length, and type II diabetes.

Carboxymethyl-lysine (CML), an advanced glycation end-product, has been linked to oxidative stress, cardiovascular and other age-related diseases. The first study investigates the association between sleep disordered breathing (SDB), a source of oxidative stress, and CML. 1002 participants with sleep data from the Cardiovascular Health Study (CHS) were analyzed. SDB was not significantly associated with CML, but after stratification by gender and hemoglobin it was found that among female subjects with higher hemoglobin levels, those with more severe SDB had a significantly higher CML concentration, while the reverse was observed among males.

Telomere length is another biomarker of cumulative exposure to oxidative stress and an indicator of aging. In the second study, 974 participants from the Framingham

Heart Study were analyzed to investigate the role of modifiable factors known to affect oxidative stress including obesity, cigarette smoking, excessive alcohol consumption, and physical inactivity, on telomere length. Age, gender, BMI, and alcohol consumption were found to significantly influence telomere length.

Diabetes and its complications have roots in oxidative stress. Sleep disturbance, especially sleep apnea, is a potential source of oxidative stress. A total of 9635 participants in the first National Health and Nutrition Examination Survey were followed from 1982 to 1992. Time to diabetes was modeled as a function of sleep quality and quantity with adjustment for potential confounders. Sleep quality, but not sleep quantity, was significantly associated with type II diabetes.

In conclusion, oxidative stress plays a vital role in aging and age-related diseases. This dissertation emphasizes the importance of good sleep hygiene, healthy weight, and moderate alcohol consumption to curb the burden of oxidative stress on the human system. These findings may be considered in the policy framework of interventions aimed at reducing age-related diseases, to promote healthier aging and longevity.

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CHAPTER ONE

BACKGROUND

1.1 Evolutionary Theories of Aging

Aging is a major contributor to Alzheimer's disease, atherosclerosis, osteoporosis and autoimmune diseases¹. Why and how organisms age is still not fully understood. Weismann et al.,² proposed that aging is a death-mechanism, an adaptive response to exclude old members of population on a schedule responsive to new propagation, primarily under selection against overcrowding. Another school of thought suggests that late-acting deleterious effects of accumulated mutations in the cells of organisms contribute to aging of the organism. This is known as the somatic mutational theory and was proposed by Smith in 1962 when he used ionizing radiation to argue that radiation causes genetic mutations and shortens lifespan, and could serve as a major force in aging³.

More recently, disposable soma theory was proposed to explain variation among species in characteristic lifespan. The theory considers lifespan a trait evolved by selection primarily on the energetic requirements of complex organisms. Under the theory, the somatic self of an organism is a system of high energy demand for both reproductive and non-reproductive activities. From birth to death the molecular processes

of DNA replication and repair, protein synthesis and replenishment, require considerable energy expenditure, and lifespan is viewed as an average balance in tradeoffs between energy costs and investments that sustain the somatic and behavioral functions of an organism, at least through reproduction. Post-reproductively selection pressure falls off, and with the passage of time, the energetic maintenance of these basic systems declines causing systemic defects to accumulate and manifest as aging, or senescence of the organism⁴⁻⁶.

1.2 The Free Radical Theory of Aging

The free radical theory of aging was first proposed by Harman in 1956 and states that lifespan is mainly dependent on the production of intracellular reactive oxygen species (ROS)⁷. This theory is based on the premise that the harmful effects of ROS on various cells and organs results in their functional deterioration, and are often expressed as general decline and/or diseases associated with advancing age. The theory was expanded later to identify mitochondrial production of $O_2^{\cdot-}$ and H_2O_2 as the main source of ROS production causing age-related damage and organ dysfunction⁸.

1.3 Mitochondrial DNA and Reactive Oxygen Species

Mitochondria are present by the hundreds in every human cell, and each mitochondrion contains multiple small circular genomes (mtDNA). Mitochondria are the sites within cells of energy production and transport, where food and oxygen intake generate chemical energy and heat for work and maintenance of body temperature. This process of oxidative phosphorylation (OXPHOS) to produce energy (ATP) occurs by oxidizing hydrogen from consumed carbohydrates and fats in an ordered sequence of

electron transport: electrons from $\text{NADH} + \text{H}^+$ or from succinate are donated to NADH or succinate dehydrogenase, then passed to coenzyme Q (CoQ) to produce ubiquinone ($\text{CoQH}^\bullet$), then ubiquinol (CoQH_2), and to cytochrome c oxidoreductase (complex III). Electrons are then transferred from complex III to IV, also known as cytochrome oxidase, where $\frac{1}{2}\text{O}_2$ is produced and finally H_2O . It is the flow of electrons through this transport chain (ETC) that releases energy which in turn pumps protons out of the inner membrane of the mitochondria creating an electrochemical gradient which also captures energy to aid ATP release. The mitochondria in turn generate (ROS) as by-products of the energy production process: these include superoxide anion, hydrogen peroxide, and highly reactive hydroxyl radical. These free radicals then inflict damage on the mitochondria, mtDNAs, and the cell with downstream pathological consequences including hypertension, atherosclerosis, and diabetes⁹⁻¹².

Retention of electrons in the initial steps of the ETC generate ROS as by-products when unpaired electrons at CoQ binding-sites of complexes I, II, and III are directly donated to O_2 to generate superoxide anion ($\text{O}_2^\bullet$), then converted to H_2O_2 in the presence of Manganese superoxide dismutase (MnSOD). Finally, H_2O_2 converts to the highly reactive hydroxyl radical ($\text{OH}^\bullet$) in the presence of iron or other transition metals^{9, 13, 14}. Retention of electrons in the ETC occurs when calories are consumed in excess of the demand for their use, or with lack of exercise. Caloric intake without compensatory exercise, ramps down ATP production, causes hyperpolarization of the electrochemical gradient, and the eventual inhibition of OXPHOS, while leaving untransported electrons as free radicals available for ROS production⁹.

These reactive oxygen species could cause mitochondrial DNA mutations and these defects in the mitochondria could lead to dysfunction and eventually death. Mitochondrial production of reactive oxygen species is significantly increased in the heart and increases cardiac aging. This is because the heart is an organ that is very rich in mitochondria because of its high metabolic demand. Reactive oxygen species production is therefore very high in the heart making the heart very susceptible to oxidative damage^{15, 16}. In support of the free radical theory of aging Schriener et al., compared mice that overexpressed human catalase localized to the mitochondria with wild-type litter mates. They found that cardiac malfunction was delayed, and H₂O₂ production and mitochondrial DNA mutations were reduced, and lifespan increased in mice overexpressing human catalase targeted to the mitochondria¹⁷. Mitochondrial reactive oxygen species have also been linked to vascular aging by induction of apoptosis and studies have shown that the rate of endothelial apoptosis was increased with oxidative stress^{18, 19}. Endothelium-dependent vasodilation has also been proposed to be the result of mitochondrial oxidative stress and can cause a drop in blood pressure sufficient enough to produce shock^{20, 21}. Moreover, defects to the mitochondria as a result of reactive oxygen species could collapse the blood-brain barrier and lead to neuroinflammation associated with aging. This is because the integrity of the blood-brain barrier depends on the proper functioning of the mitochondria and inhibition of the mitochondrial function has adverse consequences for the blood-brain barrier²².

1.4 Other Sources of Reactive Oxygen Species

Mitochondria produce most of the endogenous reactive oxygen species^{23, 24}. However various enzymes localized elsewhere within the cell are also involved in ROS

production, including NADPH oxidase at the plasma membrane, lipid oxidation within peroxisomes, and xanthine oxidase in the cytoplasm.

1.4.1 Reactive Oxygen Species Generation by NADPH Oxidase

The human immune system is comprised of phagocytes (neutrophils, monocytes, eosinophils) that fight invading pathogens with the help of antimicrobial oxidants such as $O_2^{\bullet-}$ and H_2O_2 . NADPH oxidase is responsible for the generation of these antimicrobial oxidants. NADPH oxidase is activated when cells come into contact with bacteria or other inflammatory stimuli and catalyzes the reduction of oxygen to $O_2^{\bullet-}$ to kill bacteria²⁵. The superoxide anion can undergo further reactions to form the more potent hydroxyl radical ($OH^{\bullet}$). This can happen in two ways: by the Fenton mechanism ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$) or the Haber–Weiss mechanism ($O_2^{\bullet-} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2$; catalyzed by Fe^{3+})²⁶. Release of these oxidants is essential for immune system function, but excessive release can cause inflammatory tissue injury and eventual dysfunction of the associated organs²⁷.

1.4.2 Reactive Oxygen Species Generation by Xanthine Oxidase

The phenomenon of the “oxygen paradox” proposed that post-ischemic tissue injury is mainly attributed to free radical production during reoxygenation²⁸⁻³⁰. In ischemic states like myocardial infarction and stroke there is severe restriction of blood flow to tissues and the immediate medical solution is to restore oxygen. However, depending on the duration of the ischemia it has been found that tissue damage occurs as a result of reoxygenation of the ischemic tissues. There are two processes that lead to free radical production in post-ischemic tissues. Firstly, Ischemic conditions enable the conversion of xanthine dehydrogenase to xanthine oxidase. Secondly, in ischemia there is

depletion of ATP to AMP. Catabolism of AMP eventually leads to accumulation of hypoxanthine which is the substrate for the enzyme xanthine oxidase. Xanthine oxidase then catalyzes the oxidation of hypoxanthine to xanthine and further catalyzes the oxidation of xanthine to uric acid, and molecular oxygen (in reperfusion) to $O_2^{\bullet-}$ and H_2O_2 . Further reactions in the ischemic tissue promote the formation of $OH^{\bullet}$ via the Fenton mechanism. This increased free radical production in ischemic diseases such as stroke and myocardial infarction has therefore been proposed as a major factor in reperfusion-induced injury^{26, 31}.

1.4.3 Reactive Oxygen Species Generation by Lipid Oxidation

Peroxisomes are membrane-bound organelles that are present in the cytoplasm of all eukaryotic cells. They are involved in metabolism and contain enzymes that oxidize fatty acids and amino acids. As part of the peroxisomal metabolism reactive oxygen species are produced. Peroxisomal enzymes such as acyl-CoA oxidase, D-amino acid oxidase, and xanthine oxidase produce H_2O_2 which can undergo the Fenton reaction to produce the more potent hydroxyl radical³². Peroxisomes also contain the enzyme catalase, the most abundant peroxisomal antioxidant enzyme, which decomposes H_2O_2 to water, hence peroxisomes are protective against oxidative stress in the cell. Increased risk of age-related diseases including diabetes, atherosclerosis, and cancer have been found in individuals deficient in the enzyme catalase³³. Oxidative stress occurs when the production of reactive oxygen species in the peroxisome exceeds that which can be adequately detoxified^{34, 35}. The xanthine oxidase in the peroxisome can also reduce nitrates and nitrites to $NO^{\bullet}$ ³⁶. However $NO^{\bullet}$ can react with $O_2^{\bullet-}$ to produce $ONOO^-$ which is a strong oxidizing agent and capable of rendering peroxisomal enzymes inactive³⁷.

This also leads to decrease in nitric oxide bioavailability. Nitric oxide is vasodilatory in function and therefore has antiproliferative effect on smooth muscle cells. Depletion of nitric oxides leads to vascular thickening and loss of elasticity and hence endothelial dysfunction³⁸.

1.5 Advanced Glycation End-Products

Advanced glycation end products (AGEs) are a heterogeneous group of macromolecules that are formed by the nonenzymatic glycation of proteins, lipids, and nucleic acids. AGE formation induces oxidative stress which indirectly causes more AGEs production^{39, 40}. AGEs have been implicated in pathologies such as diabetic microvascular disease, connective tissue diseases (esp. rheumatoid arthritis), Alzheimer's disease, end-stage renal disease, hypertension, atherosclerosis and many other age-related disorders³⁹. Accumulation of AGEs occurs with age and as part of normal metabolic processes. However, accelerated production is associated with diabetes due to hyperglycemia and leads to vascular damage and eventual microvascular complications such as neuropathy, nephropathy, and retinopathy^{39, 41}. Carboxymethyl-lysine (CML), a well characterized AGE, is formed on protein by combined nonenzymatic glycation and oxidation reactions, and also during metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein (lipid peroxidation). CML is characterized as a general biomarker of oxidative stress and damage in tissue proteins⁴².

1.5.1 Hypoxia, Lysyl Oxidase Activity, and Carboxymethyl-lysine

Collagen is the protein that forms connective fibers in tissues. Collagen fibers keep bones and blood vessels strong. Collagen is also required for the repair of blood

vessels. It is the main component of connective tissue, and is the most abundant protein in mammals⁴³. Elastin is a major component of the extracellular matrix. It is a protein in connective tissue that is elastic and allows tissues in the arterial wall to resume their shape after stretching or contracting. Degradation of elastin in arterial walls is a characteristic feature of atherogenesis⁴⁴. The biomechanical properties of the vessel wall are determined largely by the extracellular matrix proteins collagen and elastin. The degree of cross-linking is a critical feature of collagen and elastin fibers. The cross-links in collagen contributes to the stiffness of collagen fibers⁴⁵, whereas the cross-links in elastin are important for the rubber-like properties of elastin fibers⁴⁶.

Lysyl oxidase catalyzes the covalent cross-linking of collagen and elastin in the extracellular space by oxidizing specific lysine residues in these proteins. The cross-linking of collagen and elastin stabilizes these proteins and also helps in the formation and repair of the extracellular matrix. Thus lysyl oxidase activity is essential in maintaining the tensile and elastic features of connective tissues of many systems including the pulmonary system⁴⁷. Downregulation of lysyl oxidase has been associated with endothelial dysfunction^{48, 49}. However, overexpression of lysyl oxidase has been associated with pulmonary arterial stiffness in hypertension⁵⁰, atrial fibrillation⁵¹, and in tumor initiation and progression⁵².

It has been found that hypoxia increases lysyl oxidase gene expression levels^{50, 53}. Endothelial cells respond to hypoxia by remodeling the extracellular matrix. This implies an upregulation of lysyl oxidase, a vital enzyme in extracellular matrix assembly. Furthermore, it has been found that the hypoxia-induced increase in lysyl oxidase expression is associated with increased levels of reactive oxygen species⁵⁴.

These findings indicate that hypoxia in SDB disrupts lysyl oxidase activity. The overexpression of lysyl oxidase in the extracellular matrix is also an indication of increased lysine modifications including carboxymethyl-lysine (CML)^{55, 56}.

1.6 Oxidative Stress and Disease

When an imbalance exist between prooxidant and antioxidants such that prooxidant levels exceed that of antioxidants the situation is referred to as oxidative stress. This can result from diminished antioxidant levels (vitamins E, C, and D, flavonoids and carotenoids) and micro-nutrient levels (iron, copper, zinc, and selenium). These micro-nutrients play vital roles in the proper functioning of antioxidant enzymes (copper/zinc superoxide dismutase, catalase, or glutathione peroxidase). Depletion of these nutrients and dysfunction of antioxidant enzymes leads to increased accumulation of reactive oxygen species²⁶ from any of the mechanisms described above. Since proposed by Harman in 1956^{7, 23} oxidative stress remains the most widely accepted cause of aging and age-related diseases. Oxidative stress has been associated with aging in humans, and diseases such as diabetes, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Huntington disease, cancer, and cardiovascular disease⁵⁷⁻⁶⁴.

Reactive oxygen species ($O_2^{\bullet-}$, H_2O_2 , and $OH^{\bullet}$) impose oxidative damage on important proteins, lipids, nucleic acids⁶⁵⁻⁶⁷. The hydroxyl radical has the ability to modify DNA bases and also cause strand breaks, leading DNA damage⁶⁸. It can attach to the C-8 position of guanine to form 8-hydroxyguanine (8-OH-guanine) which has been implicated in Parkinson's disease and Alzheimer's disease^{69, 70}. Another effect of oxidative damage on DNA bases is mutation arising from $AT \leftrightarrow GC$ transition and by

GC↔TA transversion, which can result in changes in protein gene expression²⁶. In proteins, reactive oxygen species cause damage by forming protein modifications such as carbonyls, dityrosine, and nitrated and chlorinated tyrosines, and these have been found at elevated levels in inflammatory disease patients²⁶. In the presence of reactive oxygen species and oxygen, lipids undergo peroxidation in which several molecules of lipid (LH) are oxidized. The process involves the highly reactive hydroxyl radical extracting the allylic hydrogen from the lipid thereby forming a carbon-centered lipid radical (L[•]), which reacts with oxygen to form a lipid peroxy radical (LOO[•]). Lipid peroxidation of low-density lipoprotein (LDL) and subsequent accumulation of the oxidized LDL leads to the formation of foam cells from macrophages, the initial stage of Atherosclerosis^{26, 71, 72}.

1.6.1 Oxidative Stress and Type-2-Diabetes

The β -cells of the pancreas produce insulin which is important in glucose metabolism. It has been established that elevated levels of long chain saturated non-esterified fatty acids exhibit a strong cytotoxic effect on the β -cells resulting in β -cell dysfunction and apoptosis^{73, 74}. Metabolism of long chain non-esterified fatty acids in the peroxisomes leads to the formation of H₂O₂ which is thought to mediate the lipotoxicity of the β -cells⁷⁵. The β -cells are particularly vulnerable to the toxic effects mediated by H₂O₂ because β -cells lack catalase, the enzyme responsible for decomposing H₂O₂ to water^{76, 77}.

1.6.2 Oxidative Stress and Leucocyte Telomere Length

As discussed earlier oxidative stress is associated with aging⁵⁷. Telomeres have also been found to play a major role in human aging, raising discussions on the relationship between oxidative stress and telomere length⁷⁸. Telomeres are repetitive DNA sequences that cap the ends of chromosomes. In humans, the long stretches of the tandem repeats are made up of TTAGGG⁷⁹. Telomeres function to regulate the proliferation of cells in organisms. This role was made possible because telomeres shorten as the organism ages and this shortening places limits on the number of replications that the cells of the organism can undergo. The decreased proliferative potential of the cell due to telomere attrition may cause alterations in cellular metabolism and lead to apoptosis⁷⁸.

Genetic and environmental factors influence telomere length: these include physical activity, tobacco smoking, socio-economic status, body-mass index, alcohol consumption, multivitamin intake, dietary antioxidants, paternal age at time of offspring's birth, and perceived stress and adverse life events. These factors may increase or decrease oxidative stress and inflammation, which are very important in telomere length dynamics and aging⁸⁰⁻⁸⁸. Eisenberg⁷⁸ proposed the "thrifty telomere model" which states that regulation of maintenance effort in an organism is the main function of the telomeres. This suggests that telomeres are responsible for programming the allocation of resources within the organism. Hence factors like tobacco use, alcohol consumption, and obesity, which potentially place extra oxidative burden on the cells of the organism could lead to shorter telomeres because the extra oxidative burden could only be met when part of the soma is lost (in this case, telomere length reduction). As telomeres become

shorter, the replicative capacity of the cell is reduced leading to senescence. Von Zglinicki in his review of oxidative stress and telomere length acknowledged that in most cases the replicative senescence of telomeres is a stress response arising from external oxidative stress or diminished internal antioxidant defenses. His review of experiments on stress dependency of telomere shortening revealed that 19 out of 22 independent experiments from seven different laboratories, showed increased telomere shortening rate under conditions of increased oxidative stress. He further suggested that telomere length might serve as a biomarker of cumulative exposure to oxidative stress and a prognostic indicator for risk of late-life diseases⁸⁹.

1.7 Summary

In summary, reactive oxygen species have been widely accepted as a major contributor to dysfunction of biological systems resulting in aging and age-related diseases. In this dissertation, factors that encourage increased reactive oxygen species production were investigated. Sleep-disordered breathing and CML are both associated with oxidative stress; hence, this dissertation investigated the relationship between sleep-disordered breathing and serum CML concentration. CML accumulation has been associated with Alzheimer's disease, end-stage renal disease, hypertension, atherosclerosis and many other age-related disorders. Understanding its association with sleep disordered breathing could provide avenues for prevention. In addition, we investigated the role of sleep and obesity on type-2 diabetes risk. Diabetes results from oxidative damage to insulin producing cells and research suggests that sleep disturbances and obesity may be involved in this process. Lastly, we investigated the effects of alcohol consumption and obesity on telomere length. Telomere shortening has been found to be

stress dependent and alcohol consumption and obesity can induce stress. Telomere shortening has been linked to neurodegenerative diseases, infections, diabetes, malignancies, atherosclerosis, and cardiovascular disease. Understanding the sources of stress in telomere shortening is important in the wake of the harmful effects of shorter telomeres on health.

Figure 1: Mechanisms linking oxidative stress to aging and degenerative diseases

CHAPTER TWO

THE RELATIONSHIP BETWEEN SLEEP-DISORDERED BREATHING AND N ϵ -CARBOXYMETHYL-LYSINE CONCENTRATION IN THE CARDIOVASCULAR HEALTH STUDY

2.1 INTRODUCTION

Advanced glycation end-products (AGEs) have been implicated in pathologies such as diabetic microvascular disease, rheumatoid arthritis, Alzheimer's disease, end-stage renal disease, hypertension, atherosclerosis and other late-life disorders³⁹. Major AGEs include hydroimidazolone (HI), N ϵ -carboxymethyl-lysine (CML), pentosidine (PTD), and glucosepane³⁹. CML is the focus in this study because it has been found to be a general biomarker of oxidative stress and damage in tissue proteins. CML is formed on protein by combined nonenzymatic glycation and oxidation reactions, and also during metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein⁴². CML has serious implications for cardiovascular disease because it has been found to be the main contributor to atherosclerotic plaque^{42, 90, 91}. Cardiovascular disease is the leading cause of death in the United States. More than 80 million people live with cardiovascular disease of some sort, and together with stroke they account for more than \$500 billion in medical costs in 2010. It is important that the scientific community find

ways to reduce accumulation of advanced glycation end-products, including CML, as part of efforts aimed at reversing the trend of cardiovascular morbidity and mortality.

There is a possible link between sleep-disordered breathing and serum CML levels because sleep-disordered breathing is accompanied by production of free radicals and increased oxidative stress, a condition which promotes increased CML production. In sleep-disordered breathing there are repeated episodes of hypoxia which leads to energy crisis as the production of adenosine triphosphate (ATP) is impaired leading to up-regulation of glycolysis and consequent increased production of reactive oxygen species (ROS)^{24, 92}. Sleep-disordered breathing, especially obstructive sleep apnea, can be treated by use of continuous positive airway pressure (CPAP)⁹³⁻⁹⁶. This implies that if evidence exists for a causal relationship, then intensification of efforts to promote sleep hygiene and treatment of sleep-disordered breathing could be considered as part of efforts to reduce CML accumulation and hence reduce cardiovascular and other age-related diseases.

Few studies investigated the effect of sleep-disordered breathing on AGEs, broadly^{96, 97}. A pilot study has also shown that continuous positive airway pressure treatment reduces serum AGEs in patients with obstructive sleep apnea syndrome⁹⁸. The relationship between sleep disordered breathing and CML concentration however has not been studied. Moreover, there is evidence for increased lipid peroxidation biomarkers, an indication of increased state of oxidative stress, in sleep-disordered breathing⁹⁹. Since CML is formed from lipid peroxidation and known to be an excellent biomarker for oxidative stress it will be more appropriate to investigate the association between sleep-disordered breathing and CML specifically instead of AGEs as a broad category. The

main aim of this study is to investigate the association between sleep-disordered breathing and serum CML levels in the Cardiovascular Heart Study.

2.1.1 Study objectives

1. To investigate the cross-sectional relationship between sleep disordered breathing (SDB) assessed by apnea-hypopnea index (AHI), and carboxymethyl-lysine (CML) concentration in the Cardiovascular Heart Study (CHS) cohort enrolled in the Sleep Heart Health Study (SHHS).
2. To investigate the potential modification effects of gender and hemoglobin on the association between sleep disordered breathing and CML.

2.1.2 Hypotheses

1. Study subjects with SDB will have higher serum concentrations of CML.
2. The effect of SDB on CML concentration will vary by gender and will be higher in participants with higher hemoglobin levels.

2.1.3 Justification of the study

Carboxymethyl-lysine (CML), a well characterized advanced glycation end-product has been associated with degenerative diseases including Alzheimer's disease, end-stage renal disease, hypertension, atherosclerosis and many other age-related disorders. Pharmaceutical interventions to reduce CML accumulation have shown promising results but are yet to be investigated using larger populations. Continuous

positive airway pressure (CPAP) treatment for sleep-disordered breathing, especially obstructive sleep apnea, has been established. Evidence for a causal relationship between sleep-disordered breathing and CML could provide avenue for intervention within the wide range of possible interventions.

2.2 METHODS

2.2.1 Study Design: Population-based cross-sectional study of the association between sleep-disordered breathing and serum carboxymethyl-lysine.

2.2.2 Study Participants: The subjects for this study are CHS cohort members who were enrolled in the SHHS. The CHS is a population-based longitudinal study designed to determine the risk factors for development and progression of cardiovascular disease (CVD) in older adults (≥ 65 years) in the United States. The CHS recruited 5,888 adults from four U.S communities: Forsyth County, North Carolina; Sacramento County, California; Washington County, Maryland; and Pittsburgh, Pennsylvania. The SHHS is a prospective cohort study designed to investigate obstructive sleep apnea (OSA) and other sleep-disordered breathing (SDB) as risk factors for the development of cardiovascular disease. Participants in SHHS were recruited from already existing cohort studies: Atherosclerosis Risk in Communities Study, Cardiovascular Health Study, Framingham Heart Study, Strong Heart Study, New York Hypertension Cohorts, and Tucson Epidemiologic Study of Airways Obstructive Diseases and the Health and Environment Study. Participants in the SHHS underwent home polysomnography to assess the

presence of OSA and other SDB¹⁰⁰. This study focuses on over 1000 participants that were recruited from the CHS into the SHHS.

2.2.3 Assessment of Sleep Disordered Breathing

SDB was diagnosed by polysomnography and measured by apnea-hypopnea index (AHI). AHI is the number of respiratory events scored per hour of sleep. Apneas and hypopneas were classified according to the American Academy of Sleep Medicine (AASM) criteria. According to AASM criteria an event is classified as apnea if there is a drop in peak thermal sensor excursion by $\geq 90\%$ from baseline, for 10 seconds or greater, and an event is classified as hypopnea if there is a $\geq 30\%$ drop in the nasal pressure excursion for 10 seconds or greater associated with $\geq 4\%$ desaturation^{101, 102}. AHI was used as a continuous variable and cut-points were also be considered. AHI cut points for diagnosis are: $< 5/\text{hr}$ (normal), ≥ 5 and $< 15/\text{hr}$ (Mild), ≥ 15 and $\leq 30/\text{hr}$ (moderate), and $\text{AHI} > 30/\text{hr}$ (severe)¹⁰³.

2.2.4 Polysomnography

Polysomnography (PSG) or a sleep study is a test used to diagnose sleep disorders. It is considered to be the “gold standard” for diagnosing SDB and other sleep disorders. PSG monitors sleep stages and cycles to identify disruptions in sleep patterns. PSG is recommended when the following are suspected: sleep-related breathing disorder, periodic limb movement disorder, narcolepsy, unusual behaviors during sleep, and unexplained chronic insomnia. PSG is a noninvasive test. However the adhesive used to attach the test sensors to the skin could cause skin irritation. Alcohol and caffeine may

make symptoms of some sleep disorders worse and it is recommended that they are avoided at least 12 hours before the sleep study.

There are two critical components of PSG: (1) determination of sleep-wake stage, and (2) identification of related bodily processes. Sleep-wake determination involves electroencephalography (EEG; records surface electrical activity of the brain), electrooculogram (EOG; records eye movements during sleep and wakefulness), and electromyography of the submentalalis (EMG; recordings are essential for scoring sleep stages)¹⁰⁴. The placement of these electrodes is shown by Appendices 1, 2, and . Related bodily processes measured in PSG include heart rate, blood pressure, and respiratory function. Respiratory air flow is determined by measuring inhaled and exhaled air temperatures by a thermistor. Thermosensors are best in determining apneas because they are very sensitive to even minor air flow. They are however poor detectors of partial flow limitations (hypopneas) because even slight air flow produces large amplitude deflections due to their high sensitivity. Apart from thermosensors, nasal pressure can be measured by piezoelectric pressure sensors which are very sensitive to hypopneas. Appendix 4 shows the placement of nasal pressure cannulae^{104, 105}.

Relevant parameters measured in PSG include total recording time, total sleep time, sleep period time, time in bed, sleep efficiency, sleep latency and REM sleep latency, and sleep stage distribution. A summary of the entire night's PSG data can be presented in a graphic form called a sleep hypnogram. Appendix 5 shows a sleep hypnogram of normal and fragmented sleep patterns¹⁰⁵.

This study used sleep study data of subjects who underwent home PSG. Apnea-hypopnea index (AHI) was scored according to the guidelines of the American Academy of Sleep Medicine¹⁰¹.

2.2.5 Formation of Advanced Glycation End-products (AGEs)

AGEs are formed when reducing sugars react non-enzymatically with amino groups in proteins, lipids, and nucleic acids. This involves series of reactions in which Schiff bases undergo Amadori rearrangement to form the Amadori product. The Amadori product undergoes further reactions to generate AGEs. This process is also called the Maillard reaction (browning reaction) and was identified in 1912 when it was found that a characteristic yellow-brown color was developed when amino acids were heated in the presence of reducing sugars⁴¹. CML is one of the dominant AGEs and known to be a glycoxidation product because it is formed by both oxidation and glycation reactions¹⁰⁶. CML is also formed as a product of lipid peroxidation⁴². Glycoxidation and lipoxidation reactions are accompanied with reactive oxygen species production; hence CML has been characterized as a general biomarker of oxidative stress. A schematic presentation of AGE formation pathways is shown in Appendix 6.

2.2.6 Measurement of CML

CML was measured as described by Barzilay et al¹⁰⁷. Briefly, fasting samples were stored at -80 degrees Celsius and CML measured using a competitive enzyme-linked immunosorbent assay (ELISA); AGE-CML ELISA, Microcoat, Penzberg, Germany.

2.2.7 Selection and Definition of Covariates

The following variables were collected for use in this study because previous research has shown their association with either sleep disordered breathing (SDB) or CML accumulation: age, sex, race, smoking, alcohol intake, BMI, hypertension, diabetes, household income, physical activity, general health status, insomnia, estimated glomerular filtration rate (eGFR), C-reactive protein, hemoglobin, HDL cholesterol, prevalent MI, CHD, stroke, and claudication. Some of these variables were used as continuous variables and others were used as categories. Age as a categorical variable was classified as 64-69, 70-74, 75-79, and 80 years or more. Race and ethnicity was classified as “White”, “African American or Black”, and “other”. The “other” class includes American Indian or Alaskan native, Asian or Pacific Islander. BMI was categorized according to the World Health Organization guidelines as “normal” (BMI $<25.0 \text{ kg/m}^2$), “overweight” (BMI 25-29.9 kg/m^2), or “obese” (BMI $\geq 30 \text{ kg/m}^2$). Smoking status was categorized as “nonsmoker”, “former smoker”, or “current smoker”, and blood pressure was categorized into “normal”, “prehypertension”, or “hypertension” according to the Seventh report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure (JNC VII) criteria. According to this guideline a normal blood pressure has systolic pressure of less than 120 mmHg and a diastolic pressure of less than 90 mmHg, prehypertension is systolic blood pressure of 120 to 139 mmHg or diastolic pressure of 80 to 89 mmHg, and hypertension is systolic pressure of 140 and above or diastolic pressure of 90 and above. Self-reported health status was categorized as “excellent”, “very good”, “good”, “fair”, or “poor” and diabetes status was defined based on the American Diabetes Association guidelines. The

2-hour glucose test (oral glucose tolerance test-OGTT) was used and diabetes was defined as 2 hour blood glucose of greater than or equal to 200 mg/dl, impaired fasting glucose (prediabetes) was defined as 2 hour blood glucose of ≥ 140 to < 200 mg/dl, and normal was defined as 2 hour blood glucose of less than 140 mg/dl. The status of conditions such as MI, CHD, CHF, stroke, Claudication, and insomnia were coded as “yes” or “no”, and exercise intensity was categorized as “none”, “low”, “moderate”, or high. Household income of participants was put into 5 classes: $< \$12,000$, $\$12,000$ - $\$24,999$, $\$25,000$ - $\$49,999$, $\$50,000$ - $\$75,000$, and $> \$75,000$.

2.2.8 Inclusion and Exclusion Criteria

A total of 2287 participants in the CHS have CML records but since the exposure of interest is sleep disordered breathing (SDB) assessed by apnea-hypopnea index (AHI), we excluded all participants without AHI records. A total of 1285 participants without AHI records were excluded leaving a sample size of 1002 that was used in this analysis.

2.2.9 Statistical Analysis

CML was measured between 1996 and 1997 (CHS year 9) around the same time that polysomnography was conducted on the participants. Linear regression was used to test the cross-sectional relationship between SDB (assessed by AHI) and CML concentration. CML distribution was skewed to the right and was log transformed before analysis. SDB (AHI) was the exposure variable and serum CML concentration was the outcome variable. The following were adjusted for: age, sex, race, smoking, alcohol intake, BMI, hypertension, estimated glomerular filtration rate (eGFR), C-reactive protein, hemoglobin, HDL cholesterol, prevalent MI, CHD, stroke, and claudication. This parsimonious set of variables was arrived at through a model building technique

recommended originally by Hosmer and Lemeshow¹⁰⁸, and described further by Jewell¹⁰⁹. It involves examining risk factors in order of their primary interest in terms of relationships with the outcome or main predictor, and checking for possible interactions. Stratified analyses based on gender and hemoglobin were performed with adjustment for the confounders mentioned above. Measurements from previous years were used to impute missing values. All analyses were performed using SAS version 9.3. Levels of significance of all analyses were based on p-value of 0.05.

2.2.10 Power Calculation (SDB and CML)

2.2.11 Prevalence of sleep apnea in the study population

More than 18 million adults in America have sleep apnea according to the National Sleep Foundation (NSF)¹¹⁰. The NSF also found that individuals ≥ 40 years are at increased risk of sleep apnea. Using the 2010 U.S census¹¹¹, approximately 124 million residents were ≥ 40 years old. Hence 18 million adults with sleep apnea will amount to approximately 15% of the population ≥ 40 years. The CHS cohort is made up of individuals that are ≥ 65 years of age, and moderate to severe ($AHI \geq 15$) sleep disordered breathing was found in 35.2% of the sample. With 1002 participants this results in an exposed group of 353 and unexposed group of 649.

2.2.12 Prevalence of high CML among exposed and unexposed groups

CML is a metabolic by-product and increased accumulation increases the risk of health problems. An investigation into the effect of CML on the risk of hip fracture in the CHS showed that all but the first quartile showed increased risk of hip fracture¹⁰⁷. This

implies the 2nd, 3rd, and 4th quartiles of CML could be regarded as “high” (that is, 75% of the population, and in this case ≥ 65 year olds). In another study the association between CML (categorized as “Low” or “High” at the median) and carotid diameter was investigated and it was found that “high” CML was associated with enlarged carotid diameter¹¹². In this case 50% of the study participants were classified as having “high” CML.

With 95% confidence level, 35.2% prevalence of sleep apnea, 75% “high” CML in the study population (unexposed), and 83% “high” CML in the study population (exposed), the power of the study is more than 80% as presented in table 1.

Table 1: Power calculation for the association between sleep disordered breathing and carboxymethyl-lysine (based on 75% of study population with high CML)

Power for Cross-Sectional Studies (SDB and CML)		
	Exposed Group	Unexposed Group
Sample Size	353	649
Prevalence (or) Coverage (%)	83	75
Power based on:		
Normal approximation		83.04%
Normal approximation with continuity correction		80.91%

With 95% confidence level, 35.2% prevalence of sleep apnea, 50% “high” CML in the study population (unexposed), and 59.4% “high” CML in the study population (exposed), the power of the study is at least 80% as presented in table 2.

Table 2: Power calculation for the association between sleep disordered breathing and carboxymethyl-lysine (based on 50% of study population with high CML)

Power for Cross-Sectional Studies (SDB and CML)		
	Exposed Group	Unexposed Group
Sample Size	353	649
Prevalence (or) Coverage (%)	59.4	50
Power based on:		
Normal approximation		81.4%
Normal approximation with continuity correction		79.54%

2.3 RESULTS

A total of 1002 subjects were involved in this study. The mean age was 77.5 and 28% were 80 years and above [Table 3]. The sample was predominantly Caucasian (81%), and made up of 57% females. The mean BMI of participants was 27.2 kg/m² with normal, overweight, and obese subjects forming 31.4%, 46.9%, and 21.7%, respectively. Nondrinkers constitute 56.7% of the sample and among drinkers the average alcohol consumption per day was 10gm. Non-smokers represent 48.6% of the sample and 6.3% of the sample were current smokers. Participants had mean hemoglobin of 13.8 g/dl, and by gender it was 13.3 g/dl and 14.4 g/dl for females and males respectively. The mean C-reactive protein (CRP) concentration was 5.0 mg/L, and the mean estimated glomerular filtration rate (eGFR- cystatin C) was 82.4 mL/min/1.73 m². The percent of sleep time of SaO₂ below 90% was on average 4.3 among the study participants and about 21 arousals were experienced on average per hour of sleep. The mean CML concentration among study participants was 6161.2 ng/ml (6.4 ng/ml after log transformation), and participants experienced on average 11.5 apneas and hypopneas per hour of sleep. Participants who earned \$50,000 or more, had moderate to high physical activity, high blood pressure ($\geq 140/90$), self-reported good health or better, and impaired fasting glucose or diabetic formed 16.3%, 53.9%, 52.3%, 83.6%, and 24.4%, respectively, of the sample. Participants who had MI, CHD, CHF, stroke, claudication, and insomnia formed 9.8%, 24.0%, 6.8%, 4.0%, 2.8%, and 18.9%, respectively, of the sample [Table 3].

In unadjusted analyses, BMI, % sleep time of SaO₂ below 90%, arousal index, HDL, and eGFR- cysC, were significantly associated with sleep disordered breathing (SDB) measured by Apnea-Hypopnea Index (AHI) [Table 3]. Whilst increasing values of BMI, % sleep time of SaO₂ below 90%, and arousal index were associated with increasing severity of SDB, decreasing values of HDL and eGFR- cysC, were associated with increasing severity of SDB. Hemoglobin has a borderline significance with SDB ($p=0.069$) in this unadjusted model and higher levels were associated with increasing severity of SDB. Other variables that were significantly associated with SDB in the unadjusted model include race/ethnicity, gender, prevalent MI status, and prevalent CHF status. African Americans, males, MI patients, and CHF patients were more likely to have severe SDB [Table 3]. Variables which significantly influenced CML accumulation in the unadjusted models were age, BMI, alcohol use, HDL, triglycerides, hemoglobin, eGFR- cysC, arousal index, and smoking status. Higher levels of CML were associated with increasing age, and HDL but with decreasing BMI, alcohol use, triglycerides, hemoglobin eGFR- cysC, and arousal index. Current smoking status was significantly associated with lower CML levels in the unadjusted analysis [Table 4].

Sleep disordered breathing was not associated with CML in either the unadjusted or adjusted models [Table 5]. However, modification effects by gender and hemoglobin on the association between SDB and CML were identified [Table 6]. The variables in the main effects model include apnea-hypopnea index, age, BMI, gender, race, alcohol, smoking, stroke, claudication, CHD, MI, diabetes, hypertension, HDL, hemoglobin, CRP, and eGFR. As shown in table 4, the model with the 3-way interaction appears to be the best model for this analysis. Taking the 3-way interaction into consideration, it was found

that among males with hemoglobin concentration greater than 14.29 g/dl, there was an inverse relationship between SDB and CML concentration, while among females with the same hemoglobin level there was a direct relationship between SDB and CML concentration [Table 7]. Interaction effects by diabetes and insomnia were investigated but were not significant.

2.3.1 Males with hemoglobin concentration >14.29 g/dl

Compared to male subjects with no SDB, males with severe SDB have 16.1% lower CML concentration if hemoglobin is >14.29 g/dl ($p=0.0146$). There was no significant association between SDB and CML concentration among males with hemoglobin ≤ 14.29 g/dl [Table 7].

2.3.2 Females with hemoglobin concentration >14.29 g/dl

Compared to female subjects with no SDB, females with severe SDB have 28.1% higher CML concentration if hemoglobin is >14.29 g/dl ($p=0.0336$). Also, compared to female subjects with no SDB, females with mild SDB have 19.0% higher CML concentration if hemoglobin is >14.29 g/dl ($p=0.0061$). There was no significant association between SDB and CML concentration among females with hemoglobin ≤ 14.29 g/dl [Table 7].

2.3.3 The effect of hemoglobin (irrespective of gender) on the SDB-CML association

Considering the fact that an interaction between SDB (AHI) [Table 6] and hemoglobin was detected and that hemoglobin is highly influenced by gender, an alternative approach was to find those hemoglobin cut-points where the influence of SDB on CML becomes clinically important. This was done by performing analyses with

different hemoglobin cut-points until the association between SDB and CML becomes significant. The following cut-points have been identified and apply to both males and females: <12.7 g/dl, 12.7 to <14.6 g/dl, and ≥ 14.6 g/dl.

2.3.4 The effect of hemoglobin <12.7 g/dl (irrespective of gender) on the SDB-CML association

There was no significant association between SDB and CML concentration for hemoglobin <12.7 g/dl [Table 8].

2.3.5 The effect of hemoglobin 12.7 - 14.59 g/dl (irrespective of gender) on the SDB-CML association

Among subjects with hemoglobin concentration between 12.7 and <14.6 g/dl, it was found that subjects with severe SDB were more likely (9.1% ; $p=0.0318$) to have higher CML concentration compared to subjects with no SDB. It was also found within the same hemoglobin class that subjects with mild SDB were more likely (5.9% ; $p=0.0223$) to have higher CML concentration compared to subjects with no SDB [Table 8].

2.3.6 The effect of hemoglobin ≥ 14.6 g/dl (irrespective of gender) on the SDB-CML association

Among subjects with hemoglobin concentration ≥ 14.6 g/dl, it was found that subjects with severe SDB have significantly lower CML concentration (13.8% ; $p=0.0364$) compared to subjects with no SDB [Table 8].

2.4 DISCUSSION

The multivariable models did not detect significant association between sleep disordered breathing (SDB) and carboxymethyl-lysine (CML) concentration. It was found however that the association between SDB and CML was modified by gender and hemoglobin. At high hemoglobin levels females with SDB have higher levels of CML whilst males with SDB have lower levels of CML. This study is the first of its kind with no previous studies to compare the findings to. However, a few studies investigated the association between SDB and advanced glycation end-products broadly.

In one study conducted by Lam et al.,⁹⁶ among 105 males (mean age 43.5 years) without diabetes, significantly higher serum advanced glycation end-product (AGE) levels were observed in subjects with SDB ($AHI \geq 5$), compared to subjects without SDB ($AHI < 5$). They also found that among subjects with moderate to severe SDB who underwent CPAP treatment, serum levels of advanced glycation end-products were decreased indicating a direct relationship between SDB and advanced glycation end-products. This is contrary to the lower CML concentration in severe SDB found among males with high hemoglobin in our study. The difference in findings could arise from the differences between CML (a specific AGE) and AGEs in general. In addition, we stratified on hemoglobin but Lam et al. did not. Furthermore, Lam et al. studied a younger population (mean age: 43.5 years) while we studied a much older population (mean age: 77.5 years) which could potentially contribute to differences in the findings.

Tan et al.⁹⁷ investigated the association between SDB and advanced glycation end-products in a case-control study involving 119 nondiabetic patients with SDB and 234 age-matched healthy controls. They found that markers of oxidative stress increased

in SDB patients and serum levels of advanced glycation end-products increased. The studies by Tan et al., and Lam et al. were consistent in establishing a direct relationship between SDB and advanced glycation end-products. We found a direct relationship among females but not males, and only after hemoglobin stratification. CML is a well characterized advanced glycation end-product, and has been described as the general biomarker of oxidative stress. Hence a direct relationship between SDB and CML is expected but our data only partially supports that hypothesis.

2.4.1 Mechanisms linking sleep disordered breathing to CML concentration

Revisiting our hypothesis of free radical production thorough the xanthine oxidase pathway and how that might affect CML accumulation, it has been established that SDB is characterized by intermittent hypoxia and re-oxygenation after each hypoxic episode generates free radicals. Overproduction of free radicals leads to a state of oxidative stress and could accelerate CML production. The modification effect by gender is plausible because in premenopausal women estrogen is very active in scavenging free radicals, and also has the ability to stimulate enzymes to detoxify free radicals. This implies that in premenopausal women, one could expect reduced CML accumulation compared to males even in sleep disordered breathing situations. In this study the mean age was 73.5 years with minimum of 64 years and a maximum of 95 years. Therefore the women in this study are all postmenopausal and unless they all use estrogen replacement therapies, one should not expect the protective effect of estrogen on free radicals among women in this study. Significant effects of SDB on CML concentration was found but only at high hemoglobin levels and these effects differ by gender. Among females with hemoglobin concentration >14.29 g/dl, increased CML concentration was associated with mild and

severe SDB [Table 7] and this supports our hypothesis that increased free radical production in SDB could lead to increased CML accumulation; this was not the case for males. Focusing more on this result among females, what could be the possible reasons why the effect of SDB on CML was only significant at the higher spectrum of hemoglobin?

Circulating ferrous hemoglobin participates in chemical reactions that could be toxic in living organisms. Among these reactions is autoxidation of oxy-hemoglobin. Oxy-hemoglobin undergoes a slow, but spontaneous intramolecular redox reaction, in which the heme iron is oxidized to the ferric form and oxygen reduced to superoxide¹¹³⁻¹¹⁶.

In the presence of adequate antioxidant defense systems, the superoxide will be dismutated to oxygen and hydrogen peroxide by superoxide dismutase:

In the absence of antioxidant enzymes these reactions could promote hydroxyl radical formation via the following reactions²⁶:

Fenton reaction: $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^{\bullet} + \text{OH}^-$ or the

Haber–Weiss mechanism: $\text{O}_2^{\bullet -} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^{\bullet} + \text{OH}^- + \text{O}_2$; catalyzed by Fe^{3+}

Intermittent hypoxia followed by re-oxygenation which occurs in sleep disordered breathing (SDB) generates superoxide^{28, 29} and hemoglobin serves as the source of iron which catalyzes the reactions leading to the more potent hydroxyl radical¹¹³. CML is a biomarker of oxidative stress and accelerated CML production as a result of increased free radicals at high hemoglobin levels is plausible. The increased CML production at high hemoglobin levels occurred mainly in females and not in males because in postmenopausal women (as in this study), iron stores tend to increase rather than decrease (Appendix 7)¹¹⁷. Ferritin, an intracellular protein that stores iron and controls its release has been found to increase whiles estrogen decrease during the menopausal transition (Appendix 8)¹¹⁸.

The increase in body iron in females after menopause has oxidative burden consequences. This is because menopause is accompanied with increase in body weight, decreased antioxidant defenses and chronic inflammation¹¹⁹. This results in increased free radical production and reduced antioxidant defenses in postmenopausal women, a situation of oxidative stress. Oxidative stress from increased free radicals in SDB at high hemoglobin levels in postmenopausal women is a plausible cause of the increased CML concentration found in these individuals. A schematic presentation of iron increase during menopause and oxidative stress is shown in Appendix 9.

Among males SDB was significantly inversely associated with CML concentration at hemoglobin >14.29. In other words SDB (severe) was found to be associated with lower CML concentration. This is the first study examining the relationship between SDB and CML hence there are no comparative studies for this observation. However, there are plausible explanations for this association. Males have

higher hemoglobin concentration than females and are expected to generate more reactive oxygen species in the presence of SDB and hence increased CML accumulation. However, the reverse was observed and may partly be due to unmeasured carboxyhemoglobin (COHb) in males. Cigarette smoking raises the blood levels of COHb resulting in reduced hemoglobin which interrupts oxygen transport from the lungs to the rest of the body¹²⁰. Hence unlike females whose iron stores increase after menopause, older males are more likely to be anemic due to tobacco use and that could influence the effect of hemoglobin on the SDB and CML association. Results from this study have shown that males were significantly likely than females to be smokers ($p < .0001$) [Figure 2] which adds evidence to the COHb hypothesis.

Figure 2: Percent of males and females in each smoking category

Apart from the hindrance of hemoglobin function by COHb, older males could actually suffer reduced hemoglobin due to colorectal cancer^{121, 122}. An estimated 71,830

men and 65,000 women will be diagnosed with colorectal cancer and 26,270 men and 24,040 women will die of the disease in 2014, according to the National Center for Health Statistics¹²³. Females can build up their iron stores after menopause but males cannot and the associated iron deficiency in males could play an important role in reducing oxidative stress and hence the observed lower CML concentration with SDB¹²⁴.

Physical activity has been found in many studies to protect against oxidative stress, especially in older individuals and could also account for the association between SDB and CML concentration in males with hemoglobin >14.29g/dl. A study by Traustadóttir et al.,¹²⁵ tested the hypothesis that physically fit older adults would have less oxidative stress than unfit age-matched controls. They recruited 37 fit individuals with an average age of 65 years, and 35 unfit individuals with an average age of 67.7 years, and found that fit individuals had significantly lower oxidative stress markers compared to the unfit individuals. Our results have shown that males were more likely than females to engage in moderate to high physical activity ($p=0.0028$), as shown on figure 3. More specifically, it was found that males with hemoglobin above 14.29 g/dl were significantly more likely than females in the same hemoglobin group, to engage in moderate to high physical activity ($p=0.0391$), as shown on figure 4. These findings might explain the protective effect of SDB on CML concentration found among males with hemoglobin >14.29 g/dl.

Figure 3: Percent of males and females in each exercise intensity category

Figure 4: Percent of males and females by exercise intensity: hemoglobin >14.29 g/dl

These findings are also consistent with findings by Karolkiewicz et al.,¹²⁶ who studied the relationship between markers of oxidative stress and physical activity among men (mean age: 73.4 years). They found that compared with less active men, more active men have higher plasma levels of glutathione (GSH), indicating favored antioxidant potential, and lower thiobarbituric acid reactive substance (TBARS) concentration, indicating inhibited lipid peroxidation. Pierce et al.,¹²⁷ also came to the conclusion that physical activity protects against the harmful effects of oxidative stress when they compared older men who perform habitual aerobic exercise with their sedentary counterparts.

The lower CML concentration found among males with severe SDB at high hemoglobin levels may not necessarily be a protective effect of severe SDB. Life expectancy for males and females in the United States is approximately 77 and 82 years, respectively, and we studied a population with mean age of 77.5 years. It is likely that males with severe SDB and who by our hypothesis should have high CML levels have died before CML was measured for this analysis. Hence we may be looking at males who even with SDB have survived for reasons beyond the scope of this study, and their CML values may not be reflective of what would be observed if a younger population were studied.

2.4.2 The effect of hemoglobin on the association between SDB and CML

The 3-way interaction between gender, hemoglobin, and SDB on the CML concentration has been discussed above. However in table 7 we sought to find hemoglobin levels at which the effect of SDB on CML concentration would be clinically important. New hemoglobin cut-points have been created for the entire sample without

gender stratification. Mild and severe SDB were significantly associated with higher CML concentrations in the normal hemoglobin class (12.7-14.59 g/dl). This normal hemoglobin class involved more than half of the entire study sample and divided among males and females as 37% and 63%, respectively. Although this hemoglobin level appears to be important clinically in terms of CML accumulation and consistent with our hypothesis, the unequal representation of gender raised concerns. This is because female gender has been associated with increased CML concentration in the presence of SDB (at hemoglobin >14.29 g/dl) [Table 6] and since females have dominated this normal hemoglobin class (63%), it is likely that the association observed in this hemoglobin class is due to female gender. In the high hemoglobin class (≥ 14.6 g/dl) [Table 8] severe SDB was associated with lower CML concentration. This high hemoglobin class was made up of 77.6% males. It is also likely that male gender influenced the direction of the association between SDB and CML concentration. Nevertheless, these results further strengthen the importance of the gender effect observed in table 7. The hemoglobin cutpoints in table 6 will need to be tested with a much larger sample such that each class has adequate representation of males and females. If significant associations disappear after equal representation of gender in the hemoglobin classes, then the 3-way interaction between gender, hemoglobin, and SDB (as done in table 7) will be the best approach for a study examining the relationship between SDB and CML concentration.

2.4.3 Strengths and Limitations

Strengths of this paper include firstly, the reliability of the data. This study was conducted in the cardiovascular health study (CHS) initiated by the National Heart, Lung and Blood Institute, and has extensive clinical data with participants coming from four

U.S communities. More than 1000 research papers have been published in peer reviewed journals since the cohort began giving credibility to the dataset and hence our findings. Secondly, our findings support the hypothesis that oxidative stress from SDB affects CML accumulation. Although the association between SDB and CML has not been studied, our findings are consistent with studies that investigated the effects of factors like smoking, physical activity, and hemoglobin on oxidative stress. Limitations of this study include its cross-sectional nature. Ethical reasons will not allow for a prospective study between SDB and CML concentration since SDB can be treated. However, a second or more CML measurements with time may help to retrospectively study longitudinal effects of SDB on CML accumulation. This study included only subjects with AHI records limiting our sample size to 1002. A larger sample may be instrumental in establishing the validity of the findings. Lastly, there are no studies on the association between SDB and CML for comparison. Availability of such a study could provide some useful clues on the best modelling approach for the association. Nevertheless, the approaches used in this study are consistent with published literature and would pave the way for future researchers.

2.4.4 Recommendations for Future Research

Future studies would be needed to further investigate the inverse relationship between SDB and CML accumulation that was observed at high hemoglobin levels among males. A larger sample size will be useful to confirm such an association. Also, it will be important to measure carboxyhemoglobin in males to verify if the interaction between hemoglobin and SDB persists and if SDB protects against increased CML concentration as was observed in this study at high hemoglobin levels. Lastly, future

studies to test the association between SDB and CML concentration at hemoglobin levels of <12.7 g/dl, 12.7-14.29 g/dl, and >14.29 g/dl, will be important in establishing the role of gender in the association between SDB and CML concentration.

2.5 CONCLUSION

CML levels have been linked to disease, particularly cardiovascular disease. Findings from this study have indicated that CML level depends on a complex interplay of factors such as gender, hemoglobin, and sleep disordered breathing. Sleep disordered breathing is characterized by production of free radicals and hemoglobin serves as the source of iron that catalyzes the reactions resulting in the increased CML concentration found at high hemoglobin levels. The protective effect of SDB found among males could be attributed to physical activity which is common among older men resulting in reduced oxidative stress and hence lower CML. Cigarette smoking among males has also been hypothesized to reduce oxidative stress because more carboxyhemoglobin is produced inhibiting the reactions of hemoglobin in promoting free radicals. These findings have public health implications because SDB can be treated and antioxidant defenses systems can be upregulated through physical activity to lower CML accumulation and hence reduce diseases that arise from oxidative damage.

Table 3: Characteristics of up to 1002 participants from the Cardiovascular Health Study by severity of SDB

Variable	N	AHI				F (P-value)
		Normal	Mild	Moderate	Severe	
Age (years), mean (s.d)	1002	77.2 (4.4)	77.7 (4.5)	77.7 (4.3)	77.8 (4.9)	2.3 (0.1318)
BMI (kg/m ²)	990	26.7 (4.3)	27.2 (4.2)	27.9 (4.2)	28.7 (5.5)	20.3 (<.0001)
Alcohol (GM)	988	3.4 (6.6)	5.2 (11.4)	4.6 (10.9)	4.5 (11.7)	2.5 (0.1159)
Cholesterol(mg/dl)	968	203 (36.5)	200 (41.0)	202 (40.9)	193.4(37.5)	3.0 (0.0843)
Adjusted LDL (mg/dl)	963	128 (34.1)	127 (34.4)	130 (29.9)	129.4(28.4)	0.4 (0.5504)
HDL (mg/dl)	982	55.1 (15.2)	53.2 (13.9)	51.2 (13.9)	51.1 (12.3)	10.9 (0.0010)
Triglyceride (mg/dl)	985	141 (79.8)	147 (100)	149 (88.1)	139.0(99.1)	0.11 (0.7401)
Hemoglobin (g/dl)	978	13.7(1.4)	13.7 (1.4)	13.9 (1.3)	14.0 (1.2)	3.3 (0.0686)
C-reactive protein (mg/L)	972	5.2 (9.9)	4.6 (8.9)	5.7 (10.2)	4.1 (4.6)	0.2 (0.6381)
eGFR-cysC	925	83.6(19.0)	83.7 (18.8)	78.0 (17.3)	79.4 (16.6)	8.7 (0.0033)
% Sleep time SaO ₂ < 90%	1002	1.8 (8.3)	3.8 (11.6)	6.7 (12.6)	13.3 (15.8)	79.7 (<.0001)
Arousal index all	959	17.2 (9.3)	19.2 (8.8)	25.0 (11.3)	36.5 (15.1)	235.1(<.0001)
Age	N (%)					X² (P-value)
<75	263	114 (28.8)	88 (24.9)	41 (25.3)	20 (22.0)	3.07 (0.8001)
75-79	455	174 (43.9)	160 (45.3)	75 (46.3)	46 (50.6)	
≥80	284	108 (27.3)	105 (29.8)	46 (28.4)	25 (27.5)	
Gender						
Male	429	130 (32.8)	157 (44.5)	85 (52.5)	57 (62.6)	37.3 (<.0001)
Female	573	266 (67.2)	196 (55.5)	77 (47.5)	34 (37.4)	
Race						
White	816	313 (79.0)	294 (83.3)	141 (87.0)	68 (74.7)	0.0330*
Black/AA	179	82 (20.7)	56 (15.9)	20 (12.4)	21 (23.1)	
Other	7	1 (0.3)	3 (0.8)	1 (0.6)	2 (2.2)	
BMI (kg/m²)						
Normal	311	147 (37.5)	101 (29.2)	41 (25.5)	22 (24.2)	20.9 (0.0019)
Overweight	464	175 (44.6)	173 (50.0)	78 (48.5)	38 (41.7)	
Obese	215	70 (17.9)	72 (20.8)	42 (26.0)	31 (34.1)	
Smoking Status						
Non-smoker	485	196 (49.5)	162 (46.4)	85 (52.5)	42 (46.7)	8.9 (0.1799)
Former smoker	449	169 (42.7)	170 (48.7)	71 (43.8)	39 (43.3)	
Current smoker	63	31 (7.8)	17 (4.9)	6 (3.7)	9 (10.0)	
Income						
<12,000	168	68 (18.0)	63 (18.8)	21 (13.8)	16 (19.3)	18.0 (0.1159)
12,000 - <25,000	333	145 (38.4)	98 (29.2)	61 (40.1)	29 (34.9)	
25,000 - <50,000	293	112 (29.6)	104 (30.9)	50 (32.8)	27 (32.5)	
50,000 - 75,000	89	28 (7.4)	45 (13.4)	9 (5.9)	7 (8.4)	
>75,000	66	25 (6.6)	26 (7.7)	11 (7.2)	4 (4.8)	

Table 3: Characteristics of up to 1002 participants from the Cardiovascular Health Study by severity of SDB (Continued)

Variable	N	Normal	Mild	AHI		Severe	X ² (P-value)
				Moderate			
Exercise Intensity							
No Exercise	71	25 (6.3)	32 (9.2)	7 (4.4)	7 (7.7)		10.3 (0.3263)
Low	388	149 (37.7)	148 (42.5)	57 (35.4)	34 (37.4)		
Moderate	393	161 (40.8)	122 (35.1)	71 (44.1)	39 (42.9)		
High	143	60 (15.2)	46 (13.2)	26 (16.2)	11 (12.1)		
Blood Pressure Status							
Normal	351	140 (35.5)	126 (35.8)	56 (34.6)	29 (31.9)		3.0 (0.8060)
Prehypertension	126	50 (12.7)	48 (13.6)	15 (9.3)	13 (14.3)		
Hypertension	522	204 (51.8)	178 (50.6)	91 (56.2)	49 (53.9)		
General Health Status							
Excellent	44	19 (4.8)	19 (5.4)	3 (1.9)	3 (3.3)		17.6 (0.1279)
Very Good	315	123 (31.1)	109 (31.0)	62 (38.3)	21 (23.1)		
Good	477	194 (49.0)	166 (47.3)	71 (43.8)	46 (50.5)		
Fair	153	56 (14.1)	56 (16.0)	23 (14.2)	18 (19.8)		
Poor	11	4 (1.0)	1 (0.3)	3 (1.8)	3 (3.3)		
MI Status							
No	904	370 (93.4)	314 (89.0)	141 (87.0)	79 (86.8)		8.3 (0.0395)
Yes	98	26 (6.6)	39 (11.0)	21 (13.0)	12 (13.2)		
CHD							
No	761	317 (80.0)	262 (74.2)	117 (72.2)	65 (71.4)		6.5 (0.0907)
Yes	241	79 (20.0)	91 (25.8)	45 (27.8)	26 (28.6)		
CHF							
No	934	375 (94.7)	327 (92.6)	153 (94.4)	79 (86.8)		7.8 (0.0493)
Yes	68	21 (5.30)	26 (7.4)	9 (5.6)	12 (13.2)		
Stroke							
No	962	380 (96.0)	339 (96.0)	158 (97.5)	85 (93.4)		0.4592*
Yes	40	16 (4.0)	14 (4.0)	4 (2.5)	6 (6.6)		
Claudication							
No	974	386 (97.5)	346 (98.0)	154 (95.1)	88 (96.7)		0.2724*
Yes	28	10 (2.5)	7 (2.0)	8 (4.9)	3 (3.3)		
Diabetes Status (ADA)							
Normal	754	316 (80.2)	260 (74.1)	111 (68.9)	67 (73.6)		10.2 (0.1159)
IFG	112	38 (9.6)	43 (12.2)	20 (12.4)	11 (12.1)		
Diabetic	131	40 (10.2)	48 (13.7)	30 (18.6)	13 (14.3)		
Insomnia							
No	797	310 (80.3)	275 (79.3)	133 (83.1)	79 (87.8)		4.0 (0.2643)
Yes	186	76 (19.7)	72 (20.7)	27 (16.9)	11 (12.2)		

AHI CUTPOINTS (events/hr of sleep): Normal (<5); Mild (5 to <15); Moderate (15 to 30); Severe (>30)

*P-value from Fisher's Exact Test.

Table 4: Characteristics of up to 1002 participants from the Cardiovascular Health Study by quartiles of CML

		CML					
Variable	N	Quartile 1	Quartile 2	Quartile 3	Quartile 4	F (P-value)	
Age (years), mean (s.d)	1002	76.3 (3.8)	77.5 (4.4)	77.9 (4.5)	78.4 (4.8)	29.2(<.0001	
BMI (kg/m ²)	990	28.5 (4.5)	27.5 (4.5)	26.5 (4.2)	26.4 (4.2)	35.7(<.0001	
Alcohol (GM)	988	5.5 (12.1)	4.5 (8.5)	3.3 (8.4)	4.0 (9.4)	4.6 (0.0327)	
Cholesterol (mg/dl)	968	205 (39.0)	200 (35.6)	199 (37.4)	200 (43.5)	2.2 (0.1426)	
Adjusted LDL (mg/dl)	963	129 (34.1)	127 (33.0)	128 (31.8)	128 (33.5)	0.12(0.7262)	
HDL (mg/dl)	982	52.1 (13.8)	52.6 (13.5)	53.3 (14.4)	55.7 (15.5)	8.0 (0.0047)	
Triglyceride (mg/dl)	985	157 (78.5)	140 (74.6)	143 (103.0)	136 (100.7)	5.9 (0.0151)	
Hemoglobin (g/dl)	978	14.0 (1.2)	13.9 (1.4)	13.8 (1.2)	13.5 (1.5)	19.3(<.0001	
C-reactive protein (mg/L)	972	4.6 (5.0)	5.3 (10.9)	5.9 (12.9)	4.2 (5.6)	0.1 (0.8282)	
eGFR-cysC	925	84.9 (17.3)	82.6 (17.9)	81.4 (18.5)	80.5 (20.3)	7.0 (0.0081)	
% Sleep time of SaO2 < 90%	1002	4.7 (10.9)	4.3 (11.2)	4.7 (12.5)	3.7 (11.5)	0.8 (0.3855)	
Arousal index all	959	21.9 (13.2)	21.3 (11.2)	20.2 (10.2)	20.1 (11.0)	4.0 (0.0473)	
Age	N (%)					X ² (P-value)	
<75	263	84 (33.5)	62 (24.8)	62 (24.8)	55 (21.9)		
75-79	455	121 (48.2)	118 (47.2)	109 (43.6)	107 (42.6)		
≥80	284	46 (18.3)	70 (28.0)	79 (31.6)	89 (35.5)	22.7(0.0009)	
Gender							
Male	429	96 (38.3)	113 (45.2)	115 (46.0)	105 (41.8)		
Female	573	155 (61.7)	137 (54.8)	135 (54.0)	146 (58.2)	3.9 (0.2776)	
Race							
White	816	209 (83.3)	199 (79.6)	208 (83.2)	200 (79.7)		
Black/AA	179	41 (16.3)	49 (19.6)	39 (15.6)	50 (19.9)		
Other	7	1 (0.4)	2 (0.8)	3 (1.2)	1 (0.4)	0.6931*	
BMI (kg/m ²)							
Normal	311	42 (16.9)	76 (30.9)	97 (39.3)	96 (38.5)		
Overweight	464	139 (56.1)	111 (45.1)	105 (42.5)	109 (43.8)		
Obese	215	67 (27.0)	59 (24.0)	45 (18.2)	44 (17.7)	37.3(<.0001	
Smoking Status							
Non-smoker	485	110 (44.0)	104 (41.9)	134 (53.8)	137 (54.8)		
Former smoker	449	116 (46.4)	128 (51.6)	98 (39.4)	107 (42.8)		
Current smoker	63	24 (9.6)	16 (6.5)	17 (6.8)	6 (2.4)	21.7 (0.0014	
Income							
<12,000	168	49 (20.6)	40 (17.3)	34 (14.1)	45 (18.8)		
12,000 - <25,000	333	84 (35.3)	73 (31.6)	94 (39.0)	82 (34.3)		
25,000 - <50,000	293	70 (29.4)	77 (33.3)	75 (31.1)	71 (29.7)		
50,000 - 75,000	89	23 (9.7)	22 (9.5)	22 (9.1)	22 (9.2)		
>75,000	66	12 (5.0)	19 (8.2)	16 (6.6)	19 (8.0)	7.9 (0.7924)	

Table 4: Characteristics of up to 1002 participants from the Cardiovascular Health Study by quartiles of CML (cont'd)

Variable	N	CML				X ² (P-value)
		Quartile 1	Quartile 2	Quartile 3	Quartile 4	
Exercise Intensity						
No Exercise	71	24 (9.6)	18 (7.3)	16 (6.4)	13 (5.2)	5.6 (0.7836)
Low	388	94 (37.8)	101 (40.9)	94 (37.8)	99 (39.6)	
Moderate	393	98 (39.4)	91 (36.8)	100 (40.2)	104 (41.6)	
High	143	33 (13.3)	37 (15.0)	39 (15.6)	34 (13.6)	
Blood Pressure Status						
Normal	351	88 (35.3)	97 (38.8)	88 (35.3)	78 (31.1)	6.8 (.3394)
Prehypertension	126	38 (15.3)	30 (12.0)	31 (12.5)	27 (10.8)	
Hypertension	522	123 (49.4)	123 (49.2)	130 (52.2)	146 (58.2)	
General Health Status						
Excellent	44	13 (5.2)	8 (3.2)	16 (6.4)	7 (2.8)	9.7 (0.6280)
Very Good	315	80 (31.9)	84 (33.7)	74 (29.7)	77 (30.7)	
Good	477	121 (48.2)	112 (45.0)	122 (49.0)	122 (48.6)	
Fair	153	35 (13.9)	43 (17.3)	35 (14.1)	40 (15.9)	
Poor	11	2 (0.8)	2 (0.8)	2 (0.8)	5 (2.0)	
MI Status						
No	904	225 (89.6)	227 (90.8)	229 (91.6)	223 (88.8)	1.3 (0.7367)
Yes	98	26 (10.4)	23 (9.2)	21 (8.4)	28 (11.2)	
CHD						
No	761	196 (78.1)	193 (77.2)	190 (76.0)	182 (72.5)	2.5 (0.4811)
Yes	241	55 (21.9)	57 (22.8)	60 (24.0)	69 (27.5)	
CHF						
No	934	238 (94.8)	235 (94.0)	231 (92.4)	230 (91.6)	2.5 (0.4714)
Yes	68	13 (5.2)	15 (6.0)	19 (7.6)	21 (8.4)	
Stroke						
No	962	244 (97.2)	238 (95.2)	239 (95.6)	241 (96.0)	1.5 (0.6863)
Yes	40	7 (2.8)	12 (4.8)	11 (4.4)	10 (4.0)	
Claudication						
No	974	243 (96.8)	244 (97.6)	247 (98.8)	240 (95.6)	5.0 (0.1751)
Yes	28	8 (3.2)	6 (2.4)	3 (1.2)	11 (4.4)	
Diabetes status (ADA)						
Normal	754	174 (69.3)	183 (73.5)	197 (79.8)	200 (80.0)	11.7(0.0678)
IFG	112	34 (13.6)	32 (12.9)	25 (10.1)	21 (8.4)	
Diabetic	131	43 (17.1)	34 (13.7)	25 (10.1)	29 (11.6)	
Insomnia						
No	797	194 (79.8)	194 (79.5)	201 (81.7)	208 (83.2)	1.4 (0.6976)
Yes	186	49 (20.2)	50 (20.5)	45 (18.3)	42 (16.8)	

LOG(CML) Quartiles (ng/ml): (1) ≤6.1972; (2) >6.1972-6.3599; (3) 6.3599-6.5341; (4) >6.5341

*P-value from Fisher's Exact Test;

Table 5: The effect of SDB (AHI) on CML (N=1002)

Parameter	N	Estimate	Standard Error	t Value	Pr > t
AHI (events/hr):		-0.0003	0.0007	-0.32	0.7218
AHI (events/hr):		-0.0004	0.0008	-0.56	0.5770
SDB Severity: Model 1					
Normal	396	Referent			
Mild	353	0.0209	0.0205	1.02	0.3080
Moderate	162	0.0040	0.0260	0.15	0.8769
Severe	91	0.0089	0.0325	0.27	0.7842
SDB Severity: Model 2					
Normal	396	Referent			
Mild	353	0.0314	0.0214	1.47	0.1425
Moderate	162	0.0040	0.0275	0.15	0.8842
Severe	91	0.0004	0.0348	0.01	0.9915

Model 1 was unadjusted; **Model 2** adjusted for age, gender, BMI, smoking, alcohol, HDL, C-reactive protein, hemoglobin, eGFR-Cystatin C, CHD, MI, Claudication, Stroke, diabetes, hypertension, race.

Table 6: Interaction checks and model selection

Model #	Model	DF	LR	P-	Comments
1	Main Effects (No interaction)	24	319.4	-	
2	A*H	27	333.1	0.0033	b/n model 2 & 1
3	A*G	27	327.1	0.0526	b/n 3 & 1
4	H*G	25	324.9	0.0190	b/n 4 & 1
5	(A*H) (A*G)	28	339.2	0.0135	b/n 5 & 2
6	(A*H) (A*G) (H*G)	29	344.6	0.0201	b/n 6 & 5
7	(A*H) (A*G) (H*G) (A*H*G)	32	354.2	0.0223	b/n 7 & 6

A=Apnea-Hypopnea Index; **H**=Hemoglobin; **G**=Gender

Table 7: The effect of SDB (AHI) on CML stratified by GENDER and HEMOGLOBIN concentration

	HEMOGLOBIN								
	<13.20			13.20 – 14.29			>14.29		
	N	β	P-value	N	β	P-value	N	β	P-value
ALL (N=978)									
SDB Severity:									
Normal (n=389)	138	Ref		132	Ref		119	Ref	
Mild (n=340)	111	0.049	0.261	124	0.037	0.220	105	0.016	0.676
Moderate(n=158)	43	0.021	0.723	58	0.033	0.387	57	-0.039	0.401
Severe (n=91)	24	0.054	0.484	31	0.026	0.619	36	-0.062	0.271
MALES (N=423)									
SDB Severity:									
Normal (n=129)	23	Ref		40	Ref		66	Ref	
Mild (n=154)	19	-0.082	0.588	51	0.068	0.166	84	-0.067	0.166
Moderate (n=83)	8	-0.075	0.679	31	0.104	0.086	44	-0.084	0.152
Severe (n=57)	8	0.010	0.962	18	0.038	0.606	31	-0.161	0.0146
FEMALES (N=555)									
SDB Severity:									
Normal (n=260)	115	Ref		92	Ref		53	Ref	
Mild (n=186)	92	0.055	0.249	73	0.041	0.328	21	0.189	0.0061
Moderate (n=75)	35	0.023	0.729	27	-0.009	0.864	13	0.050	0.553
Severe (n=34)	16	0.063	0.480	13	-0.013	0.877	5	0.281	0.0336

Models were adjusted for age, BMI, smoking, alcohol, HDL, C-reactive protein, eGFR-Cystatin C, CHD, MI, Claudication, Stroke, diabetes, hypertension, race.

Ref=Referent

Table 8: The effect of SDB (AHI) on CML by HEMOGLOBIN concentration

Parameter	N	Estimate	Standard Error	t Value	Pr > t
HEMOGLOBIN CLASS 1 (<12.7 g/dl)	185				
SDB Severity:					
Normal	78	Referent			
Mild	67	0.029	0.058	0.51	0.614
Moderate	28	0.009	0.076	0.12	0.903
Severe	12	0.019	0.108	0.18	0.858
HEMOGLOBIN CLASS 2 (12.7 – 14.59)	552				
SDB Severity:					
Normal	221	Referent			
Mild	195	0.0591	0.026	2.29	0.0223
Moderate	83	0.030	0.034	0.89	0.376
Severe	53	0.0909	0.042	2.15	0.0318
HEMOGLOBIN CLASS 3 ($\geq$14.6 g/dl)	241				
SDB Severity:					
Normal	90	Referent			
Mild	78	-0.025	0.043	-0.57	0.569
Moderate	47	-0.039	0.053	-0.74	0.458
Severe	26	-0.1381	0.065	-2.11	0.0364

Models were adjusted for age, BMI, gender, smoking, alcohol, HDL, C-reactive protein, eGFR-Cystatin C, CHD, MI, Claudication, Stroke, diabetes, hypertension, race.

CHAPTER THREE

THE JOINT EFFECT OF OBESITY AND SLEEP INADEQUACY ON RISK OF TYPE-2 DIABETES

3.1 INTRODUCTION

Diabetes is the seventh leading cause of death in the US affecting 25.8 million people (8.3% of the U.S population)¹²⁸. It is a major risk factor for hypertension, heart disease and stroke. Co-morbid effects of diabetes include kidney disease, blindness, nervous system damage, and too commonly nontraumatic lower-limb amputation. Several epidemiological studies have indicated an association between poor patterns of sleep and diabetes risk but the results have not been conclusive¹²⁹⁻¹³⁴. Moreover, sleep duration and sleep quality have often been analyzed separately in both longitudinal and cross-sectional studies, and only very few studies have incorporated aspects of both sleep duration and quality into their analyses^{135, 136}. Sleep duration is measured by the number of hours a person sleeps in a day, and does not take into account the nature of the sleep. Sleep quality on the other hand describes the total sleep pattern of an individual. Major factors deleterious to sleep quality include sleep apnea, anxiety (depression), insomnia, snoring, and medical conditions such as COPD, heartburn, lung problems, chronic pain,

and enlarged prostate¹³⁷⁻¹⁴⁰. These factors can lead to difficulty either in initiating sleep or in maintaining sleep^{141, 142}.

Obesity plays an important role in the association between sleep and diabetes. Obesity prevalence is known to be higher among those with short sleep duration¹⁴³⁻¹⁴⁶, and obesity is also known to elevate the risk of diabetes^{147, 148}. Sleep problems such as obstructive sleep apnea (OSA) and snoring have also been found to increase the risk of diabetes independent of obesity¹⁴⁹, making it unclear what role obesity plays in the association between sleep and diabetes. Previous studies have generally adjusted for obesity or BMI but have not specifically investigated the potential complex role obesity plays in the relationship between sleep insufficiency and diabetes^{129, 134, 150}. It is possible that associations between sleep and diabetes are modified or confounded by obesity.

Furthermore, sleep disorders, obesity, and diabetes are all entangled in web of oxidative stress. It is however not clear whether it is the oxidative stress from sleep-disordered breathing that contributes to the development of diabetes, or if sleep problems are the results of diabetes. The association gets even more complicated in the presence of obesity. This study investigates the joint effect of sleep and obesity on the risk of diabetes with the aim of identifying relationships that may be useful in explaining some of the complication and provide basis for sound public health practice.

3.1.1 Objectives

1. To investigate the association between sleep and diabetes using sleep quantity and quality measures
2. To investigate the effect of possible interaction between sleep quantity and sleep quality on the diabetes
3. To investigate the joint effect of sleep and obesity on the risk of diabetes

3.1.2 Hypothesis

We hypothesize that sleep quantity and sleep quality are independently associated with the risk of diabetes and their interaction with obesity exacerbates the risk.

3.1.3 Justification

Sleep duration among Americans has been decreasing since the industrial revolution. Type-2 diabetes, a major risk factor for cardiovascular disease (CVD), has also been on the rise during the same period. Obesity, a risk factor for type-2 diabetes and CVD, has now reached epidemic level in the United States raising health concerns for this and future generations. Apart from physical activity and dietary recommendations which are yet to have full impacts, the search for opportunities to reduce type-diabetes and CVD is an ongoing process. Understanding the relationship between sleep problems and type-2 diabetes and how obesity changes the dynamics could fill the gaps in knowledge of type-2 diabetes and move us closer to ensuring a healthy nation.

3.2.0 METHODS

3.2.1 Study Design: Prospective cohort study of the First National Health and Nutrition Examination Survey (NHANES 1).

3.2.2 Study Participants: Participants were selected from the Epidemiologic Follow-up Study of the First National Health and Nutrition Examination Survey (NHANES 1) between 1982 and 1992. NHANES is a survey designed to assess the health and nutritional status of the non-institutionalized population of the United States¹⁵¹. There were a total of 12,220 records at baseline (1982-1984) which indicated a response to at least one of the questions on sleep quality or quantity. However, among these 12,220 records are included some responses collected by proxy for individuals deceased between 1982 and 1984, and since this is a longitudinal study, only participants who were alive at baseline (n=10,523) were included. Further exclusions include participants who were diabetic at baseline (n=888). This resulted in sample size of 9635.

3.2.3 Exposure Assessment

The following questions were used to assess sleep quality: “(1) how often do you have trouble falling asleep? (2) how often do you have trouble with waking up during the night? (3) how often do you have trouble waking up too early and not being able to fall asleep? (4) how often do you get so sleepy during the day or evening that you have to take a nap?”. Responses to each of these questions were based on a 5-point scale (“Never”, “Rarely”, “Sometimes”, “Often”, and “Almost always”). These responses were summed up across the 4 sleep problem items to form the sleep quality variable. Our

preliminary analyses did not show significant differences between responses “Never” and “Rarely” and diabetes and between “Often”, and “Almost always” and diabetes. Hence to ensure adequate sample size in our response categories for valid results these categories were combined. The resulting sleep quality classes are: (1) “good sleep” (subjects who “never” or “rarely” have sleep problems); (2) “moderate sleep” (subjects who “sometimes” have sleep problems); (3) “poor sleep” (subjects who “often”, or “almost always” have sleep problems). A sleep quantity indicator was created based on a single question, “how many hours of sleep do you usually get per night?” The sleep quantity indicator was categorized as <7 hours of sleep, 7 to 9 hours of sleep, and >9 hours of sleep.

3.2.4 Diabetes Assessment

Incidence of diabetes from baseline (1982-1984) to 1992 was ascertained from self-reports by subjects (physician diagnosed), and from primary recorded cause of death (ICD-9: 250-250.93) for those who were deceased during follow-up, but did not report a diagnosis of diabetes prior to death.

3.2.5 Assessment of Covariates

Age of subjects at baseline was categorized into 4 groups (<45, 45-54, 55-68, and >68). The cut-points were set to ensure an adequate number of subjects in all classes, and to take into account the effect of age on both sleep and diabetes. Race/ethnicity was categorized as “white”, “black” or “other”, where “other” includes Asian or Pacific

Islanders, American Indians, and Eskimos. BMI was categorized as “normal” (<25.0), “overweight” ($25-29.9$), and “obese” (≥ 30). Blood pressure was categorized as “normal”, “prehypertension”, and “hypertension” according to the Seventh report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure (JNC VII) criteria ¹⁵². Self-reported cigarette smoking was classified as “never smoker”, “former smoker”, and “current smoker”, while self-reported alcohol consumption was classified as “abstainer”, “light drinker”, “moderate drinker”, and “heavy drinker”. Self-reported physical activity was classified as “very active”, “moderately active”, and “quite inactive”, and educational status will be categorized as “<high school”, “high school”, and “> high school”. Self-reported coffee drinking and depression were assessed as “yes or no”.

3.2.6 Statistical Analyses

Chi-square tests were used to test whether categorical variables differed across the levels of sleep quantity and quality. Associations between continuous covariates with sleep quantity and quality were estimated using analysis of covariance. Lastly, Cox proportional hazards models were used to estimate the association between sleep quantity and sleep quality and time to onset of diabetes in the study sample. Various models were constructed: Model 1 was unadjusted, Model 2 adjusted for age, and Model 3 adjusted for Age, BMI, Sex, Race, Hypertension, Cigarette Smoking, Alcohol Consumption, Depression, Coffee Drinking, Physical Activity, and Education. Model variables were selected because they are known to influence both sleep and diabetes. A final model included an interaction term for BMI and sleep (quantity and quantity). A likelihood ratio

test was used to check for possible interaction between BMI and sleep. A significance level of 0.05 was used to determine all significant associations. All analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC).

3.2.7 Power Calculation (Sleep Inadequacy and Type-2 Diabetes)

3.2.8 Prevalence of sleep problems in the U.S population

An estimated 70 million Americans suffer sleep problems (that is, about 23% of the U.S population by 2010 census)¹⁵³. This include sleep restriction, primary sleep disorders (various forms of sleep disorders affecting people of all ages) , and secondary sleep disorders (common in people with a chronic disease, or an alcohol or substance abuse disorder, accompanied with poor sleep quality and excessive daytime sleepiness). Of 9284 subjects who self-reported their sleep quality, 39.9% of them reported poor sleep quality. This gives an exposed group of 3640 and unexposed group of 5644 in terms of sleep quality.

3.2.9 Prevalence of type-2 diabetes among exposed and unexposed groups

11.3% of Americans ≥ 20 years have diabetes¹⁵⁴. In this study however, the proportion of subjects with poor sleep quality who are diabetic is 6.3%, and the proportion of subjects with good or moderate sleep who are diabetic is 4.1%. Assuming 95% confidence level, the power of the study is more than 80% as shown in table 9.

Table 9: Power calculation for the association between sleep inadequacy and type-2-diabetes

Power for Cohort Studies (Sleep Inadequacy and Type-2-Diabetes)		
	Exposed Group	Unexposed Group
Sample Size	3640	5644
Prevalence (or) Coverage (%)	6.3	4.1
Power based on:		
Normal approximation		99.75%
Normal approximation with continuity correction		99.71%

3.3 RESULTS

Over the 10 year follow-up period, there were 465 incident cases of diabetes (4.8%). The mean age and BMI of study subjects were 56 years and 26.0, respectively. Females formed 62.8% of the study sample and most of the subjects were white (86.8%). 53.8% of the subjects were either overweight or obese and 71.1% were either prehypertensive or hypertensive. Current cigarette smokers form 28.4% of the sample and moderate to heavy drinkers form 11.6%. The study sample was also characterized by high school education or more (63.1%), and physically very active individuals form 28.2%. Subjects who had an income of \$25,000 or more form 36.9% of the sample, coffee drinkers 86.2%, and depressed subjects 2.5%.

Covariates more likely to lead to poor sleep quality were increasing age, overweight/obesity, white race, female sex, high blood pressure, depression, less than

\$25,000 of income, physical inactivity, and less than high school educational status. On the other hand, current cigarette smoking and light to moderate alcohol consumption appear to promote good sleep quality [Table 10]. Sleep quality and quantity were treated in this study as independent predictors of diabetes because a test of interaction between them did not reach statistical significance ($p=0.3751$). Results from the Cox proportional hazards model showed that poor sleep quality was significantly associated with diabetes in the unadjusted model ($HR=1.63$, $95\%CI=1.26-2.10$). The association remained significant in the age-adjusted ($HR=1.36$, $95\% CI=1.04-1.76$) and multivariable adjusted ($HR=1.47$, $95\%CI=1.09-1.99$) models. Compared with 7 to 9 hours of sleep, subjects who slept for less than 7 hours and those who slept for more than 9 hours are significantly at risk of diabetes in unadjusted and age adjusted models. The association however did not reach statistical significance when all relevant covariates were adjusted for [Table 12].

A joint effect of BMI and sleep quantity on diabetes was considered but was not significant ($p=0.5879$). A significant interaction effect between BMI and sleep quality on diabetes however was found ($p=0.0151$). It was found that obese subjects who have poor sleep quality were at a much greater risk of diabetes compared to those with normal BMI and good sleep patterns ($HR=31.11$, $95\%CI=11.37 - 85.17$) after adjusting for age, sex, race, hypertension, cigarette smoking, alcohol consumption, depression, coffee drinking, physical activity, educational status, and sleep quantity [Table 13]. In order to find absolute risk estimates for the effect of sleep quality on diabetes, 1 unit change in sleep quality was evaluated within each category of BMI. Within the obese BMI category 1 unit change in sleep quality was associated with 1.270 (1.037-1.556) risk of diabetes; 1

unit change in sleep quality does not appear to have an effect on diabetes among overweight subjects (HR=0.996; 95%CI=0.795-1.248); and within the normal BMI category, 1 unit change in sleep quality was associated with 1.557 (1.107 – 2.191) risk of diabetes [Table 14].

3.4 DISCUSSION

Previous investigations of the effect of sleep duration on diabetes risk have found U-shaped relationships; with too little or too much sleep leading to an increased risk of diabetes. The mechanisms by which sleep deprivation could contribute to diabetes have been established but little is known about how excess sleep could increase the risk of diabetes. Possible explanations for the association between excess sleep and diabetes include: (1) other medical conditions associated with diabetes resulting in longer time in bed, or (2) less physical activity due to more time spent in bed. The effect of sleep quantity on diabetes has demonstrated this U-shaped relationship with <7 hours and >9 hours of sleep having higher risk estimates compared to the reference group 7-9 hours of sleep [Table 12]. In our multivariable model without considering interactions, we found elevated risks for diabetes for both short (HR=1.06; 95% CI=0.85-1.32) and long sleep (HR=1.46; 95%CI=0.93-2.30) when compared with the referent (7-9 hours), although these were not significant after adjusting for confounding factors [Table 12].

Gangwisch et al¹²⁹ found an odds ratio of 1.47 (1.03-2.09) for the effect of sleep duration of ≤ 5 hours on diabetes, and 1.52 (1.06-2.17) for the effect of ≥ 9 hours of sleep on diabetes, with 7 hours of sleep as the reference group. The main difference between

the effect estimates of sleep duration from this study and that of Gangwisch et al., is that whilst Gangwisch et al., made 7 hours as their reference group and estimated risks for ≤ 5 , 6, 8, and ≥ 9 hours of sleep, this study estimated the effects for <7 and >9 hours of sleep with 7-9 hours of sleep as referent. This was done to conform to recommendations by the National Sleep Foundation (NSF) that adults need 7-9 hours of sleep per night for proper functioning. Therefore estimating risk for sleep quantity below and above the recommended amount could be easily interpreted within the guidelines of the NSF. Rafalson et al.,¹⁵⁵ in a nested case-control study, found a threefold increased risk of diabetes (OR=3.0; 95%CI=1.05 - 8.59) for sleep duration <6.0 hrs compared to 6 to 8 hrs. As already explained, the difference in estimates may be due to differences in the sleep duration classifications adopted. Longitudinal studies by Yaggi et al.,¹³², Ayas et al.,¹⁵⁶ and Tuomilehto et al.,¹⁵⁷ did not find significant associations between short sleep duration and diabetes. Since ours was also a longitudinal analysis, it raises questions as to which study design best estimates the effect of sleep duration on diabetes.

Longitudinal studies on sleep quality and diabetes have received much attention because many of them found significant risk estimates. Kawakami et al.,¹⁵⁸ found a threefold increased risk of diabetes (HR=2.98; 95%CI=1.36 – 6.53) among subjects who have “high” compared to “low” difficulty initiating sleep. They also found that “high” difficulty maintaining sleep doubles the risk of diabetes compared to “low” (HR=2.23; 95%CI=1.08 – 4.61). In our fully adjusted model, poor sleep quality significantly increased the risk of diabetes (HR=1.47; 95%CI=1.09-1.99). Our effect estimate was different than those reported by Kawakami et al., and may be due to the fact that they studied only males and did not adjust for number of hours of sleep. Moreover, our sleep

quality variable was formed from four different questionnaire items whilst estimates by Kawakami et al., were based on either difficulty initiating sleep or difficulty maintaining sleep. Meisinger et al.,¹³³ also found “difficulty maintaining sleep” to be significantly associated with diabetes (Males: HR=1.60, 95%CI=1.05 – 2.45; Females: HR=1.98, 95% CI=1.20 – 3.29). Meisinger et al., did not consider duration of sleep and males and females were analyzed separately, making their estimates to be slightly different from what we observed.

Sleep quality issues can be attributed to many factors including but not limited to depression, excessive alcohol consumption, physical inactivity, and sleep apnea. These factors could independently or in combination disrupt sleep and contribute to diabetes. Longitudinal studies by Marshall et al.,¹⁵⁰ Botros et al.,¹³⁰ and Celen et al.,¹³¹ have all found significant associations between obstructive sleep apnea and diabetes while Al-Delaimy et al.,¹⁵⁹ found that occasional and regular snoring increased the risk of diabetes. Our estimate is similar to those of Botros et al., (HR= 1.43, 95%CI: 1.10-1.86) and Al-Delaimy et al., (occasional snoring: RR=1.41, 95% CI: 1.22 - 1.63; regular snoring RR=2.03, 95%CI: 1.71 - 2.40), but very different from those by Marshall et al., (moderate- to- severe OSA: OR= 13.45, 95% CI=1.59 - 114.11) and Celen et al., (OSA in women; OR=11.78, 95%CI: 1.14-121.7). Marshall et al., had only 9 incident diabetes cases out of 295 subjects over a 4 year period which explains their wide confidence interval, and Celen et al., had only 31 women involved in their study out of which 7 had diabetes during follow-up leading to a wide confidence interval as well. The longitudinal study by Bjorkelund et al.,¹³⁵ combined some components of sleep duration and quality. Their risk estimate however was insignificant although similar to ours in

magnitude (HR=1.35; 95%CI= 0.89 – 2.10). Bjorkelund et al., combined three items (1) sleep complaints, (2) use of sleeping pills, and (3) sleep <6h in 24hrs, and categorized the composite sleep problem variable as “yes” or “no”. This dichotomization may cause loss of vital information rendering the statistical test insignificant.

While risk estimates from longitudinal studies remain conflicting, those from cross-sectional studies have been consistent. Cross-sectional studies by Kachi et al.,¹⁶⁰, Lou et al.,¹⁶¹, Chao et al.,¹⁶², Hsieh et al.,¹⁶³, Tuomilehto et al.,¹⁶⁴, Chaput et al.,¹⁶⁵, and Gottlieb et al.,¹⁶⁶ have all found significant associations between short sleep duration and risk of diabetes. Cross-sectional studies measure both exposure and outcome at the same time and it may be possible that the short sleep duration was as a result of some other health condition or even as a result of diabetes. In addition, these studies estimated only the effect of short sleep duration on diabetes without considering the effect of sleep quality. In contrast, the cross-sectional study by Jongoh Kim et al.,¹⁶⁷ did not find any association between sleep duration and diabetes in the entire study sample of 1,652 Korean men. However, after stratification by abdominal obesity, they did find an increased risk of diabetes among 1,241 of the men without abdominal obesity and who sleep duration of ≤ 5 hours per day. We did not find significant association between sleep duration and diabetes and we did not perform stratified analyses of sleep duration by BMI because our test for interaction between sleep duration and BMI was not significant. However, the pattern is similar if short sleep duration is taken to be a proxy for poor sleep quality.

Cross-sectional studies by Ronksley et al.,¹⁶⁸ and Reichmuth et al.,¹⁶⁹ found that sleep apnea significantly increased the risk of diabetes in both males and females. This is

consistent with findings from our longitudinal analysis that poor sleep quality significantly increased the risk of diabetes. The cross-sectional study by Vgontzas et al.,¹³⁶ did not find individual associations between insomnia and diabetes, or sleep duration and diabetes, but after examining the joint effects of insomnia and sleep duration, they found that compared to normal sleeping subjects (sleep duration of >6 hours), those with insomnia and with short sleep duration (<5 hours) were at increased risk of diabetes. Our sleep quality variable was composed of items other than insomnia and although we did not stratify on number of hours of sleep, we found a significant relationship between poor sleep quality and diabetes after adjusting for confounders including sleep duration. Mahmood et al.,¹³⁴ however did not find significant association between obstructive sleep apnea and diabetes after adjusting for confounding variables. The study by Mahmood et al., was predominantly African Americans (67%), whereas our study was predominantly Caucasian (87%). This vast difference in ethnicity between the two studies could be a reason for differences in effect estimates. Most of the cross-sectional associations have been consistent but they cannot show a causal direction. We have found in our prospective study a risk estimate of 1.47 for the effect of poor sleep quality on diabetes risk, and it is statistically significant.

Obesity is a known risk factor for diabetes, but the effect of its interaction with sleep on diabetes as reported here has not been specifically investigated and reported. We found 31 fold increased risk of diabetes among obese subjects with poor sleep quality when compared to normal weight subjects with good sleep quality [Table 13]. The hazard ratio for the effect of poor sleep quality on diabetes was only 1.47 [Table 12] when BMI served as confounder. Effect sizes of around 1.47 have been common because researchers

often treat BMI as a confounding variable which makes it impossible for the full public health impact of sleep and obesity on diabetes to be assessed. Poor sleep quality could cause changes in appetite regulating hormones, leptin and ghrelin, which may lead to weight gain and obesity, and subsequently diabetes¹⁴⁸. Other mechanisms by which poor quality sleep increases diabetes risk include overactivity of the sympathetic nervous system, increased inflammation, elevated evening cortisol levels, all of which leads to reduced insulin sensitivity and diabetes. Poor sleep has also been proposed to result in reduced energy expenditure which could also affect weight status and ultimately diabetes¹⁴⁹. Among normal BMI subjects 1 unit change in sleep quality (good-moderate-poor) was associated with 1.557 (1.107-2.191) risk of diabetes. Among obese subjects 1 unit change in sleep quality was associated with 1.270 (1.037-1.556) risk of diabetes. Sleep quality did not appear to influence the risk of diabetes among overweight subjects [Table 14]. In other words overweight subjects have a baseline risk of diabetes which is higher than that of normal weight subjects [Table 13] but once that point is reached then the effect of sleep does not seem to matter.

We were able to show in this study that sleep duration and sleep quality have independent influences on diabetes risk. Furthermore, we found that BMI modify the effect on sleep quality on diabetes. The prospective nature of the study also enhances our analysis because we could establish temporality. Only subjects who were alive at the start of the study were included to eliminate errors that arise from proxy responses. Our study has limitations as well. All the information provided in this document on sleep was self-reported by subjects and could potentially bias the findings; a sleep study on the subjects can solve this problem. However it has been shown that the use of self-reported sleep,

characterized by the dimensions used in this study, provides just as good a measure as when monitored ¹⁷⁰. Also, only a single record of sleep documented at baseline (1982-1984) was used, instead of multiple records. Sleep habits may change over time and cause misclassification of subjects on the exposure. However, it is not likely these misclassifications would change findings significantly because they would be non-differential.

3.5 CONCLUSION

Poor sleep quality has been found to significantly increase the risk of diabetes in the NHANES 1 Epidemiologic Follow-up Study. When the joint effect of obesity was considered, the effect of sleep quality on diabetes was found to be exacerbated among normal weight subjects. Obese subjects already have higher baseline risk of type-2-diabetes and although sleep quality does matter, the difference was not as large as found among normal weight subjects. Sleep cycles influence hormone levels, such as cortisol and growth hormone, as well as inflammation processes evidenced by effects on markers of inflammation, such as TNF- α and IL-6. Therefore, poor quality sleep could disrupt hormone and inflammation responses, so that poor sleep over time may lead to impaired glucose tolerance and diabetes. Poor sleep patterns also influences energy expenditure, in part through the disruption of hunger suppressing and stimulating hormones which are also thought to be contributing factors to obesity and hence diabetes. The risk estimates of interaction between poor sleep quality and BMI found in this study warrants some attention. Thus, we recommend weight management efforts to be intensified and sleep

problems reduced with every available resource in order to reduce the incidence of diabetes. Additional studies are needed to examine the effect of sleep on diabetes risk among overweight individuals and to explain possible biologic mechanisms why sleep quality may not be an important risk factor for diabetes among overweight individuals.

Table 10: Baseline Characteristics by sleep quality

Baseline Characteristics	N	Sleep Quality			F (P-value)
		Good N=2180	Moderate N=3464	Poor N=3640	
Age, y: Mean (SD)	9284	51.0 (13.3)	54.2 (14.0)	60.9 (14.6)	743.04 (<.0001)
BMI: Mean (SD)	9129	26.0 (4.8)	26.0 (4.8)	26.1 (4.9)	1.51 (0.2195)
Age Category, n(%)					X²(P-value)
<45	2579	887 (34.4)	1085 (42.1)	607 (23.5)	699.36(<.0001)
45-54	2213	564 (25.5)	881 (39.8)	768 (34.7)	
55-68	2194	430 (19.6)	828 (37.7)	936 (42.7)	
>68	2298	299 (13.0)	670 (29.2)	1329 (57.8)	
BMI Category, n(%)					9.13 (0.0580)
<25.0	4217	1020 (24.2)	1617 (38.3)	1580 (37.5)	
25-29.9	3270	771 (23.6)	1193 (36.5)	1306 (39.9)	
>29.9	1642	366 (22.3)	600 (36.5)	676 (41.2)	
RACE					43.23 (<.0001)
White	8063	1811 (22.5)	3021 (37.5)	3231 (40.1)	
Black	1132	345 (30.5)	402 (35.5)	385 (34.0)	
Other	89	24 (27.0)	41 (46.1)	24 (27.0)	
SEX					42.95 (<.0001)
Male	3457	939 (27.2)	1252 (36.2)	1266 (36.6)	
Female	5827	1241 (21.3)	2212 (38.0)	2374 (40.7)	
HYPERTENSION					112.26 (<.0001)
Normal	2601	744 (28.6)	1038 (39.9)	819 (31.5)	
Prehypertensive	3645	836 (22.9)	1365 (37.5)	1444 (39.6)	
Hypertensive	2756	552 (20.0)	971 (35.2)	1233 (44.7)	
SMOKING					26.47 (<.0001)
Never	4183	950 (22.7)	1567 (37.5)	1666 (39.8)	
Former	2464	545 (22.1)	884 (35.9)	1035 (42.0)	
Current	2636	685 (26.0)	1013 (38.4)	938 (35.6)	
ALCOHOL					55.07 (<.0001)
Abstainer	3902	827 (21.2)	1382 (35.4)	1693 (43.4)	
Light Drinker	4282	1077 (25.2)	1647 (38.5)	1558 (36.4)	
Moderate Drinker	999	251 (25.1)	403 (40.3)	345 (34.5)	
Heavy Drinker	76	18 (23.7)	25 (32.9)	33 (43.4)	
PHYSICAL ACTIVITY					186.27 (<.0001)
Very Active	2601	773 (29.7)	979 (37.6)	849 (32.6)	
Moderately Active	5148	1135 (22.1)	2023 (39.3)	1990 (38.7)	
Quite Inactive	1488	264 (17.7)	449 (30.2)	775 (52.1)	
COFFEE					5.26 (0.0721)
Yes	7998	1865 (23.3)	2961 (37.0)	3172 (39.7)	
No	1276	314 (24.6)	499 (39.1)	463 (36.3)	

Table 10: Baseline Characteristics by sleep quality (Continued)

Baseline Characteristics	N	<u>Sleep Quality</u>			X ² (P-value)
		Good N=2180	Moderate N=3464	Poor N=3640	
DEPRESSION					
Yes	229	26 (11.4)	62 (27.1)	141 (61.6)	51.33
No	8973	2138 (23.8)	3369 (37.6)	3466 (38.6)	
INCOME, \$					
<10000	2389	369 (15.5)	810 (33.9)	1210 (50.6)	305.14
10,000-24,999	3036	733 (24.1)	1088 (35.8)	1215 (40.0)	
25,000-49,000	2374	699 (29.4)	982 (41.4)	693 (29.2)	
>50,000	805	250 (31.1)	323 (40.1)	232 (28.8)	
EDUCATION					
<HS	3404	653 (19.2)	1147 (33.7)	1604 (47.1)	157.52
HS	3456	862 (24.9)	1352 (39.1)	1242 (35.9)	
>HS	2372	656 (27.7)	945 (39.8)	771 (32.5)	
DIABETES					
Yes	447	82 (18.3)	150 (33.6)	215 (48.1)	16.52
No	8837	2098 (23.7)	3314 (37.5)	3425 (38.8)	
SLEEP QUANTITY (hrs)					
<7	6095	1592 (26.1)	2545 (41.8)	1958 (32.1)	393.0
7-9	2599	453 (17.4)	729 (28.1)	1417 (54.5)	
>9	293	63 (21.5)	91 (31.1)	139 (47.4)	

Table 11: Baseline characteristics by sleep quantity

Baseline Characteristics	N	Sleep Quantity (hrs)			F (P-value)
		<7 N=2599	7-9 N=6096	>9 N=293	
Age, y: Mean (SD)	8988	56.0 (14.5)	55.6 (14.5)	66.7 (15.0)	64.94 (<.0001)
BMI: Mean (SD)	8842	26.5 (5.0)	25.9 (4.8)	26.1 (5.1)	22.45 (<.0001)
Age Category, n(%)					X²(P-value)
<45	2491	699 (28.1)	1756 (70.5)	36 (1.4)	161.54
45-54	2149	699 (31.1)	1447 (67.3)	33 (1.6)	
55-68	2118	598 (28.2)	1456 (68.8)	64 (3.0)	
>68	2230	633 (28.4)	1437 (64.4)	160 (7.2)	
BMI Category, n(%)					28.14 (<.0001)
<25.0	4092	1084 (26.5)	2884 (70.5)	124 (3.0)	
25-29.9	3158	942 (29.8)	2114 (67.0)	102 (3.2)	
>29.9	1592	528 (33.2)	1013 (63.6)	51 (3.2)	
RACE					67.04 (<.0001)
White	7834	2181 (27.8)	5421 (69.2)	232 (3.0)	
Black	1067	383 (35.8)	624 (58.5)	61 (5.7)	
Other	87	36 (41.4)	51 (58.6)	-	
SEX					4.41 (0.1102)
Male	3334	972 (29.1)	2237 (67.1)	125 (3.8)	
Female	5654	1627 (28.8)	3859 (68.2)	168 (3.0)	
HYPERTENSION					51.13 (<.0001)
Normal	2527	637 (25.2)	1834 (72.6)	56 (2.2)	
Prehypertensive	3535	1068 (30.2)	2366 (66.9)	101 (2.9)	
Hypertensive	2666	811 (30.4)	1735 (65.1)	120 (4.5)	
SMOKING					22.31 (0.0002)
Never	4067	1093 (26.9)	2823 (69.4)	151 (3.7)	
Former	2377	697 (29.3)	1606 (67.6)	74 (3.1)	
Current	2543	809 (31.8)	1666 (65.5)	68 (2.7)	
ALCOHOL					44.0 (<.0001)
Abstainer	3814	1074 (28.2)	2565 (67.2)	175 (4.6)	
Light Drinker	4122	1207 (29.3)	2822 (68.4)	93 (2.3)	
Moderate Drinker	954	283 (29.7)	650 (68.1)	21 (2.2)	
Heavy Drinker	75	31 (41.3)	42 (56.0)	2 (2.7)	
PHYSICAL ACTIVITY					54.09 (<.0001)
Very Active	2517	765 (30.4)	1694 (67.3)	58 (2.3)	
Moderately Active	4991	1365 (27.4)	3480 (69.7)	146 (2.9)	
Quite Inactive	1440	461 (32.0)	898 (62.4)	81 (5.6)	
COFFEE					10.66 (0.0048)
Yes	7750	2247 (29.0)	5269 (68.0)	234 (3.0)	
No	1229	349 (28.4)	821 (66.8)	59 (4.8)	

Table 11: Baseline Characteristics by sleep quantity (Continued)

		<u>Sleep Quantity</u>			
		1	2	3	
Baseline Characteristics	N	N=2599	N=6096	N=293	X ² (P-value)
DEPRESSION					
Yes	222	83 (37.4)	127 (57.2)	12 (5.4)	12.58 (0.0019)
No	8687	2497 (28.7)	5912 (68.1)	278 (3.2)	
INCOME, \$					
<10000	2332	739 (31.7)	1447 (62.0)	146 (6.3)	150.64 (<.0001)
10,000-24,999	2926	840 (28.7)	2011 (68.7)	75 (2.6)	
25,000-49,000	2310	617 (26.7)	1665 (72.1)	28 (1.2)	
>50,000	773	220 (28.4)	548 (70.9)	5 (0.7)	
EDUCATION					
<HS	3302	1012 (30.7)	2101 (63.6)	189 (5.7)	129.24 (<.0001)
HS	3341	970 (29.0)	2306 (69.0)	65 (2.0)	
>HS	2294	602 (26.2)	1659 (72.3)	33 (1.5)	
DIABETES					
Yes	427	141 (33.0)	263 (61.6)	23 (5.4)	11.40 (0.0034)
No	8561	2458 (28.7)	5833 (68.1)	270 (3.2)	
SLEEP QUALITY					
Good	2108	453 (21.5)	1592 (75.5)	63 (3.0)	393.0 (<.0001)
Moderate	3365	729 (21.7)	2545 (75.6)	91 (2.7)	
Poor	3514	1417 (40.3)	1958 (55.7)	139 (4.0)	

Table 12: Hazard Ratios for Sleep Quality and Quantity as risk factors for Type-2 Diabetes

Sleep Quality				
	Good	Moderate	Poor	
Baseline		HR (95%CI)	HR (95%CI)	P-value
Model 1	Referent	1.16 (0.89-1.52)	1.63 (1.26-2.10)	0.0001
Model 2	Referent	1.09 (0.83-1.43)	1.36 (1.04-1.76)	0.0356
Model 3	Referent	1.28 (0.95-1.72)	1.47 (1.09-1.99)	0.0392
Sleep Quantity (hours)				
	7-9	<7	>9	P-value
Model 1	Referent	1.26 (1.03-1.55)	1.93 (1.26-2.96)	0.0022
Model 2	Referent	1.25 (1.02-1.53)	1.56 (1.02-2.41)	0.0244
Model 3	Referent	1.06 (0.85-1.32)	1.46 (0.93-2.30)	0.2570

Model 1 was unadjusted. **Model 2** was Age-adjusted. **Model 3** was adjusted for Age, Sex, BMI, Race, Hypertension, Cigarette Smoking, Alcohol Consumption, Depression, Coffee Drinking, Physical Activity, and Education. The model 3 for sleep quality was adjusted for sleep quantity; likewise the model 3 for sleep quantity was adjusted for sleep quality.

Table 13: Effect of the interaction of BMI and Sleep Quality on Type-2 Diabetes

	HR (95% CI) Good Sleep	HR (95% CI) Moderate Sleep	HR (95% CI) Poor Sleep
Normal BMI	(Referent)	4.70 (1.66-13.33)	4.55 (1.61-12.90)
Overweight	8.97 (3.12-25.50)	8.28 (2.97-23.10)	8.74 (3.15-24.22)
Obese	19.88 (7.00-56.49)	23.32 (8.45-64.34)	31.11 (11.37-85.17)

Model adjusted for age, sex, race, hypertension, cigarette smoking, alcohol consumption, depression, coffee consumption, physical activity, education, and sleep quantity.

Table 14: Effect of 1-unit change in sleep quality on type-2 diabetes within BMI classes

	Hazard Ratio	95% CI
Normal BMI	1.557	1.107 – 2.191
Overweight	0.996	0.795 – 1.248
Obese	1.270	1.037 – 1.556

Model adjusted for age, sex, race, hypertension, cigarette smoking, alcohol consumption, depression, coffee consumption, physical activity, education, and sleep quantity.

CHAPTER FOUR

FACTORS AFFECTING TELOMERE LENGTH: THE ROLE OF OXIDATIVE STRESS

4.1 INTRODUCTION

It has been established that telomere shortening decreases the proliferative potential of the cells of the organism and causes aging of the organism. Aging has long been known to be associated with decreased immune system function which implies that shorter telomeres may be involved in compromising immune system function leading to some of the late-life diseases in humans. Research has subsequently established that telomere length is a biomarker of cumulative exposure to oxidative stress and a prognostic indicator for risk of late-life diseases^{89, 171}. Telomere shortening has been associated with neurodegenerative diseases, infections, diabetes, malignancies, autoimmune diseases, hypertension, atherosclerosis, cardiovascular disease, and mortality especially from cardiovascular disease¹⁷²⁻¹⁷⁸. It is therefore of importance to public health to identify factors associated with oxidative stress that contribute to telomere length shortening with the aim that modifying these factors may reduce telomere attrition and hence protect health. Factors such as obesity, physical inactivity, excessive use of

alcohol, and use of tobacco products have been associated with increased oxidative stress but research is limited on which of these factors actually contribute to telomere shortening^{80, 81, 83, 84, 86, 88}. This research is focused on identifying factors that influence telomere length in order to contribute to available literature on telomere biology which is important in improving human health.

4.1.1 Aim

This study investigates the effect of oxidative stress on telomere length in the Framingham Heart Study (FHS). Factors associated with oxidative stress such as body mass index, physical activity, tobacco use, and alcohol use will be investigated as to their influence on telomere length.

4.1.2 Hypothesis

We hypothesize that factors that increase oxidative stress such as increasing BMI (obesity), high tobacco and alcohol use, and physical inactivity will be associated with shorter telomeres.

4.1.3 Justification

Telomere length shortening has been found in previous studies to be the cumulative effect of oxidative stress and inflammation. Leucocyte telomere length therefore is a dependable predictor of past metabolic history in biological organisms¹⁷¹. Shorter telomeres have been linked to aging and age-related diseases and the search for

ways to reduce telomere length shortening cannot be overemphasized. Identifying factors that increase oxidative stress and therefore accelerate telomere length shortening is important to ensuring good health.

4.2 METHODS

4.2.1 Study Design: Cross-sectional study of the association between telomere length and body mass index, physical activity, tobacco use, and alcohol use.

4.2.2 Study Participants: This study enrolled subjects from the offspring cohort of the Framingham Heart Study (FHS). The FHS is a health research project started in 1948 under the direction of the National Heart, Lung, and Blood Institute (NHLBI) with the objective to identify the common factors that contribute to CVD. A total of 5,209 men and women between the ages of 30 and 62 from the town of Framingham, Massachusetts, were enrolled in the study. A second generation cohort made up of 5,124 offspring of the participants of the original cohort was enrolled in 1971. The participants underwent extensive physical examinations and lifestyle interviews at baseline, and followed up every two years thereafter. The FHS is one of the most well-organized cohorts in scientific research and has extensive information on major CVD risk factors such as high blood pressure, smoking, obesity, diabetes, and physical inactivity. Studies based on the FHS have contributed immensely to scientific literature over the last 50 years, and the use of this cohort for our present research provides us with the advantage of quality medical

and physical examinations, and laboratory tests, which are important in conducting valid research¹⁷⁹.

4.2.3 Inclusion Criteria

The offspring cohort was used for this study. The ages of the participants of the offspring cohort at baseline ranged from 5 to 70 years (n=5,124). Participants without history of any overt cardiovascular disease were included in this study. In addition, only participants with telomere length records were included.

4.2.4 Exclusion Criteria

Participants whose telomere lengths could not be determined either due to insufficient amount of DNA or poor DNA quality were excluded from this study (345 of such exclusions were made in previous studies^{180, 181}). A resultant sample of 974 subjects was used for this study.

4.2.5 Measurement of Telomere Length

This study used leukocyte telomere length data of participants provided by FHS. An independent laboratory that was blinded to all characteristics of the leukocyte donors conducted the telomere length measurements by Southern blot analyses and the results transmitted electronically to FHS only by ID numbers^{174, 180, 181}.

4.2.6 Assessment and Definition of Covariates

The FHS after obtaining informed consent, collected detailed physical and medical information on all study participants. For this study, hypertension status was defined according to the seventh report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure (JNC VII) as either “Normal”: systolic <120mmHg and diastolic <80mmHg; “Prehypertension”: systolic 120-139mmHg and diastolic 80-89mmHg; “Hypertension”: systolic $\geq$ 140mmHg and diastolic $\geq$ 90mmHg. Body Mass Index (BMI) was calculated as weight (kg)/height (m)² and categorized as normal (BMI <24.99), overweight (BMI 25-29.99), and obese (BMI $\geq$ 30). Self-reported smoking was coded as “never smoker”, “former smoker”, or “current smoker”. Energy expenditure was used to assess physical activity and was used as quartiles. Alcohol consumption was categorized as “nondrinker” “light”, “moderate”, or “heavy” drinker based on number of drinks per week. The alcohol variable used in the analyses was a combination of beer, red wine, white wine, and others including highballs and cocktails. Light drinking comprised of taking 1-5 drinks per week, moderate drinking was 6-13 drinks per week, and 14 or more drinks per week was categorized as heavy drinking.

4.2.7 Statistical Analysis

Generalized linear models (GLM) were used to model the effect of physical activity, body mass index, alcohol consumption, and tobacco use on telomere length. Telomere length was normally distributed for this sample and did not call for any transformation. Adjustments were made for age, gender, and hypertension to prevent

confounding by these variables. All analyses were performed using SAS version 9.3. Level of significance of all analyses was based on p-value of 0.05.

4.2.8 Power Calculation (factors affecting telomere length)

4.2.9 Prevalence of short telomeres among exposed and unexposed groups

For the purpose of estimating power for this study, telomere lengths were set at median with “short” and “long” telomeres recorded as below and above median, respectively. This implies 50% “short” telomere prevalence in the study sample.

4.2.10 Overweight/Obesity

More than 50% of U.S adults are overweight or obese¹⁸². Among the 974 Framingham cohort participants with telomere records, overweight or obese individuals formed 69.9% resulting in exposed and unexposed samples of 681 and 293, respectively. At 95% confidence level and 60% prevalence of short telomere among overweight or obese participants, there is more than 80% power for this study [Table 15].

4.2.11 Cigarette Smoking

19.0% of all adults (aged 18 years or older), in the United States smoke cigarettes¹⁸³. Among the 974 Framingham cohort participants with telomere records, we found 13.7% current smokers resulting in exposed and unexposed groups of 124 and 784 respectively. At 95% confidence level and 64% prevalence of short telomere among Cigarette smoking participants, there is more than 80% power for this study [Table 15].

4.2.12 Alcohol Use

Heavy drinking is defined as consuming more than 1 drink per day on average for women and more than 2 drinks per day on average for men. 24% of men and 12% of women binge drink. Binge drinking is defined as a pattern of alcohol consumption that brings the blood alcohol concentration level to 0.08% or more which usually corresponds to 5 or more drinks on a single occasion for men or 4 or more drinks on a single occasion for women, within hours¹⁸⁴. In this study 33% of subjects consume 6 or more drinks per week. Using 33% prevalence of excessive alcohol consumption for both males and females and with a total of 974 Framingham cohort participants with telomere records, the exposed and unexposed samples were 317 and 644 respectively. At 95% confidence level and 59.9% prevalence of short telomere among excessive alcohol users, there is more than 80% power for this study [Table 15].

4.2.13 Physical Inactivity

More than 50% of Americans do not meet the Physical Activity Guidelines for aerobic physical activity¹⁸⁵, and 25% of adults are completely inactive¹⁸⁶. We categorized physical activity into quartiles, and with the lowest quartile representing physically inactive subjects, the exposed and unexposed samples will be 225 and 675 respectively. At 95% confidence level and 61.1% prevalence of short telomere among physically inactivity participants, there is more than 80% power for this study [Table 15].

Table 15: Power Calculations (factors affecting telomere length)

Variable	Sample Exposed	Sample Unexposed	Prevalence of short telomere in exposed	Prevalence of short telomere in unexposed	Power with continuity correction
Overweight/Obesity	681 (69.9%)	293	60%	50%	80.6%
Current smoking	124 (13.7%)	784	64%	50%	80.1%
Alcohol use	317 (33.0%)	644	59.9%	50%	80.7%
Physical Inactivity	225 (25.0%)	675	61.1%	50%	80.4%

4.3 RESULTS

A total of 974 subjects were analyzed and the mean age was 58.2 years. Age distributions for <50, 50-69, and >69 years were 15.3%, 72.5%, and 12.2%, respectively. The mean BMI for the sample was 28.0 with overweight and obese subjects constituting 69.9%. Females formed 51.4%, and current smokers formed 13.7% of the sample. Subjects who did not use alcohol formed 35.4% of the sample, and heavy drinkers (≥ 14 drinks/week) formed 13.0%. Subjects who were classified as hypertensive ($\geq 140/90$) constituted 40.8% of the sample [Table 16]. Telomere length was normally distributed with a mean of 6.99kbp (6.99 kilo base-pairs). Unadjusted analyses have shown that increasing age, BMI, male gender, and hypertension were significantly associated with telomere length shortening, while cigarette smoking, energy expenditure, and alcohol consumption were not.

In the multivariable model age, gender, BMI, and alcohol consumption were significantly associated with telomere length [Table 17]. Hypertension and energy expenditure were not found to influence telomere length or confound the association between telomere length and the other variables in the model and were eliminated from further analyses. Cigarette smoking although not significantly associated with telomere length in both the unadjusted and multivariable models, it has been found to confound the association between alcohol use and telomere length in the multivariable model [Table 18]. The effect estimate between alcohol use and telomere length was attenuated by the presence of cigarette smoking in the model making alcohol use not quite statistically significant ($p=0.0983$) [Table 18]. Age, gender, and BMI were still significantly associated with telomere length when cigarette smoking was added to the model. A unit

increase in age was associated with 21.1bp (21.1 base-pairs) decrease in telomere length ($p < .0001$) [Table 18]. Compared to subjects below 50 years old, subjects 70 years or older have 596bp shorter telomeres ($p < .0001$) [Table 19]. A unit increase in BMI was associated with 8.5bp decrease in telomere length ($p = 0.0164$) [Table 18]. Overweight subjects have 73.8bp shorter telomeres compared to normal weight subjects but was nonsignificant ($p = 0.1024$), and obese subjects have 113bp shorter telomeres compared to normal weight subjects ($p = 0.0193$) [Table 19]. There was no difference between non-drinkers and light drinkers (1-5 drinks/per week). The non-drinker and light drinker classes have therefore been collapsed for comparison with moderate and heavy drinking classes. Moderate drinking was associated 18.2 bp shorter telomere length compared to the none or light drinker group but the association was nonsignificant ($p = 0.6999$), and heavy drinking was associated with 93.1bp shorter telomeres compared to the none or light drinker group ($p = 0.0976$) [Table 19].

Males were significantly more likely to have shorter telomeres (108.5bp shorter) compared to females ($p = .0041$) [Table 18]. This estimate was attenuated by about 5bp but remained significant ($p = 0.0073$) when categories of the other variables were considered instead of interval [Table 19]. Current smoking was associated with 69.2bp shorter telomeres compared to no smoking but the association did not reach statistical significance ($p = 0.2551$).

4.4 DISCUSSION

The results of the study have shown that age, gender, and BMI have significant influence on telomere length. The association between aging and telomere attrition has long been established. Telomeres shorten at every cell division and one would expect that when all other factors are held constant older people should have shorter telomeres compared to younger people. During the formation of peripheral leucocytes there is successive division of hematopoietic stem cells and progenitor cells contributing to the telomere shortening with age. In this sample, comparing subjects above 50 years old to subjects less than 50 years have shown that telomeres shorten at a rate of approximately 200bp for every 10 years after the age 50. Telomere length therefore has been proposed by some researchers to be an indicator of biological age¹⁸⁷. Cell division is necessary for growth but does not tell the whole story about the shortening of telomeres with age. Hence characterizing telomere length as a biological age indicator has been argued against by other researchers¹⁸⁸. Other proposals put forth to explain the shortening of telomeres with age include inflammation and oxidative stress^{174, 175, 189}. Inflammation increases the demand for cell replication and hence accelerated shortening of telomeres. The number of telomere repeats lost per cell replication has also been found to increase due to oxidative stress¹⁹⁰⁻¹⁹². Inflammation and oxidative stress play important roles in aging and late-life diseases^{23, 193} and their association with telomere shortening explains the increased incidence and prevalence of late-life diseases, particularly cardiovascular disease among individuals with shorter telomeres^{175, 178, 194, 195}. The rate of telomere shortening with age was investigated by Vaziri et al.,¹⁹⁶ among 140 individuals (0-107 years old) including 21 Down syndrome (DS) patients. They found that among 119

unrelated normal individuals, mean telomere length of peripheral blood T lymphocytes declined at a rate of 41 bp per year ($P < .00005$; $r = .83$). They also found that the rate of loss in DS patients was threefold higher than in age-matched normal individuals (rate of among DS patients: 133 ± 15 bp/year; rate of loss among age-matched normal individuals: 43 ± 7.7 bp/year; $P < .0005$). Down syndrome is characterized by immune function loss which implies that accelerated loss of peripheral blood T lymphocytes could be a reflection of immune cell senescence in DS patients. In our study, the rate of telomere attrition was 21 bp per year after adjusting for gender, body mass index, alcohol use, and cigarette smoking [Table 18]. These adjustments could be a source for the differences in estimates between our study and that of Vaziri et al. Moreover, Vaziri et al., were more specific, that is, they measured T lymphocytes rather than a broad category of leucocytes. Furthermore, our analyses involved over 900 subjects whilst results by Vaziri et al., were based on 140 individuals including 21 DS patients.

Our analyses also showed a significant difference in telomere length between males and females (Males vs. females: $\beta = -103$ bp, $p = 0.0073$) [Table 19]. This result also conforms to published literature about telomere length differences by gender^{196, 197}. In the study by Vaziri et al., males have a faster rate of loss in telomeric DNA compared to females although the difference was not significant (50 ± 4.2 vs. 40 ± 3.6 bp/year, $P = .1$). Bekaert et al.,¹⁹⁸ in their investigation into the association between telomere length and CVD risk factors also showed that females have significantly longer telomeres compared to males. Meta-analyses by Gardner et al.,¹⁹⁷ from 36 cohorts (36,230 participants) provided further evidence that females on average have longer telomeres compared to males. There are many schools of thought concerning the gender difference between

males and females. The study by Aubert et al.,¹⁹⁹ found that females have longer telomeres at birth compared to males, but the difference was not statistically significant. Okuda et al.,²⁰⁰ found no evidence of telomere length difference by gender at birth, and suggested that longer telomeres in females than males that occur in adulthood could be due to slower rate of telomere attrition in females. Slower rate of telomere attrition in females could mean reduced oxidative stress because in telomere biology, the influence of free radicals is a major component of telomere attrition⁸⁹. In support of this it has been found that females produce fewer reactive oxygen species than men. This is possible through the many actions of estrogen in scavenging free radicals, or inhibiting their formation, and ability to stimulate enzymes to detoxify free radicals²⁰¹. Thus, the antioxidant properties of estrogen serve to preserve telomeres of females or at least slow the rate of attrition. It is also thought that estrogen might stimulate telomerase to add telomere repeats to the ends of chromosomes given that an estrogen-response element is present in the telomerase reverse transcriptase²⁰¹. Estrogen has also been found to stimulate nitric oxide production in vascular endothelial cells, which leads to stimulation of telomerase in these cells and could protect against endothelial dysfunction that characterize oxidative stress²⁰².

Obesity is another important factor in telomere biology. It is currently a global epidemic and has been implicated in many disease processes. Obesity is characterized by a state of high-systematic oxidative stress and inflammation²⁰³ and because telomeres are very vulnerable to attack by reactive oxygen species²⁰⁴, it has been postulated that telomere attrition could be accelerated by obesity. We found in our analysis that a unit increase in BMI is associated with 8.5 bp (0.0085 kbp) decrease in telomere length

($p=0.0164$) [Table 18]. When obese subjects ($BMI \geq 30$) were compared with normal subjects ($BMI < 25$), it was found that obesity is associated with 113 bp shorter telomeres ($p=0.0193$) [Table 19]. The study by Fitzpatrick et al.,¹⁷⁵ in the Cardiovascular Health Study showed similar results when they compared overweight subjects with normal subjects ($\beta=-117$ bp, $p=0.06$). Their overweight class however included obese subjects. Our sample size was more than twice theirs (419) and may be the reason for their p -value of 0.06. Lee et al.,²⁰⁵ also showed that BMI was inversely associated with telomere length although they used the ratio of telomere repeat copy number (T) and single-copy gene copy number (S), T/S ratio, to estimate telomere length while we used terminal restriction fragments (TRF) method.

The study by Diaz et al.,²⁰⁶ involving 317 subjects between the ages of 40 and 64 did not show any association between BMI and telomere length. Our sample of 974 is 3 times bigger and could be among reasons for the varying results. A recent meta-analysis by Muezzinler et al., gave credence to our results. They found that a unit increase in BMI is associated with 8.0 bp (0.008 kbp) decrease in telomere length ($p=0.058$). Although their p -value was only marginally statistically significant, we got approximately the same estimate for the effect of BMI on telomere length and this is worth noting. Obesity begins with high caloric intake and less physical activity. The result is accumulation of excess fat in the adipose tissue, leading to low-grade inflammation, hypoxia and increased free radical production^{207, 208}. This alters the secretion of adipokines (adiponectin and leptin) and cytokines ($TNF\alpha$, $IL-1\beta$, and $IL-6$), by the adipose tissue. This then manifests as health problems particularly, insulin resistance, diabetes, and atherosclerosis. Obesity therefore, is associated with hyperglycemia, high levels of circulating free fatty acids,

decreased antioxidant defenses and chronic inflammation. The resultant enhanced oxidative stress could explain the association between obesity and telomere attrition.

Alcohol consumption and cigarette smoking were not found to significantly influence telomere length in our study. Alcohol use was marginally associated with telomere length when smoking was not considered [Table 17] but after adjustment for cigarette smoking the effect was no longer significant [Table 18]. A study of business men in Helsinki²⁰⁹ found that compared with non-drinkers, subjects who drank more than one drink per day have significantly shorter telomeres. One drink per day was not found to be significantly associated with telomere length and conforms to the protection offered by light to moderate drinking as found by some studies. In this study, light drinkers and non-drinkers were combined because these groups were not different in their effects on telomere length. In another study involving Caucasian males living in Northeastern Italy, it was found that alcohol abusers have significantly shorter telomeres (almost halved) compared to controls. The association was also found in their linear model with decreasing telomere lengths associated with increasing amount of drinks per day²¹⁰. In Japan, specimens of the esophagus were collected from 26 alcoholic patients with mean age of 61.2 years, and compared with specimens of normal esophageal mucosa obtained from 24 autopsy cases with mean age of 73.3 years. When telomeres in the basal cells of the esophageal epithelium were measured it was found that those from the alcoholics were significantly shorter than the normal controls²¹¹. This study focused on telomere length from the esophageal mucosa whilst we measured leucocyte telomere length. Nevertheless, both studies are comparable in terms of highlighting the harmful effects of excessive alcohol consumption. It has been suggested that some alcohol use may be

beneficial and the study by Sun et al.,⁸⁴ has provided evidence. They analyzed 5,862 women in the Nurses' Health Study and found that moderate drinking (1 drink/week to 2 drinks/day), was associated with longer telomere length although the association was not statistically significant. They further put together all low-risk factors such as non-current smoking, maintaining a healthy body weight, engaging in regular moderate or vigorous physical activities, drinking alcohol in moderation, and eating a healthy diet, and found that the higher the number of low-risk factors, the longer the telomeres ($p = 0.015$). Light to moderate drinking has also been found to reduce cardiovascular risk in a meta-analysis involving 84 studies. The pooled adjusted relative risk for CVD mortality was 0.75 (0.70 to 0.80) for alcohol drinkers compared to non-drinkers. For coronary heart disease mortality, it was found that 1-2 drinks a day provided the lowest risk and for stroke mortality the lowest risk was found for drinking ≤ 1 drink per day²¹². Another meta-analysis involving 23 studies has also found that moderate alcohol intake is protective against dementia and Alzheimer's disease²¹³. A prospective study of U.S. adults aged 55 and older in the Health and Retirement Study found that moderate drinking (1 drink/d) was strongly associated with less mortality and better risk factor profiles, compared to no drinking²¹⁴. With all the proposed benefits of light to moderate alcohol use, the mechanisms influencing telomere shortening are not clearly defined. As outlined earlier, telomeres are vulnerable to oxidative stress and it is plausible that alcohol-induced increase in oxidative stress, inflammation, and impaired antioxidant systems are the main mechanisms linking excessive alcohol use to telomere attrition.

Cigarette smoking has been linked with telomere shortening although we did not find a significant association. Valdes et al.,⁸¹ found a dose-response relationship between

smoking and telomere length where each pack-year of cigarette smoked was equivalent to an additional 5 bp of telomere length lost compared with the rate in the overall cohort ($p=0.017$). Our study categorized smoking as “never”, “former”, and “current” rather than pack years and our analysis comprised of males and females together whiles Valdes et al., studied only women. In a cross-sectional study of 20 current smokers and 20 non-smokers, a higher mean pack year of cigarette was found in subjects with negative telomerase reverse transcriptase (hTERT) expression in the skin ($P < 0.01$)²¹⁵.

Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by adding the telomere repeat “TTAGGG”. The telomerase reverse transcriptase (hTERT) is a catalytic subunit of the enzyme telomerase. Hence negative action of smoking on hTERT expression implies shorter telomeres. Active smoking was found in the Prevention of Renal and Vascular End-stage Disease (PREVEND) study, to almost triple the rate of telomere attrition per year ($P < 0.0001$)²¹⁶. It is biologically plausible that smoking is associated with telomere length which could translate into health related states linked to both smoking and shortened telomeres. Cigarette smokers are at higher risk of developing several chronic disorders including buildups in arteries, some types of cancer, and chronic obstructive pulmonary disease. It has been estimated that 30% of all cancer deaths, 20% of all coronary heart diseases and strokes, and 80% of all chronic obstructive pulmonary disease are attributable to cigarette smoking. Smoking has been found to be the main contributor to lung cancer, atherosclerosis, and myocardial infarctions²¹⁷⁻²¹⁹. Smoking could induce oxidative stress and accelerate telomere attrition because cigarette smoke contains a large variety of compounds, including oxidants and free radicals that are capable of initiating and promoting oxidative damage to tissue proteins.

It has been shown in this research that age, gender, and BMI have significant effects on telomere length. The associations were strong and conform to existing literature on the association between these factors and telomere length. The Framingham Heart Study (FHS) from which we performed these analyses is known for high data quality which reflects on the validity of our study. Our study however is not devoid of limitations. Firstly, we performed a cross-sectional analysis to study the associations presented. Prospective studies are better because they can establish temporality, but telomere shortening, according to the biology occurs with age and highly influenced by stress from oxidation. Hence although we conducted a cross-sectional study, we have no reason to suspect the direction and validity of our estimates. Secondly, the sample involved mainly Caucasian (99%) and results may not apply to other race/ethnicities. Thirdly, a much larger sample than we used (974) could be helpful in bringing out certain unseen effects and make the results more reliable. However, our results were comparable to those from other studies with even much smaller sample size. Fourthly, the effect of alcohol on telomere length could be even clearer if had analyzed each alcoholic beverage separately. We analyzed all alcoholic beverages together because of very low numbers reported per alcohol type. Our results were however comparable to other studies which used the same approach. It is important that future studies have samples large enough to increase reliability of results and also take advantage of the useful information to be gained by analyzing each alcohol type separately.

4.5 CONCLUSION

Telomeres consist of nucleotide repeats that cap the ends of chromosomes and are essential to maintaining chromosomal integrity. Telomere shortening has been implicated in disease including Down syndrome, Alzheimer's disease, some cancers, coronary heart disease, stroke, myocardial infarctions, and atherosclerosis. Healthy lifestyle factors such as non-current smoking, maintaining a healthy body weight, engaging in regular moderate or vigorous physical activities, drinking alcohol in moderation, and eating a healthy diet have been found to reduce telomere attrition. The opposite of these promote increased oxidative stress and inflammation, which enhances white blood cell turnover resulting in increased rate of telomere erosion per cell division. Although age and gender have strong effects on telomere length as seen in this analysis, they are not modifiable. However the rate of attrition can be reduced by lifestyle choices that will minimize the oxidative burden on telomeres. In light of these results, a lot needs to be done in educating the public to take seriously those activities that lower the risk of telomere loss and promote a healthy life.

Table 16: Characteristics of Study Participants by Leucocyte Telomere length

VARIABLE	N	<u>Leucocyte Telomere Length (kbp)</u>				F (P-value)
		Quartile 1 N=243	Quartile 2 N=244	Quartile 3 N=243	Quartile 4 N=244	
BMI (mean, s.d)	974	28.6 (5.4)	28.0 (5.2)	27.5 (4.7)	27.7 (5.5)	4.88 (0.0274)
AGE (mean, s.d)	973	61.4 (8.7)	59.3 (8.7)	57.9 (8.2)	54.1 (8.0)	94.25 (<.0001)
BMI						X² (P-value)
Normal	293	63 (21.5)	67 (22.9)	74 (25.3)	89 (30.4)	10.11 (0.1200)
Overweight	399	98 (24.6)	107 (26.8)	105 (26.3)	89 (22.3)	
Obese	282	82 (29.1)	70 (24.8)	64 (22.7)	66 (23.4)	
AGE						
<50	149	20 (13.4)	29 (19.5)	30 (20.1)	70 (47.0)	100.96(<.0001)
50-59	429	84 (19.6)	100 (23.3)	121 (28.2)	124 (28.9)	
60-69	276	88 (31.9)	81 (29.4)	71 (25.7)	36 (13.0)	
≥70	119	51(42.9)	34 (28.6)	21 (17.7)	13 (10.9)	
SEX						
Male	473	135 (28.5)	130 (27.5)	111 (23.5)	97 (20.5)	15.01 (0.0018)
Female	500	108 (21.6)	114 (22.8)	132 (26.4)	146 (29.2)	
BLOOD PRESSURE						
Normal	276	50 (18.1)	68 (24.6)	70 (25.4)	88 (31.9)	17.41 (0.0079)
Prehypertension	301	77 (25.6)	71 (23.6)	79 (26.2)	74 (24.6)	
Hypertension	397	116 (29.2)	105 (26.4)	94 (23.7)	82 (20.7)	
CIGARETTE						
Non-smoker	270	69 (25.6)	64 (23.7)	67 (24.8)	70 (25.9)	1.10 (0.9815)
Former smoker	514	130 (25.3)	131 (25.5)	124 (24.1)	129 (25.1)	
Current smoker	124	32 (25.8)	33 (26.6)	32 (25.8)	27 (21.8)	
ENERGY						
1 st Quartile	225	59 (26.2)	53 (23.6)	58 (25.8)	55 (24.4)	3.07 (0.9614)
2 nd Quartile	225	54 (24.0)	55 (24.4)	60 (26.7)	56 (24.9)	
3 rd Quartile	225	59 (26.2)	61 (27.1)	49 (21.8)	56 (24.9)	
4 th Quartile	225	51(22.7)	56 (24.9)	61 (27.1)	57 (25.3)	
ALCOHOL						
None	340	94 (27.7)	78 (22.9)	84 (24.7)	84 (24.7)	10.57 (0.3061)
1-5 Drinks/week	304	66 (21.7)	73 (24.0)	75 (24.7)	90 (29.6)	
6-13 Drinks/week	192	49 (25.5)	50 (26.0)	48 (25.0)	45 (23.5)	
≥14 Drinks/week	125	34 (27.2)	37 (29.6)	33 (26.4)	21 (16.8)	
BEER						
None	675	177 (26.2)	160 (23.7)	164 (24.3)	174 (25.8)	11.51 (0.2424)
1 Drink/week	94	18 (19.2)	26 (27.7)	23 (24.5)	27 (28.7)	
2-4 Drinks/week	103	24 (23.3)	23 (22.3)	35 (34.0)	21 (20.4)	
≥5 Drinks/week	97	24 (24.7)	32 (33.0)	20 (20.6)	21 (21.7)	

Table 16: Characteristics of Study Participants by Leucocyte Telomere length (continued)

		Leucocyte Telomere Length (kbp)				
VARIABLE	N	Quartile 1 N=243	Quartile 2 N=244	Quartile 3 N=243	Quartile 4 N=244	X ² (P-value)
RED WINE						
None	720	184 (25.6)	176 (24.4)	176 (24.4)	184 (25.6)	3.09 (0.9605)
1 Drink/week	102	21 (20.6)	28 (27.5)	26 (25.5)	27 (26.5)	
2-4 Drinks/week	85	20 (23.5)	23 (27.1)	24 (28.2)	18 (21.2)	
≥5 Drinks/week	65	18 (27.7)	17 (26.2)	16 (24.6)	14 (21.5)	
WHITE WINE						
None	658	173 (26.3)	164 (24.9)	159 (24.2)	162 (24.6)	5.50 (0.7887)
1 Drink/week	127	27 (21.3)	38 (29.9)	31 (24.4)	31 (24.4)	
2-4 Drinks/week	118	27 (22.9)	26 (22.0)	31 (26.3)	34 (28.8)	
≥5 Drinks/week	67	16 (23.9)	15 (22.4)	21 (31.3)	15 (22.4)	
OTHERS						
None	690	174 (25.2)	165 (23.9)	176 (25.5)	175 (25.4)	12.26 (0.1992)
1 Drink/week	105	20 (19.0)	28 (26.7)	22 (21.0)	35 (33.3)	
2-4 Drinks/week	88	22 (25.0)	28 (31.8)	23 (26.1)	15 (17.1)	
≥5 Drinks/week	83	27 (32.5)	20 (24.1)	19 (22.9)	17 (20.5)	

Leucocyte Telomere Length: Quartile 1 (<6.560); Quartile 2 (6.560- 6.953); Quartile 3 (6.954- 7.387); Quartile 4 (>7.387).

Table 17: Regression estimates of the factors affecting telomere length

Parameter	Estimate	Standard	t Value	Pr > t
Age	-0.0211	0.0020	-10.61	<.0001
Sex (M)	-0.1167	0.0361	-3.24	0.0012
BMI	-0.0080	0.0034	-2.38	0.0176
Alcohol use	-0.0047	0.0024	-2.00	0.0454

Table 18: Regression estimates of the factors affecting telomere length

Parameter	Estimate	Standard Error	t Value	Pr > t
Age	-0.0211	0.0021	-10.08	<.0001
Sex (M)	-0.1085	0.0377	-2.88	0.0041
BMI	-0.0085	0.0035	-2.40	0.0164
Alcohol use	-0.0040	0.0024	-1.65	0.0983
Smoking				
Non-smoker	Referent			
Former smoker	0.0162	0.0416	0.39	0.6969
Current smoker	-0.0669	0.0603	-1.11	0.2672

Table 19: Estimates of the factors affecting telomere length (variables as categories)

Parameter	N	Estimate	Standard	t Value	Pr > t
AGE					
<50	149	Referent			
50-59	429	-0.200	0.055	-3.67	0.0003
60-69	276	-0.425	0.059	-7.24	<.0001
≥70	119	-0.596	0.070	-8.44	<.0001
SEX					
Male	473	-0.103	0.038	-2.69	0.0073
Female	500	Referent			
BMI					
Normal	293	Referent			
Overweight	399	-0.074	0.045	-1.64	0.102
Obese	282	-0.113	0.048	-2.34	0.019
ALCOHOL					
0-5 Drinks/week	644	Referent			
6-13 Drinks/week	192	-0.018	0.047	-0.39	0.700
≥14 Drinks/week	125	-0.093	0.056	-1.66	0.098
Smoking					
Non-smoker	270				
Former smoker	514	0.009	0.042	0.22	0.826
Current smoker	124	-0.069	0.061	-1.14	0.255

CHAPTER FIVE

SUMMARY AND CONCLUSION

5.1 Oxidative Stress

All aerobic organisms require oxygen for survival. During metabolism oxygen is used to produce ATP and water. The mitochondrion is the site of metabolism and dysfunction results in impairment of the electron transport chain producing conducive environment for reactive oxygen species. Reactive oxygen species include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), hydroxyl ion (OH^-), and nitric oxide ($NO^{\bullet}$). Reactive oxygen species can be produced at other sites by different enzymes, apart from the mitochondria. These include NADPH oxidase at the plasma membrane, lipid oxidation within peroxisomes, and xanthine oxidase in the cytoplasm.

Living cells are equipped with defense mechanisms to lessen the harmful effects of reactive oxygen species. Superoxide dismutase (SOD) catalyzes the conversion of superoxide anions into hydrogen peroxide and oxygen. In the peroxisomes of eukaryotic cells, the enzyme catalase converts hydrogen peroxide to water and oxygen, completing the detoxification that was started by SOD. Catalase is more active when levels of hydrogen peroxide are low. Another group of enzymes called glutathione peroxidase catalyze the detoxification of hydrogen peroxides when present at high concentrations.

The proper functioning of these antioxidant enzymes depends on the availability of micro-nutrients such as iron, copper, zinc, and selenium. Dietary antioxidants including vitamins E, C, and D, flavonoids and carotenoids are also known to reduce or inhibit formation of reactive oxygen species. Accelerated reactive oxygen species production occurs when micro-nutrients that are important for antioxidant enzyme function, and dietary antioxidants are exhausted. This imbalance between the cell's defense systems and reactive oxygen species is known as oxidative stress.

Oxidative stress causes oxidative damage resulting in cell injury. Oxidative damage to DNA can cause DNA mutation leading to changes in protein gene expression. Protein modifications caused by reactive oxygen species have been found in elevated levels in diseased tissues in patients with inflammatory diseases. Lipids also undergo peroxidation in the presence of reactive oxygen species. Lipid peroxidation initiates the formation of plaques in the arteries with time giving rise to atherosclerosis. Lipid peroxidation is one of the most widely used indicators of oxidative stress. Oxidative stress has been implication in diabetes, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Huntington disease, cancer, and cardiovascular disease.

Lifestyle factors such as overweight and obesity, cigarette smoking, excessive alcohol consumption, and inactivity have been associated with increased oxidative stress. Obesity is accumulation of excess fat in the adipose tissue resulting in alteration in secretion of adipokines and cytokines. Obesity is characterized by high levels of circulating free fatty acids, decreased antioxidant defenses and chronic inflammation and hence enhanced oxidative stress. Cigarette smoke contains a large variety of compounds, including oxidants and free radicals, and alcohol could induce inflammation impair

antioxidant systems. Physical activity is important in utilizing ATP and prevents the harboring of electrons in the electron transport chain. Inactivity therefore promotes the harboring of excess electrons in the transport chains leading to reactive oxygen species production and hence oxidative stress.

5.2 Sleep Disordered Breathing

Sleep-Disordered breathing (SDB) describes a group of disorders characterized by inadequate ventilation or abnormal breathing during sleep. It involves total or partial collapse of the pharyngeal airway during sleep necessitating arousal to resume ventilation. The intermittent hypoxia followed by reoxygenation has been associated with reactive oxygen species production. Several studies have also shown that plasma levels of lipid peroxidation biomarkers such as thiobarbituric reactive substances (TBARS) and peroxides (PD) were significantly higher among sleep apnea patients than normal controls. Since lipid peroxidation plays a key role in the formation of atherosclerosis plaques, SDB patients have been found to be at greater risk of atherosclerosis and mortality from cardiovascular diseases. This study investigated the association between SDB and CML concentration and found that participants with SDB have higher CML levels than participants without SDB, within certain hemoglobin ranges. High CML concentration is an indication of oxidative stress. Some participants with SDB have been found to have lower CML concentration which has been attributed to their physical fitness because moderate to high physical activity has been associated with reduced oxidative stress. It should also be noted that we studied an older population (mean age 77.5 years) and most males with severe SDB may have died before CML was measured.

The lower CML values found among males who have survived may not be a protective effect of severe SDB but may be due other reasons not assessed in this study. CML values from a younger population may be important in understanding the relationship between SDB and CML concentration. Part of this dissertation also looked at sleep disturbances and type-2-diabetes. As described above sleep disturbances have oxidative stress consequences. The β -cells of the pancreas lack catalase and are vulnerable to the toxic effects mediated by reactive oxygen species. Findings from this study indicated higher risk of type-2-diabetes among subjects with poor sleep quality which was further exacerbated by obesity. Hence taken together, these observations emphasize the importance of SDB in oxidative stress and its consequences.

5.3 Carboxymethyl-lysine (CML)

Advanced glycation end-products (AGEs) are a heterogeneous group of macromolecules that are formed by the non-enzymatic glycation of proteins, lipids, and nucleic acids. CML is a dominant AGE and is formed on protein by combined non-enzymatic glycation and oxidation (glycoxidation) reactions. It is also formed during lipid peroxidation (metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein. Lipids undergo peroxidation in the presence of reactive oxygen species. Hence Lipid peroxidation is one of the most widely used indicators of oxidative stress. Since CML is formed from lipid peroxidation, it has been characterized as the general biomarker of oxidative stress. CML has therefore been associated with many of the disorders known to be related to oxidative stress including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Huntington disease, rheumatoid

arthritis, end-stage renal disease, hypertension, atherosclerosis, and cardiovascular disease. The importance of studying the association between CML and SDB is to establish grounds for preventive measures. SDB treatment by use of CPAP has been successful hence in order to reduce CML concentration and prevent disease, treatment of sleep problems could be considered among the many strategies.

5.4 Telomeres

Telomere attrition has been implicated in diseases including cancer, diabetes, hypertension, atherosclerosis, coronary artery disease, and chronic heart failure. Telomeres shorten at each cell division until a threshold is reached where no further replication can take place (cellular senescence). Telomere length therefore depends on age but shortening can be accelerated by oxidative stress. Oxidative stress and inflammation enhances white blood cell turnover resulting in increased rate of telomere erosion per cell division. Lifestyle factors such as current smoking, overweight and obesity, sedentary lifestyle, excessive alcohol consumption, and unhealthy diet have been found to increase telomere attrition. Public health messages directed towards these lifestyle factors may go a long way to promote good health and longevity.

5.5 Aging and Age-related Diseases

Since proposed by Harman in 1956, the free radical theory remained the most popular theory of aging and age-related diseases. As mentioned above, oxidative stress contributes to telomere shortening and therefore places limit on the replicative potential

of the cell. Loss of replicative potential leads to cellular senescence. CML concentrations were also higher with increased oxidative stress. According to Harman the accumulation of the impact of oxidative stress on cells over time contribute to the aging of the organism. Both high CML concentration and shortened telomeres are very dependent on age and are associated with degenerative diseases. Since the mitochondria produces most of the reactive oxygen species, it has been proposed that the higher the metabolic rate of an organism the greater the production of reactive oxygen species and hence the shorter the life span of the organism. In response to this hypothesis, a strategy like caloric restriction has been proposed as a way to reduce oxidative damage and disease. As shown in this study, sleep hygiene is something to consider in the policy framework of interventions aimed at aging and degenerative diseases. Other healthy lifestyle factors to consider include maintaining a healthy weight, smoking cessation, moderation in alcohol consumption, and moderate to high physical activity.

5.6 Recommendations for Future Research

The biology of oxidative stress and health have been received a lot of attention since the 1950s yet there is still much to be known on the topic. Inflammation and oxidative stress factors have been associated with telomere length in this study and oxidative stress from sleep disordered breathing has been associated with CML concentration. Telomere shortening and CML concentration have been implicated in degenerative diseases but the association between them is yet to be studied. Future studies may investigate this association to determine if shorter telomeres are associated with high CML concentrations or vice-versa. Further studies are also needed to verify if

higher CML concentration as a result of SDB is actually associated with a disease outcome in the CHS. Furthermore, the association between hemoglobin, SDB, and CML needs to be replicated with a much larger sample and if possible longitudinally. Specifically, a future study could focus on investigating the protective effect of SDB on CML concentration among males with high hemoglobin.

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216. Huzen, J., et al., Telomere length loss due to smoking and metabolic traits. *J Intern Med*, 2014. 275(2): p. 155-63.
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218. Howard, G., et al., Cigarette smoking and progression of atherosclerosis: The Atherosclerosis Risk in Communities (ARIC) Study. *JAMA*, 1998. 279(2): p. 119-24.
219. Meyers, D.G. and J.S. Neuberger, Cardiovascular effect of bans on smoking in public places. *Am J Cardiol*, 2008. 102(10): p. 1421-4.

APPENDICES

Appendix 1: Placement of EEG Electrodes

(Adapted from Kakkar, R.K. and G.K. Hill, Otolaryngol Clin North Am, 2007. 40(4): p. 713-43)

Appendix 2: Placement of EOG Electrodes (*Adapted from Kakkar, R.K. and G.K. Hill, Otolaryngol Clin North Am, 2007. 40(4): p. 713-43*)

Appendix 3: Placement of Submental EMG Electrodes (*Adapted from Kakkar, R.K. and G.K. Hill, Otolaryngol Clin North Am, 2007. 40(4): p. 713-43*)

Appendix 4: Nasal Pressure Cannulae (*Adapted from Kakkar, R.K. and G.K. Hill, Otolaryngol Clin North Am, 2007. 40(4): p. 713-43*)

Appendix 5: A Sleep Hypnogram (Adapted from Kakkar, R.K. and G.K. Hill, *Otolaryngol Clin North Am*, 2007. 40(4): p. 713-43)

Appendix 6: Formation of Advanced Glycation End-products (*Adapted from Singh et al., Diabetologia, 2001. 44(2): p. 129-46*)

Appendix 7: Effect of age on body iron by gender (*Adapted from Cook et al., Blood, 2003. 101(9): p. 3359-64*)

Appendix 8: Concurrent but inverse changes of ferritin versus estrogen during menopausal transition (*Jian, et al., Antioxid Redox Signal, 2009. 11(12): p. 2939-43*)

Appendix 9: Proposed mechanisms linking increased iron to increases oxidative stress (Jian, et al., *Antioxid Redox Signal*, 2009. 11(12): p. 2939-43)

Appendix 10: IRB Approval Letter: The association between sleep disordered breathing and carboxymethyl-lysine concentration in the Cardiovascular Health Study

Human Subjects Protection Program Office
MedCenter One – Suite 200
501 E. Broadway
Louisville, KY 40202-1798

Office: 502.852.5188 Fax: 502.852.2164

DATE: September 26, 2013

TO: Richard A Kerber, Ph.D.

FROM: The University of Louisville Institutional Review Board

RE: 13.0507 - Relationship between Sleep Disordered Breathing and N-carboxymethyl-lysine concentration in the Cardiovascular Health Study (CHS)

REF. NUM: 326774

This study was reviewed on 9/25/13 and determined by the chair of the Institutional Review Board that the study is exempt according to 45 CFR 46.101(b) under category (4) Research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects.

This study was also approved through 45 CFR 46.116 (D), which means that it has been granted a waiver of informed consent because it meets the following criteria:

- The research involves no more than minimal risk to the subjects.
- The waiver or alteration will not adversely affect the rights and welfare of the subjects.
- The research could not practicably be carried out without the waiver or alteration.
- Whenever appropriate, the subjects will be provided with the additional pertinent information after participation.

Documents/Attachments reviewed and approved:

. Protocol, dated 9/5/13

The study is exempt only if information that could identify subjects is not recorded.

Since this study has been approved under the exempt category indicated above, no additional reporting, such as submission of Progress Reports for continuation reviews, is needed. If your research focus or activities change, please submit an Amendment to the IRB for review to ensure that the indicated

exempt category still applies. Best wishes for a successful study. Please send all inquiries to our office email address at hsppofc@louisville.edu

Thank you for your submission.

Sincerely,

A handwritten signature in black ink that reads "Peter M. Quesada". The signature is written in a cursive, flowing style.

Peter M. Quesada, Ph.D., Chair

Social/Behavioral/Educational Institutional Review Board

PMQ/jsp

Do not reply to this email, it was auto-generated through the iRIS System.
To access this iRIS System, click on the following link: <https://iris.louisville.edu>

Appendix 11: IRB Approval Letter: Telomere length and cardiovascular disease in the Framingham Heart Study

INSTITUTIONAL REVIEW BOARDS

University of Louisville
MedCenter One, Suite 200
501 E. Broadway
Louisville, Kentucky 40202-1798
Office: 502-852-5188
Fax: 502-852-2164

To: Taylor, Kira
From: The University of Louisville Institutional Review Board (IRB)
Date: Friday, March 15, 2013
Subject: Approval Letter

Tracking #: AMEND-6696 (12.0327)
Title: Telomere length as a risk factor for cardiovascular disease in the Framingham Heart Study.

This study was re—reviewed on 03/06/2013 by the chair/vice chair of the Institutional Review Board and approved through the Expedited Review Procedure, according to 45 CFR 46.110(b), since this study falls under Expedited Category (5) Research involving materials (data, documents, records, or specimens) that have been collected, or will be collected solely for non—research purposes (such as medical treatment or diagnosis).

This study was also approved through 45 CFR 46.116 (D), which means that it has been granted a waiver of informed consent because it meets the following criteria:

- ° The research involves no more than minimal risk to the subjects.
- ° The waiver or alteration will not adversely affect the rights and welfare of the subjects.
- ° The research could not practicably be carried out without the waiver or alteration.
- ° Whenever appropriate, the subjects will be provided with the additional pertinent information after participation.

The following items have been approved:

- Research Protocol, not dated

This study now has final IRB approval from 03/06/2013 through 03/05/2014. You should complete and return the Progress Report/Continuation Request Form EIGHT weeks prior to this date in order to ensure that no lapse in approval occurs. The committee will be advised of this action at their next full board meeting.

Site Approval

If this study will take place at an affiliated research institution, such as Jewish

Hospital/St Marys Hospital, Norton Healthcare, or University of Louisville Hospital, permission to use the site of the affiliated institution may be necessary before the research may begin. If this study will take place outside of the University of Louisville Campuses, permission from the organization should be obtained before the research may begin. Failure to obtain this permission may result in a delay in the start of your research.

Privacy & Encryption Statement

The University of Louisville's Privacy and Encryption Policy requires such information as identifiable medical and health records; credit card, bank account and other personal financial information; social security numbers; proprietary research data; dates of birth (when combined with name, address and/or phone numbers) to be encrypted. For additional information: <http://security.louisville.edu/PolStds/ISO/PS018.htm>.

1099 Information (If Applicable)

As a reminder, in compliance with University policies and Internal Revenue Service code, all payments (including checks, gift cards, and gift certificates) to research subjects must be reported to the University Controller's Office. Petty Cash payments must also be monitored by the issuing department and reported to the Controller's Office. Before issuing compensation, each research subject must complete a W—9 form.

For additional information, please contact the Controller's Office at 852—8237 or contro1l@louisville.edu.

If you need assistance, please feel free to contact our office at (502) 852—5188. You may also email our service account at hsppofc@louisville.edu for assistance.

Thank you.

Board Designee: Goldman, Julie

Continue following the regulations below:

1. Unanticipated problems or serious adverse events encountered in this research study must be reported to the IRB within five (5) work days.
2. Any modifications to the study protocol or informed consent form must be reviewed and approved by the IRB prior to implementation.
3. You may not use a modified informed consent form until it has been approved and validated by the IRB.

Letter Sent By: Peek, Tamara, 3/15/2013 3:29 PM

*Full Accreditation since June 2005 by the Association for the Accreditation of
Human Research Protection Programs, Inc.*

Appendix 12: IRB Approval Letter: Telomere length and cardiovascular disease in the Framingham Heart Study (Continued Committee Approval)

Human Subjects Protection Program Office
MedCenter One – Suite 200
501 E. Broadway
Louisville, KY 40202-1798
Office: 502.852.5188 Fax: 502.852.2164

DATE: February 06, 2014

TO: Kira C Taylor

FROM: The University of Louisville Institutional Review Board

IRB NUMBER: 12.0327

STUDY TITLE: Telomere Length as a Risk Factor for Cardiovascular Disease in the Framingham Heart Study

REFERENCE #: 331763

DATE OF REVIEW: 02/06/2014

IRB STAFF CONTACT: Name: Barbara Dearing
Phone: 502-852-5987
Email: badear01@louisville.edu

The continuation request for this study was reviewed by the Chair/Vice-Chair of the Institutional Review Board (IRB) through the expedited review procedure, according to 45 CFR 46.110 and 21 CFR 56.110 since this study falls under Category 5: Research involving materials (data, documents, records, or specimens) that have been collected, or will be collected solely for non-research purposes (such as medical treatment or diagnosis).

The study now has continued committee approval from 03/06/2014 through 03/05/2015.

The following items were reviewed and approved:

Title	Version Number	Version Date	Outcome
Relevant Findings	Version 1.0	02/05/2014	Reviewed
12.0327_2013-03-06_AMEND-6696_IRB_PROTOCOL_V4.pdf	Version 1.0	null	Approved
12.0327_2012-12-12_DATA_COLLECTION_SHEET.docx	Version 1.0	null	Approved

Continuation Review Requirements

You are responsible for submitting a continuation review 30 days prior to the expiration date of your research study. Investigators who allow their study approval to expire have committed significant non-compliance with federal regulations. Such lapses may require reporting to federal agencies, a program audit by compliance auditors to ensure that subjects were not enrolled during the expired period, and may lead to findings of serious and continuing non-compliance if expiration were to occur a second time.

This action will be reported promptly to the IRB at a scheduled full Board meeting. If you have any questions, please contact the HSPPO at (502) 852-5188 or hsppofc@louisville.edu. Thank you for your submission.

Sincerely,

A handwritten signature in black ink that reads "Laura Clark M.D.". The signature is fluid and cursive, with the first name "Laura" and last name "Clark" being more prominent, and "M.D." written in a smaller, simpler script at the end.

Laura Clark, M.D., Chair,
Biomedical Institutional Review Board

CURRICULUM VITAE

PETER DZIEWORNU AHIAWODZI

3619 Fountain Drive #4
Louisville, KY 40218
(706) 254 8149

Dept. of Epidemiology and Population Health
University of Louisville
pdahia01@louisville.edu

EDUCATION

Ph.D. Epidemiology 2014

University of Louisville, Louisville, KY
Dissertation Topic: Markers and Mechanisms of Metabolic Aging
Dissertation Committee Chair: Richard A. Kerber, PhD
GPA: 3.84/4.00

Master of Public Health, Epidemiology 2010

University of Louisville, Louisville, KY
Thesis: H1N1 Hospitalizations in Louisville's Largest Hospital System,
Aug. 2009 to Jan. 2010
Advisor: Frank D. Groves, MD, MPH
GPA: 3.86/4.00

Bsc (Hons), Agricultural Economics 2001

Kwame Nkrumah University of Science and Technology, Kumasi-Ghana
Honors Thesis: An investigation into the effect of irrigation on capital accumulation
among farmers: a case study of the Afife Rice Irrigation Project in the southern part of
Ghana.
Advisor: Simon C. Fialor, Ph.D.
CWA: Second Class Upper

ACADEMIC AND RESEARCH EXPERIENCE

A. Ph.D. research activities:

1. The role of oxidative stress in aging and degenerative diseases

Aim: To explore possible pathways by which free radicals influence health status (especially in late-life).

2. The effect of telomere length on cardiovascular-related outcomes in the Framingham Heart Study

Aim: To investigate the effect of telomere length on morbidity and mortality from hypertension left ventricular hypertrophy, wall motion abnormality, stroke, and myocardial infarction in the Framingham Heart Study.

3. The interaction between sleep inadequacy and obesity on risk of diabetes in the epidemiologic follow-up study of NHANES I.

Aim: To investigate the joint effect of sleep and obesity on the increasing incidence of diabetes.

4. The relationship between Sleep-Disordered Breathing and Nε-carboxymethyl-lysine concentration in the Cardiovascular Health Study.

Aim: To investigate the relationship between sleep disordered breathing (SDB) assessed by apnea-hypopnea index (AHI), and carboxymethyl-lysine (CML) concentration in the Cardiovascular Heart Study (CHS) cohort.

5. Hypertension in young adults

Aim: To study the prevalence and incidence of hypertension among 20 to 40 year olds. The National Health and Nutrition Examination Survey (NHANES) was used and hypertension awareness, treatment and control were studied.

B. Graduate Research Assistant

2012-2014

Dept. of Epidemiology, University of Louisville

Supervisor: Richard Baumgartner, PhD

Duties:

- Literature reviews
- Web and database searches for articles to support manuscripts
- Data management and analysis
- Writing manuscripts for publication

C. Process evaluation for Susan G. Komen for the cure	2009
The investigation focused on statistics reflecting the trend of breast cancer within the Louisville Affiliate Area of the organization and surrounding areas to provide useful data for resource allocation.	

BOARD CERTIFICATION

CPH (Certified In Public Health), National Board of Public Health Examiners	2010
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ACADEMIC HONORS AND AWARDS

Ethnic Minority Tuition Award	2010-2011
Golden Key International Honors Society	2010

COMPUTER SKILLS

Proficient in Microsoft Office Suite (Word, Excel, Power Point, and Access)
 Proficient in statistical software such as SPSS and SAS
 Proficient in researching online databases and using search engines such as PubMed
 Moderate experience with Geographic Information Systems (GIS)

PROFESSIONAL MEMBERSHIP AND SERVICE

Peer Reviewer , Journal of Health Care for the Poor and Underserved (JHCPU)	
SGA Liaison : A Liaison between the doctoral students in the department of epidemiology and the student government association (SGA) of the University of Louisville	2013-2014
Member -Kentucky Public Health Association	2009-Present
Class Advisor - College of Education, Akatsi, Ghana	2003-2005
Member -Library Committee, Faculty of Agriculture, KNUST, Kumasi-Ghana	2000-2001
Organizing Secretary - Keta Secondary School Old Students Association, KNUST branch, Kumasi-Ghana	2000-2001

TEACHING EXPERIENCE

Tutor: College of Education, Akatsi-Ghana 2002-2005
Duties: Taught integrated science (Science and agriculture)

Instructor: Ohawu Agricultural College, Ohawu, Ghana 2001-2002
Duties: Taught agricultural economics and agribusiness

PUBLICATIONS

Published Abstracts

Ahiawodzi P, Hornung CA. Changes in the Prevalence of Hypertension among 21-40 Year Olds in the U.S; 2006-2008. *Am J Epidemiol.* 2011 Jun;173 Suppl 11:S174 (695-S).

in Abstracts of the 3rd North American Congress of Epidemiology. June 21-24, 2011. Montreal, Canada. *Am J Epidemiol.* 2011 Jun;173 Suppl 11:S1-331. PMID: 21661133

Ahiawodzi P, Hornung CA. Incident and prevalent pre-hypertension and hypertension among adults ≤ 40 years of age: awareness, treatment and control –NHANES 2005-2008. *Annals of Epidemiology*, Sept. 2013; 23 (9): p585 (P17-S)

PRESENTATIONS

Oral Presentations

Ahiawodzi P, Matt Zahn, Carrico RL. Groves FD. “H1N1 Hospitalizations in Louisville’s Largest Hospital System, August 2009 to January 2010” (Presented at the 62nd Annual Conference of the Kentucky Public Health Association, March 30, 2010. Louisville, Kentucky)

Poster Presentations

Ahiawodzi P, Hornung CA. Changes in the Prevalence of Hypertension among 21-40 Year Olds in the U.S; 2006-2008 (Presented at the 3rd North American Congress of Epidemiology, Montreal, Canada. June 21-24, 2011)

Ahiawodzi P, Hornung CA. Incident and prevalent pre-hypertension and hypertension among adults ≤ 40 years of age: awareness, treatment and control –NHANES 2005-2008. September 23, 2013 (Presented at the Annual Scientific Sessions of the American College of Epidemiology, Louisville, KY)