Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

[So Yeon Joyce Kong] Date

Oxidative Stress and Human Health

By

So Yeon Joyce Kong
Doctor of Philosophy

Department of Epidemiology

[Michael Goodman, M.D., M.P.H]
Advisor

[Roberd "Robin" Bostick, M.D., M.P.H]
Committee Member

[W. Dana Flanders, M.D., Sc.D.]
Committee Member

[William McClellan, M.D., M.P.H]
Committee Member

[Suzanne Judd, Ph.D., M.P.H]
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

Date

Oxidative Stress and Human Health

By

So Yeon Joyce Kong

B.S., Duke University, 2004
M.P.H., Yale University, 2008

Advisor: Michael Goodman, M.D., M.P.H.

An abstract of
a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Department of Epidemiology

2013

Abstract

Oxidative Stress and Human Health

By So Yeon Joyce Kong

The role of oxidative stress in disease causation has been the focus of research for several decades. Despite a considerable body of evidence from basic science and animal studies, observational studies and clinical trials evaluating the roles of pro- and anti-oxidant nutrients and other oxidative stress-related exposures yielded inconsistent results. We, and others, previously proposed an oxidative balance score (OBS) as a measure of combined pro- and anti-oxidant exposure status. Using this method, the individual oxidative stress-related exposures are combined such that higher OBS values reflect the relative predominance of anti-oxidant factors. The primary objective of this dissertation is to examine the association between OBS and human degenerative diseases and mortality.

In the first study, I used data from a large national prospective cohort study, Reasons for Geographic and Racial Differences in Stroke (REGARDS) to examine the association between OBS and all-cause and cause-specific mortality while exploring alternative methods of weighting the OBS components. Higher OBS was associated with reduced risk of all-cause, and particularly cancer mortality. Similar results were observed across all weighting methods. In the second study, I examined the association between OBS and incident stroke using the same methods of score weighting. I found that higher OBS had no significant effect on incident stroke or stroke mortality, irrespective of the weighting scheme. In the third study, I extended the previous analyses of questionnaire-based OBS and colorectal adenoma by assessing: 1) the association between plasma nutrient-based OBS and colorectal adenoma; 2) the association of OBS with biomarkers of oxidative stress (F_2-isoprostanes [FIP] and fluorescent oxidation products [FOP]), and with biomarker of inflammation (C-reactive protein [CRP]); and 3) the association of each of the three biomarkers with adenoma. OBS was inversely associated with colorectal adenoma, plasma FIP, and serum CRP. However, there was significant positive association between higher OBS and elevated levels of FOP. All three biomarkers were directly related to adenoma risk.

This dissertation research has important implications for epidemiologic studies evaluating the roles of oxidative stress in chronic disease etiology by showing that OBS is associated with the risks of certain (but not all) age-related degenerative conditions.

Oxidative Stress and Human Health

By

So Yeon Joyce Kong

B.S., Duke University, 2004
M.P.H., Yale University, 2008

Advisor: Michael Goodman, M.D., M.P.H.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Department of Epidemiology

2013

Table of Contents

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1. INTRODUCTION AND BACKGROUND

Introduction

Oxidative stress is defined as an imbalance between pro-oxidants and antioxidants in favor of the former, resulting in an overall increase in cellular levels of free radicals, generally known as reactive oxygen species (ROS) [1]. Oxidants are generated during the leakage of electrons from mitochondria, during the electron-transport steps of ATP production and can be a by-product of normal cellular aerobic metabolism [2]. ROS are generated by both endogenous and exogenous sources [3, 4]. Endogenous free radicals are generated from immune cell activation, inflammation, ischemia, cancer, and aging while exogenous free radicals are resulted from environmental factors such as pollution, cigarette smoke, alcohol, heavy or transition metals, certain dietary components (such as fat), and radiation [5]. Under normal physiologic conditions, cells respond to oxidative stress by up-regulating antioxidant defense mechanisms and other protective systems to restore the balance [6]. However, when these mechanisms are overwhelmed, oxidative stress can damage DNA, proteins, and lipids and lead to cell injury and death [7, 8].

In order to protect cells against oxidative stress, organisms have evolved to possess a variety of enzymatic and non-enzymatic antioxidant defense mechanisms [9]. Endogenous and exogenous antioxidants prevent and repair damages caused by oxidative stress by scavenging free radicals. Endogenous cellular antioxidant defenses are represented by enzymes (e.g., superoxide dismutase, gluthathione peroxidase, and catalase), which counterbalance oxidative microenvironments by chelating superoxide and other peroxides [4]. Non-enzymatic antioxidants are exogenously supplied through

food and/or supplements, which are represented by ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids, flavonoids, and other antioxidants [10].

For several decades, there has been a great interest in the role of oxidative stress in disease causation and prevention. Basic research has established a link between oxidative stress and pathogenesis of human illness, including cardiovascular disease and cancer, as well as, more broadly, with the process of aging [11-15]. However, despite a considerable body of evidence from basic science and animal studies supporting the role of oxidative stress in aging and human diseases, observational studies and even clinical trials evaluating the roles of antioxidants and other oxidative stress-related exposures and interventions yielded inconsistent results [16-20]. Currently, there is no clear evidence whether or not antioxidant intake delays mortality or reduces risk of chronic diseases.

One potential explanation for this discrepancy is the complex and multi-factorial mechanism by which oxidative stress may affect human health. The independent effects of individual oxidant exposures are difficult to ascertain because these effects may be highly correlated and because of the likely biological interactions involving multiple pro- and anti-oxidant factors [21]. Previous studies have shown that a combination of several risk factors may reveal overall substantial and significant increase in risk, even when associations with each individual factor are relatively weak and inconsistent [22-24]. These findings suggest that factors acting or/and interacting along a same etiologic pathway may need to be evaluated in aggregate rather than individually.

We, and others, previously proposed an oxidative balance score (OBS) as a measure of combined pro- and anti-oxidant exposure status. Using this method the individual oxidative stress-related exposures are combined such that higher OBS values

reflect the relative predominance of anti-oxidant factors. We illustrated this approach using data from two previously-conducted case-control studies of two different neoplasms – incident sporadic colorectal adenoma and prostate cancer [25-27].

Although our initial finding linked OBS to certain specific conditions, it is expected that oxidative stress may have a broader role in aging and may affect a wide array of human degenerative diseases. The objective of this dissertation is to further clarify the role of oxidative stress, using OBS method, in aging and mortality. The specific research questions are: 1) are high levels of OBS (indication of predominantly antioxidant exposures) associated with reduced mortality; 2) are high levels of OBS associated with reduced risk of stroke; and 3) is OBS associated with markers of oxidative stress (F_2-isoprostanes [FIP] and fluorescent oxidation products [FOP]) and inflammation (CRP)? These research questions will be addressed by using data from two previously-conducted case-control studies and from a national, population-based, longitudinal cohort study.

Background

Overview of Oxidative Stress and Antioxidant Defense System

In 1956, Harman proposed the "free radical theory of aging", which postulates that the process of aging and age-related diseases are caused by the accumulation of deleterious changes in the cell attributed to free radical reactions [28]. Free radicals are molecules with one or more unpaired electrons in the outer shell. Because of unpaired electrons, free radicals are very unstable and thus highly reactive. Harman's free radical theory triggered intensive research on the role of free radicals, more often known as

"reactive oxygen species" (ROS). ROS are oxygen-containing chemical species with reactive chemical properties, which include free radicals with unpaired electron such as superoxide (O_2•−) and hydroxyl radicals (HO•), as well as certain highly reactive non-radical molecules such as hydrogen peroxide (H_2O_2) [29].

ROS can be generated from either endogenous or exogenous sources (Figure 1.1). In every living organism, ROS are continually generated endogenously as by-products of normal aerobic metabolism in the mitochondria through electron leakage from electron transfer reactions. Production of endogenous ROS can also be caused by immune responses secondary to inflammation and infection, although ROS is also known to stimulate inflammation [11]. Exogenous ROS result from sources such as pollution, cigarette smoke, heavy or transition metals, fat, and radiation [10, 30-33]. At low concentrations, ROS play beneficial role in biological systems, as for example, in defense against infectious agents and in the function of a number of cellular signaling systems and mitogenic response [32]. In contrast, at high concentrations, the ROS exert harmful effects by damaging cell structures and macromolecules, a process which is termed "oxidative stress" [34].

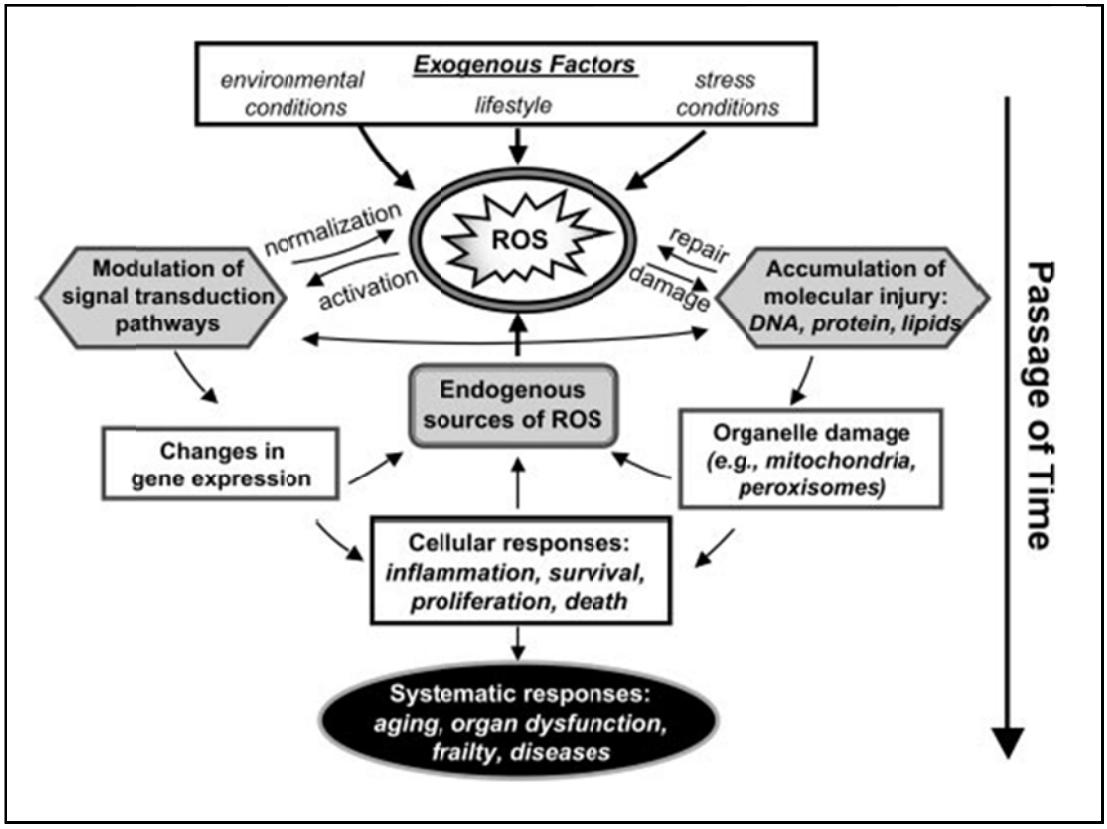

Figure 1.1. A schematic summary of proposed mechanisms by which ROS and oxidative stress could contribute the process of aging and chronic diseases [35].

In order to counterbalance the effects of free radicals and minimize oxidative stress, organisms have developed antioxidant defense systems. These defense systems involve scavenging and de-activation of ROS molecules to transform them into stable (i.e., less chemically active) molecules [4, 13, 36]. Antioxidant defense systems can act through three main mechanisms: antioxidant enzymes, chain breaking antioxidants, and transition metal binding proteins (Figure 1.2).

The *antioxidant enzymes* include catalase, glutathione peroxidases and superoxide dismutases. These enzymes are called preventive antioxidants because they prevent the uncontrolled formation of free radicals by catalysing the breakdown of free radical species [37]. When a free radical interacts with another molecule, secondary, often more

active and more damaging, radicals are often generated. This process is referred to as "free radical chain reaction." The chain reaction continues until two radicals combine to form a stable product or the radicals are neutralized by a *chain breaking antioxidant* [38]. The chain breaking antioxidants can act either in a lipid or an aqueous phase. Lipid phase antioxidants, such as vitamin E, carotenoids, and flavonoids, scavenge radicals in membranes and lipoprotein particles and play crucial role in preventing lipid peroxidation. Examples of chain breaking antioxidants that directly scavenge radicals in the aqueous compartment include vitamin C, uric acid, and glutathione.[33].

The hydroxyl radical (•OH) is a highly reactive free radical, which is probably the final mediator of most free radical induced damage [39]. The most important mechanism of hydroxyl radical formation involves transition metals (e.g., iron or copper), which catalyze decomposition of superoxide and hydrogen peroxide[40]. This process is countered by *the transition metal binding proteins* (e.g., ferritin, transferrin, lactoferrin, or caeruloplasmin), which act as antioxidants by isolating iron or copper molecules so that they are not available for the formation of hydroxyl radicals.

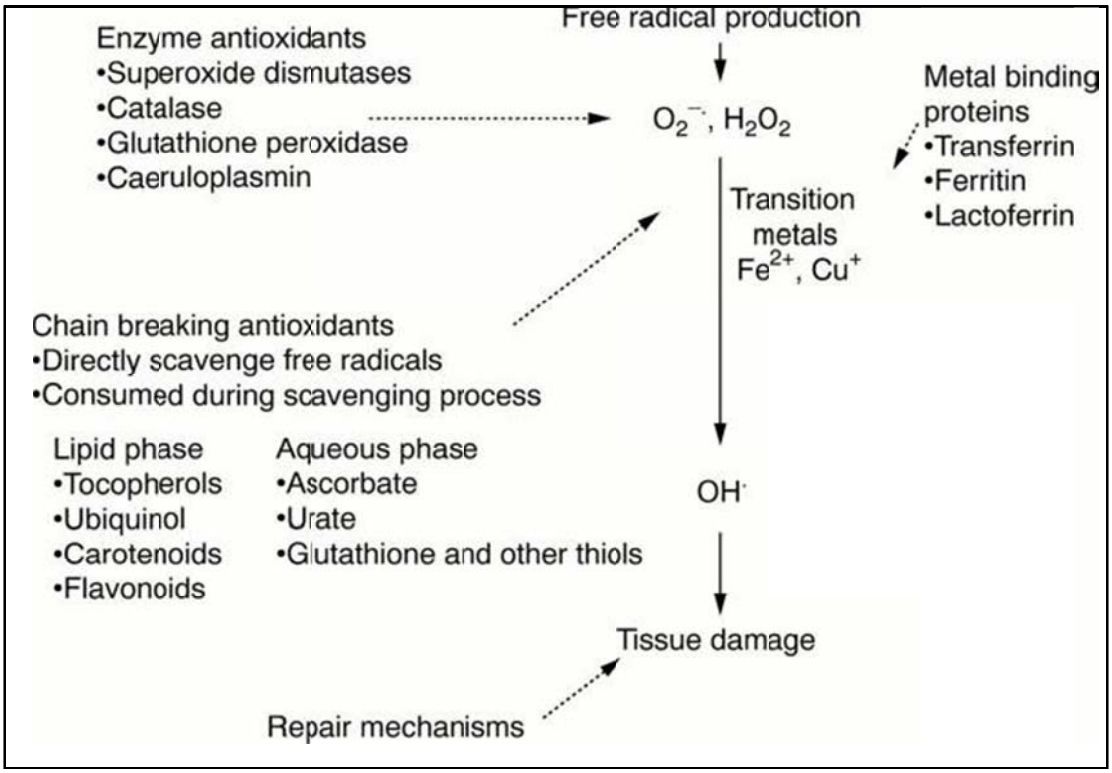

Figure 1.2. Antioxidant defense systems against free radical production [33].

Individual Pro-oxidant Factors

Iron

Many researchers have focused on metal-induced toxicity and carcinogenicity, and on the role of metals in the generation of ROS [31, 40, 41]. Iron is an important metal pro-oxidant, which is has been shown to generate oxidative stress through increasing the steady state concentration of ROS [42]. The toxicity of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) increases when they convert into the extremely reactive hydroxyl radical (•OH) in via a reaction catalyzed in the presence of iron[43]. Epidemiological evidence suggests that elevated body iron stores and dietary iron intake are both associated with risk for developing oxidative stress-related conditions including

heart disease and cancer as well as overall mortality [44-49]. Some researchers proposed that exposure to ingested iron may be a principal cause of human colorectal cancer in developed countries with high levels of meat consumption [50, 51].

Polyunsaturated Fatty Acids (PUFA)

Polyunsaturated fatty acids (PUFA) are known to be highly susceptible to free radical initiation and lipid peroxidation, particularly when the level of antioxidants, such as vitamin E, are low [52]. PUFA have methylene group between two double bonds. This bis-allylic structure makes polyunsaturated fatty acids more prone to oxidation and enables them to participate in free radical chain reactions [35]. PUFAs are classified as *n*-6 and *n*-3 based on the location of the last double bond [53, 54]. The *n*-6 and *n*-3 PUFAs are metabolically and functionally distinct and play different roles in regulation of inflammatory process [54]. The *n*-6 PUFAs are known to be pro-inflammatory while *n*-3 PUFAs are anti-inflammatory [55]. Therefore, balance of these PUFAs plays important role in development of inflammatory (and by extension oxidative stress-related) diseases. A high intake of *n*-6 PUFAs, such as through consumption of vegetable oils and sunflower oils, is a potential contributor of inflammatory process. On the other hand, high intakes of *n*-3 PUFA-rich foods, such as oily fish can help reduce inflammation [53, 56].

Smoking

Smoking is the major independent risk factor for most chronic diseases, including various types of cancers and stroke. Cigarette smoke is known to contain approximately 60 carcinogenic agents and has been considered as one of the most established

environmental sources of pro-oxidants [4]. The pro-oxidants contained in cigarette smoke can enter the bloodstream, and therefore induce DNA and tissue damage and cell death not only in the respiratory epithelium but also in other organs and tissues [57, 58]. Smoking-induced activation of inflammatory cells serves as another source of oxidative stress [59]. Cigarette smokers have been shown to have elevated levels of oxidative stress biomarkers such as F_2-isoprostanes [60] and urinary oxidized DNA products [61].

Alcohol

Chronic alcohol intake has long been shown in animal studies to increase production of ROS, such as superoxide, hydrogen peroxide, and hydroxyl radicals, during microsomal ethanol oxidation [62, 63]. In humans, the three metabolic pathways of ethanol involve enzymes alcohol dehydrogenase and catalase, and microsomal ethanol oxidation system (MEOS). Each of these metabolic pathways produces ROS and affects the antioxidant system [64]. In addition to its direct role in production of ROS, alcohol facilitates the formation of oxidative microenvironment that worsens the effects of hypoxia, endotoxemia, and cytokine release, thus creating suitable conditions for the development of oxidative stress-related chronic diseases [64-66]. Further, alcohol depletes levels of cellular antioxidant enzymes such as glutathione (GSH) [64].

Inflammation

Inflammation is known to play a critical role in production of ROS and initiation of oxidative stress [11]. Activated neutrophils can kill bacteria by imposing severe oxidative stress, therefore, if the number of activated cells is large and/or inflammation goes on for long time, serious damage may occur [67]. In 1863 Virchow hypothesized that cancer originates at the sites of inflammation. At present, there seems to be a

consensus that chronic inflammation may serve as a critical component of tumor development and progression [68]. Interaction of cellular immune system with antigens generates ROS and triggers production of pro-inflammatory cytokines and chemokines, which then induce production of ROS [69, 70]. Conversely, oxidative stress can stimulate inflammatory response by activating a variety of transcription factors, such as NF-kB, AP-1, and p53, which then lead to expression of inflammatory cytokines and chemokines [1, 71]. Thus, inflammation is both a cause and a consequence of oxidative stress. Antioxidants, such as vitamin C and β-carotene, have been known to possess anti-inflammatory properties [72-74].

Individual Anti-oxidant Factors

Carotenoids

Carotenoids are a group of lipid soluble antioxidants found in yellow and green vegetables such as carrots, spinach, and sweet potatoes. The major dietary carotenoids include α-carotene, β-carotene, lycopene, lutein, β-cryptoxanthin, and zeaxanthin. Carotenoids are known to be efficient scavengers of free radicals, and thus play an important role in preventing lipid peroxidation [75]. The antioxidant property of carotenoids is mainly due to their conjugated double-bonded structure that allows delocalizing unpaired electrons [76]. Carotenoids react with free radicals through radical addition, hydrogen abstraction from carotenoids, or electron-transfer reaction [77]. In addition to their direct antioxidant action certain carotenoids also act as precursors of other more powerful antioxidants [78].

Vitamin C

Vitamin C, also known as ascorbic acid, is a major aqueous-phase antioxidant. Under physiological conditions, it functions as a potent free radical scavenger in the plasma. [79, 80]. The antioxidant property of vitamin C is attributed to its ability to form relatively stable ascorbate radicals [81]. Humans normally acquire vitamin C from a variety of dietary sources, such as acid fruits and green vegetables [82, 83].

Vitamin E

Vitamin E is an important micronutrient essential for human health and one of the most well studied antioxidants [84]. It is a mixture of fat-soluble, naturally occurring compounds that include four tocopherols and four tocotrienols designated as α-, β-, γ-, and δ- each with its own biological activity and functional use [6, 85]. Tocopherols are most commonly found in vegetable oils and nuts and tocotrienols are found in palm oil, oat, barley, and rye [86]. α-Tocopherol is the most predominant and bioactive form of vitamin E in humans [87]. The oxidative modification of low-density lipoprotein (LDL) is considered to be a key step in the initiation and progression of cardiovascular disease [88-90]. Vitamin E, especially α-tocopherol, is thought to inhibit the oxidation of LDL thereby reducing the risk of cardiovascular disease [91].

Selenium

Selenium is an essential trace element with important clinical effects [92-94]. There are two forms of selenium in tissues: selenomethionine, the major form of dietary selenium, which account for at least 50% of the selenium in the diet, and selenocysteine, which accounts for the biological activity of selenium in the selenoproteins [95, 96].

Selenocysteine is an integral part of the active center of the glutathione peroxidase enzyme (GSH-Px), which is one of the principal antioxidant systems. A deficiency in selenium leads to profound reduction in the activity of GSH-Px in several tissues, resulting in oxidative stress [97].

Consequences of Oxidative Damage

DNA Damage

Permanent modification of genetic material resulting from oxidative damage is known to be the first step in mutagenesis, carcinogenesis, and ageing [10]. Oxidative stress can elicit a wide variety of DNA damage including strand breaks, sister chromatid exchange, DNA-DNA and DNA-protein cross-links, and base modifications [98]. Two most common by-products of DNA base modifications are 8-oxo-7,8-dihydroguanine (8-OH-dG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyAde) [3, 99]. Of those, 8-OHdG is notable as the most commonly used biomarker for DNA damage.

Lipid Peroxidation

Oxidative damage of lipids occurs when polyunsaturated fatty acids (PUFA) in cell membranes are exposed to ROS. The bis-allylic structures of PUFA make them very sensitive to oxidation and have been found to be frequent targets of ROS-induced damage [6, 35]. Excess free radicals can damage cell membranes and lipoproteins by lipid peroxidation. Lipid peroxidation can lead to altered cell membrane structure, impaired function, and cell loss [100]. Moreover, lipid peroxidation products may inflict secondary damage through DNA adduction, and inhibition of enzyme function [98]. The

most common products of lipid peroxidation are isoprostanes and malondialdehyde (MDA), which act as useful biomarkers of oxidative stress-induced damage.

Protein Damage

Extensive studies have demonstrated that oxidative stress also modifies proteins eventually leading to loss of cellular structure and function [101-104]. The modification of proteins is mainly initiated by reactions with hydroxyl radical (•OH), and during these reactions, oxidized amino acid residue side chains form protein-protein cross-linkages. In addition, oxidation of the protein backbone may result in protein fragmentation [101]. Most of the oxidized amino acids are eliminated, used as carbon sources for ATP synthesis, or reutilized for protein synthesis. Therefore, the level of oxidized protein reflects the balance between the rate of protein oxidation and the rate of oxidized protein degradation, which is dependent upon the balance between pro-oxidant and antioxidant factors [98]. This process can be monitored by measuring circulating levels of nitrotyrosine, which is a useful biomarker of oxidative stress-induced protein damage [105].

Oxidative Stress and Cancer

Cancer is a multistep process that involves multiple molecular and cellular carcinogenesis mechanisms [106]. Oxidative stress has long been known as a trigger for tumor development. Cells in every organism are exposed to various oxidizing agents both endogenous and exogenous sources. It is estimated that each human cell is exposed to approximately 10^5 oxidative attacks a day from hydroxyl radical and other reactive oxygen species [107-109].

As illustrated in Figure 1.3, oxidative stress may cause DNA, RNA, protein, and/or lipid damage, leading to chromosomal instability, genetic mutations, and/or modulation of cell growth that result in cancer [106]. Oxidative DNA damage is a major source of DNA mutations, with over one hundred oxidative adducts having been identified [110, 111]. It is estimated that on average there are several thousands of DNA alterations per day in each cell caused by both endogenous and exogenous oxidative stress [112]. The most well-established and abundant oxidative DNA base lesion is 8-hydroxydeoxy guanosine (8-OH-dG) [113].

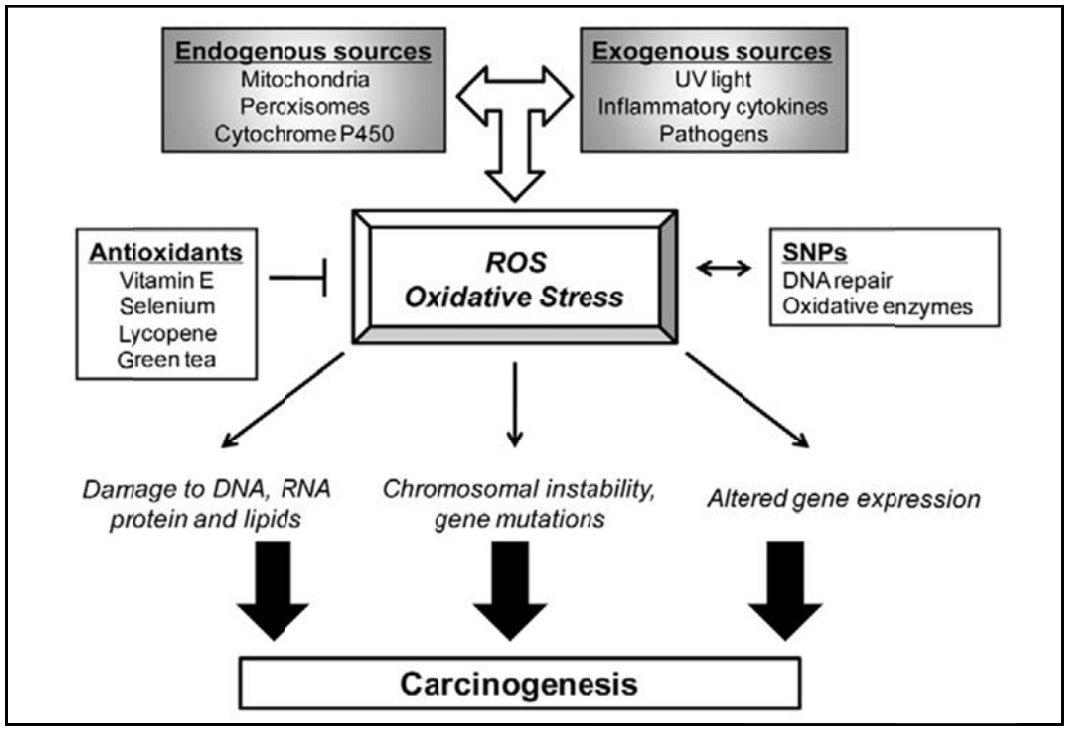

Figure 1.3. Reactive oxygen species (ROS) and their role in the development of human cancer [106].

Considerable evidence supports oxidative stress and DNA damage as critical factors in the development of various cancers as shown in Table 1.1. For example, Nyaga *et al.*[114] showed that breast cancer cells are highly susceptible to oxidatively

induced DNA damage, supporting the implication of the oxidative stress in the etiology of breast cancer. Kondo *et al.* [115] demonstrated that all clinical stages of colorectal adenoma cells are exposed to more oxidative stress than non-tumor epithelial cells and tumor cells have the capacity to adjust oxidative stress to a level sufficient to stimulate tumor proliferation. Lee *et al.* [116] found the total mean levels of 8-OH-dG were significantly higher in gastric cancer patients than in normal populations, suggesting that patients with gastric cancer are exposed to a higher level of oxidative stress. Other studies have demonstrated that elevated 8-OH-dG levels are associated with prostate, esophageal, and liver cancers [117-119].

Table 1.1. Findings of elevated levels of oxidative stress and/or DNA damage in human malignancies [3].

Type of Cancer	Study Model	Findings
Breast	Human breast cancer cell lines	Accumulation of oxidatively induced DNA damage in human breast cancer cell lines following treatment with hydrogen peroxide [114]
	Breast cancer patients	Mean levels of 5-hydroxymethyl-2'-deoxyuridine, one form of oxidative DNA damage, were significantly higher in blood of women with high risk or invasive break lesions vs. women with benign lesions [120]
Colorectal	Colorectal tumor patients	Colorectal carcinoma were exposed to more oxidative stress (significantly higher levels of 8-oxodG in nuclear DNA of primary adenocarcinoma) than their corresponding non-tumorous epithelial cells [115]
Gastric	Gastric cancer patients	Significantly higher levels of 8-oxodG in DNA from tumor-adjacent and tumor adenocarcinoma tissues than in normal tissue ($p<0.001$) of gastric cancer patients [116]

Gynecologic	Female cancer patients	Significantly higher ($p \leq 0.05$) levels of urinary 8-oxodG in patients with gynecological cancer compared to control subjects [121]
Lung	Lung cancer patients	Lymphocyte DNA levels of 8-oxo-dG significantly elevated ($p<0.05$) in patient with lung cancer compared to controls [122]
Prostate	Prostate cancer patients	Significantly higher urinary 8-OHdG to Creatine (8-OHdG/Cr) in patient with prostate cancer compared to controls ($p<0.05$) [118]

Oxidative Stress and Stroke

Atherosclerosis is characterized by the accumulation of cholesterol deposits in arteries, This process leads to a proliferation of certain cell types within the arterial wall and gradually reduces blood flow and oxygen supply to target organs such as the heart and the brain [123]. The disturbance in brain function as a result of impairment of blood supply is called stroke. Stroke is the third leading cause of death and the number one cause of disability in the United States. Each year, approximately 795,000 people experience a new or recurrent stroke, of these 87% are produced by ischemia, 10% by intracerebral hemorrhage, and 3% by subarachnoid bleeding.

A considerable body of evidence indicates that oxidative stress is a fundamental mechanism of brain injury in stroke [124]. Brain tissue is particularly susceptible to ROS-induced damage because it contains high concentrations of peroxidisable lipids, has low levels of protective antioxidants, is characterized by high oxygen consumption, and possesses high levels of iron which is a potent pro-oxidant [125-128]. Reactive oxygen species have significant effects on both cellular and vascular brain function. Cellular

effects of ROS include lipid peroxidation, protein denaturation, DNA modification, damage to the cytoskeletal structure, and chemotaxis, which are consequences of oxidative stress [124]. At vascular level, ROS exert their effects at very low concentrations, leading to increased vasodilation, platelet aggregation, increased endothelial permeability, altered reactivity to vasodilators, and formation of focal lesions in endothelial cell membrane [129].

Oxidative Stress and Aging

The free-radical theory of aging postulates that the process of aging process is the result of cumulative damage induced by free radical production in aerobic organisms [28]. This theory is based on the fact that the random deleterious effects of free radicals produced during aerobic metabolism accumulate over time, leading damage to DNA, protein, and lipids [130]. Under normal physiological conditions, electrons are constantly leaking from the electron transport chain and interact with oxygen to produce superoxide radicals [131]. The primary site of radical oxygen damage from these superoxide radicals is mitochondrial DNA (mtDNA). As the mtDNA damage accumulates over time, mitochondria are eventually shutting down, causing cells to die, and organisms to age [131]. Such accumulating damage is believed to be crucial in the process of aging process [132, 133].

Disappointing Results from Epidemiological Studies of Anti-oxidants

While a considerable body of evidence from basic science and animal studies supports the profound role of oxidative stress in pathogenesis of chronic diseases, outcomes of large, prospective, randomized clinical trials on the association between anti-oxidant supplements and these diseases have remained largely inconclusive. The

following sections summarize the largest, most highly publicized trials of antioxidant supplements conducted to date

The ATBC (Alpha-Tocopherol Beta-Carotene) [134] trial was one of the first large, randomized clinical trials showing that supplemental α-tocopherol and β-carotene have no preventive effect on the risk of cancer or cardiovascular diseases. The ATBC trial was conducted among 29,133 male smokers in Finland, and found no reduction in CHD morbidity and mortality with vitamin E (50 mg daily) and/or β-carotene (20 mg daily) supplementation. Furthermore, there was a significant 18% increase in the incidence of lung cancer among those with β-carotene supplements. Importantly, the β-carotene dosage of 20 mg/day was substantially higher than that of typical Finnish diet [32].

Unexpected increases in risk of both lung cancer and cardiovascular disease mortality were also observed in the CARET (Beta-Carotene and Retinol Efficacy Trial), a multicenter, randomized, double-blinded, placebo-controlled trial conducted in the United States [135]. In the CARET study, a total of 18,314 current and former smokers, and workers exposed to asbestos were randomized to receive 30 mg of β-carotene and 25,000 IU of retinol (vitamin A) versus placebo. After an average four years of supplementation, the active-treatment group with both β-carotene and retinol had a relative risk of lung cancer of 1.25 (95% CI: 1.07 – 1.57), as compared with the placebo group. In the active-treatment group, the relative risks of death were 1.17 (95% CI: 1.03 – 1.33) for any cause, 1.46 (95% CI: 1.07 – 2.00) for lung cancer, and 1.26 (95% CI: 0.99 – 1.61) for cardiovascular disease.

SELECT (Selenium and Vitamin E Cancer Prevention Trial) [136] was a randomized trial of selenium (200 μg/day), vitamin E (400 IU/day), or both as chemoprevention agents against prostate cancer in 35,533 men in multiple participating sites in the United States. After a median follow-up of 5.5 years, the rate ratios for prostate cancer were 1.13 (99% CI: 0.95 – 1.35) for vitamin E, 1.04 (99% CI: 0.87 – 1.24) for selenium, and 1.05 (99% CI: 0.88 – 1.25) for combination of selenium and vitamin E when compared with those with placebo. There were also no significant differences ($p > 0.15$ for all) in any other cancer end points.

The Women's Health Study (WHS) [137] investigated the effects of vitamin E supplementation on risks of cardiovascular diseases and cancer. In this randomized, double-blind, placebo-controlled, 2x2 factorial trial, 39,876 apparently healthy US women aged at least 45 years were randomly assigned to received vitamin E (600 IU) or placebo and aspirin or placebo on alternate days. After average of 10.1 years of follow-up, there was no significant effects of vitamin E on the incidences of myocardial infarction (RR = 1.01; 95% CI: 0.82 – 1.23) or stroke (RR = 0.98; 95% CI: 0.82 – 1.17). Stratification of results by type of stroke (ischemic or hemorrhagic) also showed no effect of intervention. There was no significant effect on the incidences of total cancer (RR = 1.01; 95% CI: 0.94 – 1.08), as well as specific types of cancer, such as breast (RR = 1.00; 95% CI: 0.90 – 1.12), lung (RR = 1.09; 95% CI: 0.83 – 1.44), or colon cancers (RR = 1.00; 95% CI: 0.77 – 1.31). The authors concluded that the data from this large trial indicated that vitamin E provided no overall benefit for major cardiovascular events or cancer.

Another randomized, double-blinded, placebo-controlled trial, the Women's Antioxidant Cardiovascular Study (WACS) [138], evaluated the effects of three antioxidant agents, vitamins C (500 mg daily), E (600 IU every other day), and beta-carotene (50 mg every other day), on prevention of cardiovascular diseases among 8,171 female health professionals at high risk. After an average of 9.4 years of follow-up, there was no overall effect of vitamin C (RR = 1.02; 95% CI: 0.92 – 1.13), vitamin E (RR = 0.94; 95% CI: 0.85 – 1.04), or beta-carotene (RR = 1.02, 95% CI = 0.92 – 1.13) on a combined end point of cardiovascular disease morbidity and mortality, or on the individual outcomes of myocardial infarction, stroke, coronary revascularization, or cardiovascular disease death.

While some observational studies on dietary antioxidants support a role of antioxidant in reducing risks of chronic diseases, results are on balance inconclusive. A recent meta-analysis [139] critically reviewed the evidence to determine whether foods rich in lycopene, β-carotene, α-carotene, and β-cryptoxanthin were associated with a reduced risk of cancer in cohort studies. None of the carotenoids showed a significant reduction in risk of any cancer with either increased carotenoid intake or higher circulating levels. The pooled estimates of risk of any cancer with increased intake/circulation level of β-carotene, lycopene, α-carotene, and β-cryptoxanthin were 1.01 (95% CI = 0.88 – 1.16), 0.99 (95% CI: 0.94 – 1.05), 0.91 (95% CI: 0.78 – 1.05), and 1.08 (95% CI: 0.95 – 1.23), respectively.

Similar findings were reported in another recent meta-analysis of cohort studies evaluating the association between antioxidant intake and the risk of coronary heart disease (CHD) [140]. This meta-analysis combined results from 15 prospective cohort

studies, comprising a total of more than 370,000 participants. In the dose-response meta-analysis, each 30 mg/day increase in vitamin C, 30 IU/day increase in vitamin E, and 1 mg/day increase in β-carotene yielded the estimated overall relative risk of 1.01 (95% CI = 0.99 - 1.02), 0.96 (95% CI: 0.94 -0.99), and 1.00 (95% CI: 0.88 -1.14), respectively.

Oxidative Balance Score (OBS)

Despite the solid molecular and mechanistic theory of oxidative stress and its role in chronic diseases, most clinical trials and observational studies failed to show the beneficial role of antioxidants in preventing chronic diseases. One potential explanation for this discrepancy is the complex and multi-factorial mechanism by which oxidative stress may affect human health. The independent effects of individual oxidant exposures may not offer complete insight into the pathogenesis of human disease because of the likely high correlations among various factors and because of biological interactions involving multiple pro- and anti-oxidants [21]. Data from *in vitro* and animal studies suggest that there are biochemical interactions among antioxidants. For example, it has been shown that the antioxidant activity of selenium depends on the presence of vitamin E [141, 142]. Further, vitamin C is thought to affect the activity of vitamin E by regenerating its reduced form [5]. Most antioxidant micronutrients, such as vitamin E, vitamin C, and carotenoids, when consumed as part of the diet, do not act in isolation, but as part of a package along with multiple other antioxidants. Therefore, a complex interplay among pro- and anti-oxidants, makes it difficult to predict how an individual antioxidant will function [33].

To deal with difficulties of analyzing independent effects of oxidative stress-related exposures, which may be highly inter-correlated several authors proposed

combining individual pro- or anti-oxidants into a single index or score. The term "oxidative balance score" (OBS) was first proposed by van Hoydonck and colleagues in 2002 [143]. The authors combined intakes of dietary antioxidants (vitamin C and β-carotene) and a pro-oxidant (iron) to investigate whether the oxidative balance of their dietary pattern affected mortality risk in 2,814 male Belgian smokers. In this study, male smokers with a diet relatively low in vitamin C and β-carotene and/or high in iron (highest OBS group) had a 44% higher risk for all-cause mortality and 62% higher risk in total cancer mortality when compared with those in the lowest OBS group. We previously illustrated the OBS approach by using data from two previously conducted case-control studies: a colonoscopy-based colorectal adenoma study (markers of adenomatous polyps, or MAP) and a population-based prostate cancer study (markers of prostate cancer, or MPC) [26]. Using previously collected data from these two studies, we developed a summary OBS by including 12 *a priori* selected antioxidants (tocopherol, carotene, vitamin C, lycopene, lutein/zeaxanthin, β-cryptozanthin, use of aspirin and NSAID, and selenium) and pro-oxidants (saturate fat, iron, and smoking history). The OBS was calculated by combining points assigned for each individual OBS component and categorized into equal intervals. Unlike van Hoydonck et al study [144], the higher OBS values in our analyses reflected predominance of anti-oxidant (versus pro-oxidant exposures) and thus were expected to be associated with lower disease risk. We observed a substantial decrease in risk associated with a high OBS category for both colorectal adenoma and prostate cancer. There were approximately 55 - 70% reductions in both colorectal adenoma and prostate cancer risks when the highest category of OBS compared to lowest category, although the test for trend was only significant in the MAP

study. By contrast, we observed no discernible pattern in the individual OBS components.

We further extended our previous analysis by substituting questionnaire-based measures with systemic biomarkers of pro- and anti-oxidant exposures using same case-control studies of MAP and MPC [27]. When OBS was treated as a continuous variable, there was a statistically significant 10% reduction in risk of both sporadic colorectal adenoma and prostate cancer (OR = 0.90; 95% CI = 0.83 – 0.97) for each additional score point. When the OBS was divided into three approximately equal intervals, there was about 70% reduction in risk of both neoplasms with adjusted ORs of 0.34 (95% CI = 0.13 – 0.88) and 0.34 (95% CI = 0.14 – 0.86) for adenoma and prostate cancer, respectively. These results further supported our hypothesis that combined measures of pro- and antioxidant exposures may be associated with oxidative stress-related conditions such colorectal neoplasia and prostate cancer.

More recently, the method of OBS was adopted by Agalliu and colleagues [144] to further investigate the association between oxidative balance score and risk of prostate cancer in a case-cohort study (661 cases and 1,864 subcohort) nested within the Canadian Study of Diet, Lifestyle, and Health cohort. In that, participants completed self-administered lifestyle and food frequency questionnaires (FFQ), which assessed usual intake over the past year of 166 food items at baseline. The OBS was calculated by combining individual scores from five pro-oxidant (smoking, alcohol consumption, intake of polyunsaturated fats, daily red meat intake, and total iron intake) and eight anti-oxidant exposures (β-carotene, vitamins C and E, β-cryptoxanthin, lycopene, and lutein and zeaxanthin, daily consumption of cruciferous vegetables, and selenium supplements)

with higher values indicating higher antioxidant status. Agalliu found that there was no association between OBS and overall risk of prostate cancer with hazards ratios (HRs) of 1.00, 1.02. 1.03, 0.97, and 1.01 for increasing quintiles of the score (P_{trend} = 0.71). Similar associations were found when the analysis was stratified by the stage of the diseases and restricted to incident cases that arose after two years of follow-up.

DISSERTATION RESEARCH PLAN

Objectives, Specific Aims and Study Hypotheses

The primary objective of this dissertation is to investigate associations of oxidative balance score (OBS) with risk of mortality and stroke incidence. Furthermore, I will investigate the association between OBS and markers of oxidative stress (F_2-isoprostanes [FIP] and fluorescent oxidation products [FOP]) and inflammation (C-reactive protein [CRP]). The objectives of this study will be achieved by addressing three specific aims and by testing the following hypotheses.

Aim #1: Using data from the Reasons for Geographic and Racial Differences in Stroke (REGARDS) national cohort (n=30,176), investigate whether high OBS is associated with reduced all-cause and cause-specific mortality. I hypothesize that exposure to these 14 pro- and anti-oxidant is a cause of premature death. This combination of exposures is measured by a weighted oxidative balance score (OBS).

Aim #2: Using data from the Reasons for Geographic and Racial Differences in Stroke (REGARDS) national cohort (n=30,176), investigate whether high OBS is associated with reduced risk of incident stroke. I hypothesize that exposure to

these 14 pro- and anti-oxidant is a cause of stroke. This combination of exposures is measured by a weighted oxidative balance score (OBS).

Aim #3: Using data from two previously conducted case-control studies of Markers of Adenomatous Polyps (MAP) Study I and II, investigate whether OBS is associated with markers of oxidative stress (FIP and FOP) and inflammation (CRP). I hypothesize that there are inverse associations between OBS and markers of oxidative stress and inflammation.

Methods for Aims 1 and 2:

Data sources

To address the first two questions (Aim #1 and #2), I will use data from Reasons for Geographic and Racial Differences in Stroke (REGARDS), a national, population-based cohort study of approximately 30,000 African-American and white individuals over the age of 45 years. The objective of REGARDS is to identify the risk factors for the excess stroke mortality in the Southeastern US and particularly among African-Americans.

Between January 2003 and October 2007, 30,239 REGARDS participants were randomly selected and recruited through mail and telephone contacts. The cohort members were recruited from across the US with oversampling of blacks and persons from the "stroke belt" region of the US. The "stroke belt" describes the southeastern region of the United States (North Carolina, South Caroline, Georgia, Tennessee, Mississippi, Alabama, Louisiana, and Arkansas) with a high incidence and mortality of stroke [145].

Demographic and medical history data, including information on risk factors, were obtained by computer-assisted telephone interviewing (CATI). Variables included age, race, and sex of the participants, aspirin and NSAIDs use, cigarette smoking, alcohol intake, and measures of socioeconomic status (education and income). Following the telephone interview, an in-home visit was completed to collect blood and urine samples and information on risk factors, such as blood pressure, height and weight. Additional information was collected through self-administered questionnaires, including the Block 98 FFQ. The Block 98 FFQ is an 8-page form with more than 150 multiple-choice questions based on 107 food items, which was used to assess energy intake, dietary fat, and nutritional intakes. Each participant recorded nutritional intakes for 1 week before their in-home visit [146]. At every six month interval, each participant was then followed via telephone interview to identify development of stroke and other outcomes, including death [147].

Oxidative Balance Score (Main Exposure Variable)

The oxidative balance score (OBS) will be calculated by combining information from a total of 14 *a priori* selected pro- and anti-oxidant factors, including intakes of polyunsaturated fatty acids, iron, vitamin C, lycopene, α-carotene, β-carotene, lutein, β-crypoxanthin, α-tocopherol, and selenium, smoking status, alcohol consumption and regular use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) as shown below.

$$OBS = \sum_{i=1}^{14} Individual\ OBS\ Component_i$$

The OBS components are summarized in Table 1.2 below. The continuous variables reflecting pro-oxidant (unsaturated fat and iron) and antioxidant exposures (vitamin C, lycopene, α-carotene, β-carotene, lutein, β-cryptoxanthin, α-tocopherol, and selenium) will be divided into low, medium, and high categories based on each exposure's tertile values and points will be given according to tertile categorization. For anti-oxidants the first through third tertiles will be assigned 0 through 2 points, respectively, whereas the corresponding point assignment for pro-oxidants will be carried out in reverse (0 points for the highest tertile and 2 points for the lowest tertile). A similar scoring approach will be used for pro- and anti-oxidant categorical variables (selenium supplements, smoking, alcohol and use of aspirin and NSAIDs). As the impact of different score components may vary, we will construct a weighted OBS utilizing four different weighting schemes:

1. *Equal weighting*: For the equal weighting method, we will assume that each component of OBS is equally important and contribute a similar weight toward the overall OBS.
2. *Fluorescent oxidation products (FOP)-based weighting*: Plasma FOP measurements from the previously discussed MAP study, which will be described in detail in the next section, will be used to derive weights for this weighting method. The fluorescent assay measures oxidation products from several sources, including lipids, proteins, and DNA, and thus may serve as a global indicator of oxidative balance [148]. The logistic regression model will be used to estimate the association between FOP measurement and each OBS component after adjusting for other OBS

components and confounders. The odds ratio estimates for each of the components obtained from the logistic model will be used as weights for this weighting method.

3. *F_2-isoprostane (FIP)-based weighting*: This method will use plasma F_2-isoprostanes measurements from the MAP study to derive weights for each OBS component. Plasma F_2-isoprostane concentration is considered the most established marker of oxidative balance [149]. As in the FOP-based weighting we will use multivariable logistic regression models to quantify the relation between F_2-isoprostanes and each OBS component. The adjusted odds ratio estimates from these logistic models will be used as weights.
4. *Literature-based weighting*: Each OBS component will be weighted according to the results of most recent systematic reviews/meta-analysis evaluating the association between each individual OBS components and either mortality (Aim 1) or stroke (Aim 2). In the absence of a recent systematic review/meta-analysis for any of the OBS components, a *de novo* meta-analysis will be conducted. For each OBS component, weights will be calculated based on the pooled (i.e., meta-) risk estimates (meta-RR). For each pro-oxidant the weight will be equal meta-RR, while for antioxidants the corresponding weight will be calculated as 1/meta-RR.

Table 1.2. Oxidative Balance Score (OBS) assignment scheme

Oxidative Balance Score (OBS) Components	Assignment Scheme†
1. PUFA[a] intake	0 = High (3rd tertile), 1 = Medium (2nd tertile), 2 = Low (1st tertile)
2. Total* iron intake	0 = High (3rd tertile), 1 = Medium (2nd tertile), 2 = Low (1st tertile)
3. Total vitamin C intake	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
4. Total Lycopene intake	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
5. Total α-carotene intake	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
7. Total β-carotene intake	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
6. Total lutein/zeaxanthin intake	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
8. Total β-cryptoxanthin intake	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
9. Total α-tocopherol	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
10. Selenium supplements	0 = No supplement, 1 = Unknown (missing data), 2 = Supplement
11. Smoking history	0 = Current smoker, 1 = Former smoker, 2 = Never smoker
12. Regular aspirin use	0 = No regular use, 1 = missing, 2 = Regular use
13. Regular NSAID[a] use	0 = No regular use, 1 = missing, 2 = Regular use
14. Alcohol consumption	0 = 8+ drinks/week, 1 =1-7 drinks/week, 2 = <1 drink/week

†Low, intermediate, and high categories correspond to tertile values of participants
[a]Abbreviations: PUFA = Polyunsaturated fatty acid; NSAID = Non-steroidal anti-inflammatory drug
*Total intake = Dietary intake + supplemental intake (when available) from questionnaire

Dependent Variables

The primary outcome for the first question (Aim #1) is all-cause mortality. In REGARDS study, a death was reported during telephone monitoring or through database searches, and was later confirmed through death certificates. In addition, interviews with next of kin or proxies of deceased participants were conducted to confirm the death and the date of death.

The primary outcome for the second question (Aim #2) is incident stroke. If suspected stroke was reported during follow-up, medical records were requested and stroke event was adjudicated by the Events Committee members. An incident of stroke was defined as "rapid onset of a persistent neurologic deficit attributable on an obstruction or rupture of the arterial system", using the World Health Organization definition. For both study questions, the total follow-up time for each individual was

calculated as the time between first visit interview and the date of death (for Aim#1) or the date of stroke (for Aim #2), the date of the last study visit, the date of withdrawal or loss to follow-up, or March 2011, whichever came first.

Data Analysis Plan

The OBS will be divided into quartiles, with the lowest quartile (predominance of pro-oxidants) as reference. In Aim 1, the associations between OBS and both all-cause mortality and cause-specific mortality will be examined using Cox proportional hazard models, adjusting for age, sex, race, SES, region, BMI, total daily energy intake, and physical activity. From the Cox proportional hazard regression analyses, adjusted hazard ratios (HR) with corresponding 95% confidence intervals (CI) for each OBS category compared to the reference (lowest OBS category) will be calculated. Proportional hazards assumptions will be tested by comparing –ln(ln) survival curves. The collinearity among the covariates will also be tested. A condition index of 30 or greater, coupled with a variance decomposition proportion of 0.5 or greater will be considered as evidence of collinearity. Stratified analyses will be conducted to examine whether the associations between OBS (exposure) and mortality (outcome) are modified by each of the covariates. For the purposes of interaction analyses, continuous variables such as age, BMI, and total daily energy intake will be dichotomized and -2 log likelihood ratio tests for models with and without interaction terms will be used. Tests for linear trend will be conducted by taking the median values of each OBS category. Analyses for Aim 2 will be the same but the outcome of interest will be incident stroke. A two-sided p-value of less than 0.05 will be considered as evidence of statistical significance. All statistical

analyses will be performed with SAS version 9.2 (SAS Institute, Cary, NC) statistical software package.

Methods for Aim 3

Data sources

To address the last question (Aim #3), I will use pooled data from two previously conducted colonoscopy-based case-control studies of incident, sporadic colorectal adenoma in 2 different US states by the same principal investigator (RMB). The first study, Markers of Adenomatous Polyps I (MAP I), was conducted in community gastroenterology practices in Winston-Salem and Charlotte, North Carolina. The second study, Markers of Adenomatous Polyps II (MAP II), was identical in design to MAP I and was conducted at Consultants in Gastroenterology, PA, a large, private practice in Columbia, South Carolina. Participants for these two case-control studies included patients who were 30-74 years of age with no prior history of colorectal neoplasms who were scheduled to undergo outpatient, elective colonoscopy at one of the study sites. Assessment of initial participant eligibility was identical in both studies. Cases (n=235) were first incident cases of colon or rectal adenomatous polyps at the time of elective outpatient colonoscopy and controls (n=391) were free of all polyps at colonoscopy.

In both the MAP I and MAP II studies, a modified 153-item Willett Food Frequency Questionnaire was administered to obtain information on dietary intakes and use of nutritional supplements. Additional data included demographics and use of medications, such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs). Total intakes for micronutrients (iron, vitamin C, β-carotene, and α-tocopherol) were calculated based on the sum of total daily dietary intake and total supplementary dose.

Blood Samples

For both studies, blood was collected, handled, and stored in a manner to allow measurements of pro-/anti-oxidants, FIP, FOP, and CRP. The samples were drawn into red-coated, pre-chilled Vacutainer tubes, plunged into ice and shielded from light and immediately delivered to the laboratory where the blood was centrifuged in a refrigerated centrifuge. Plasma and serum were separated; aliquotted into O-ring-capped amber-colored cryopreservation vials; the air in the vials was displaced with inert gas (nitrogen in MAP I and argon in MAP II); and then immediately frozen at -70^{o} C until analysis. Plasma lycopene, α-carotene, β-carotene, lutein, β-cryptoxanthin, and α-tocopherol levels from both studies were measured using high-performance liquid chromatography (HPLC) [150, 151]. The plasma free FIP were measured by a gas chromatography-mass spectrometry (GCMS) method [152] by the Molecular Epidemiology and Biomarker Research Laboratory (MEBRL) at the University of Minnesota (Minneapolis, MN). This method, considered the gold standard for the measurement of FIP, measures a well-defined set of F_2-isoprostane isomers. The FIP were extracted from the participant's sample using deuterium (4)-labeled 8-iso-prostaglandin F_2 alpha as an internal standard. Unlabeled, purified F_2-isoprostane was used as a calibration standard.

Oxidative Balance Score (Main Exposure Variable)

The oxidative balance score (OBS) was calculated by combining information from *a priori* selected pro- and anti-oxidant factors, which are summarized in Table 1. The blood levels of pro-oxidant (iron) and antioxidant (lycopene, α-carotene, β-carotene, lutein, β-cryptoxanthin, α-tocopherol) nutrients were divided into low, medium, and high categories based on study-specific tertile values among controls. The tertile cutoffs for

FFQ-derived variables (polyunsaturated fat, vitamin C, and alcohol) were both study- and sex-specific. The participants with low (1st tertile) pro-oxidant exposures were awarded 2 points, those with medium (2nd tertile) exposures received 1 point, and those with high (3rd tertile) exposures received 0 points. For alcohol consumption, non-drinkers, moderate drinkers (below median), and heavy drinker (above median) received 2, 1 and 0 points respectively For antioxidants, low, medium, and high levels were assigned 0, 1, and 2 points, respectively. A similar scoring approach was used for categorical variables (selenium supplements, smoking, and use of aspirin and NSAIDs). Smoking status was categorized as never (2 points), former (1 point), and current (0 points). For selenium supplements, aspirin, and NSAID use, 0 points were assigned to participants with no regular use, 1 point to those with unknown or missing data, and 2 points to those with regular use. The overall OBS was then calculated by adding up the points assigned to each participant.

Data Analysis Plan

The overall OBS will be treated as either a continuous or an ordinal variable with all categories representing an approximately equal interval, with the lowest interval used as reference. The use of equal intervals instead of quantiles (*e.g.,* tertiles or quartiles) allows comparing extremes of the distribution. Logistic regression analyses will be used to examine three types of associations. First we will examine the relation between the OBS and incident sporadic colorectal adenoma, adjusting for age, race, sex, total energy intake, BMI, plasma cholesterol, hormone replacement therapy (among women), physical activity, fiber, study, and family history of colorectal cancer. Next, we will examine the associations between OBS and the markers of oxidative stress (FIP and FOP) and

inflammation (CRP), which will be dichotomized based on study- and sex-specific median among controls, adjusting for the same potential confounding factors as in the first analysis. Finally, we will examine the associations between dichotomized markers of oxidative stress and inflammation and incident sporadic colorectal adenoma. The models for the third analysis will include the same covariates as in the analysis of association between OBS and adenoma. The correlation of FIP, FOP, and CRP will also be assessed using Pearson correlation coefficients.

We will also conduct several sensitivity analyses to evaluate 1) the change in results when quartiles were used instead of equal intervals for OBS; 2) the associations between adenoma and biomarkers using biomarker quartiles and 3) the association between OBS and each biomarker when both former and never smokers are assigned 2 points while current smokers are assigned 0 points to consider the possibility that biomarkers may only be affected by current smoking status.

The results of the logistic regression analyses will be expressed as adjusted odds ratios (ORs) with corresponding 95% confidence intervals (CIs). All models will be assessed for collinearity and goodness of fit. A two-sided p-value of less than 0.05 will be considered to be statistically significant. Statistical analyses will be performed with SAS version 9.2 (SAS Institute, Cary, NC) statistical software package.

SIGNIFICANCE AND IMPACT OF THE STUDY

While there is compelling mechanistic evidence that oxidative stress plays a central role in the pathogenesis of age-related and chronic diseases, results from epidemiological studies to support this hypothesis are conflicting. The potential explanation for this discrepancy between laboratory and population-based research is the

complex and multi-factorial nature of mechanisms by which oxidative stress may affect human health and the challenge of how to correctly estimate pro-and anti-oxidant exposures (particularly those related to nutritional intakes). It is often difficult to separate out the specific effects of individual pro and anti-oxidant factors. One way of dealing with this issue is to adjust for other oxidative stress-related exposures. However, adjustment can be of little use when the factors are highly correlated. Also, when associations between individual oxidant factor and disease are assessed, the association could be difficult to interpret because the effects of individual components are examined against the background risks associated with other factors. Moreover, the independent effects of individual oxidative exposures are difficult to ascertain because of the likely biological interactions involving multiple pro- and anti-oxidant factors. Correct measurements of nutritional intakes are further challenged by misclassification either questionnaire- and biomarker-based measures.

The main innovative feature of the proposed research is the use of a composite measure which we call oxidative balance score (OBS). To my knowledge, this is the first study to investigate the association between OBS and risk of mortality and stroke using a large, national prospective cohort study in United States. Several studies have assessed associations between OBS and risk of other diseases, including various types of cancers, but these associations have only been focusing on two outcomes – colorectal adenoma and prostate cancer [21, 25-27, 143].

Another strength of this study is the use of multiple approaches to weight OBS. All of the studies reviewed here used an assumption that individual OBS components have similar effects and therefore can be assigned equal weights (as in our weighting

method #1) . We are aware of other (still unpublished) studies that attempted to weight OBS based on the association between individual score components and outcome of interest (similar to our weighting method #5). The current study will be the first attempt to take into consideration population-based data on the relation between various OBS components and biochemical markers of oxidative stress and inflammation (FIP, FOP, and CRP). Moreover, no previous study has compared associations between OBS and markers of oxidative stress or inflammation.

The results of this dissertation project may have important implications for epidemiologic studies evaluating the role of oxidative stress in chronic disease etiology. Furthermore, this dissertation may open new avenues for the development of complex multifactorial interventions aimed at prevention of age-related degenerative chronic diseases.

CHAPTER 2. OXIDATIVE BALANCE SCORE IS PREDICTOR OF ALL-CAUSE, CANCER, AND NON-CANCER MORTALITY IN THE REASONS FOR GEOGRAPHIC AND RACIAL DIFFERENCES IN STROKE COHORT

So Yeon J.Kong[1], Roberd M. Bostick[1,2], W. Dana Flanders[1], William McClellan[1], Suzanne Judd[3], Michael Goodman[1,2]

[1] Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA.

[2] Winship Cancer Institute, Emory University, Atlanta, GA.

[3] Department of Biostatistics, University of Alabama at Birmingham, AL

ABSTRACT

There is an increasing body of evidence that high antioxidant intakes are inversely associated with risk of mortality while pro-oxidant factors are positively associated with mortality. However, observational and experimental studies with any single antioxidant or pro-oxidant factor have shown inconsistent results. We previously proposed an oxidative balance score (OBS) as an overall oxidative balance status of an individual, combining both pro and anti-oxidants.

In this study, we used data from a large national prospective cohort study, Reasons for Geographic and Racial Differences in Stroke (REGARDS) to examine the relation of OBS to all-cause and cause-specific mortality while exploring alternative methods of weighting the OBS components.

Data for individual pro- and anti-oxidant exposure were collected at baseline by telephone questionnaire in the REGARDS cohort participants, who were enrolled in 2003-2007. The OBS was calculated by combining information from a total of 14 *a priori* selected pro- and anti-oxidant factors, including intakes of polyunsaturated fatty acid, iron, vitamin C, lycopene, α-carotene, β-carotene, lutein, β-crypoxanthin, α-tocopherol, selenium, smoking status, alcohol, and regular use of aspirin and NSAID. The overall OBS was divided into quartiles with the lowest quartile (predominance of pro-oxidants) as reference. Each OBS component was included in the overall score using four weighting methods: 1) equal weights; 2) literature-based weights; 3) fluorescent oxidation products (FOP)-based weights; and 4) F_2-isoprostanes (FIP)-based weights. Cox proportional hazard models were used to estimate adjusted hazard risks (HR) and 95% confidence intervals (CI) for each OBS category compared to the reference.

Over a median follow-up period of 5.8 years, 2,079 of the 21,031 participants died. Higher OBS was associated with reduced risk of all-cause, cancer- and non-cancer mortality ($p_{trend} < 0.01$ for all). After adjustment for age, sex, race, BMI, total energy, education, income, region, and physical activity, the hazard ratios (95% CI) for all-cause, cancer, and non-cancer mortality for highest quartile were: 0.70 (0.61 – 0.81), 0.50 (0.37 – 0.67), and 0.78 (0.67 – 0.91), respectively when compared with those with lowest OBS quartile. Very similar results were observed across all weighting methods.

To our knowledge, this is the most comprehensive oxidative balance score constructed to date and one of the first studies to evaluate whether oxidative balance status is associated with all-cause and cause-specific mortality in the US population. Findings from this study suggest that OBS might be a useful tool for evaluating the roles of oxidative stress-related lifestyle factors, including diet, as determinants of morbidity and mortality.

INTRODUCTION

In 1956, Denham Harman proposed the "free radical theory of aging", which postulates that the process of aging and the development of age-related diseases are caused by the accumulation of deleterious changes in the cell attributed to free radical reactions [28]. Since then, the free radical theory triggered intensive research on the role of free radicals, more often known as "reactive oxygen and nitrogen species" (RONS). RONS can be generated from either endogenous or exogenous sources. At low concentrations, RONS play beneficial role in biological systems, as for example, in defense against infectious agents or in cell signaling and mitogenic response [32]. At

high concentrations, however, the RONS exert harmful effects by damaging cell structures and macromolecules, a process which is termed “oxidative stress” [34].

Oxidative stress, defined as the disruption of the balance between pro- and antioxidants, has been implicated in the etiology and pathophysiology of many chronic diseases, which in turn act as main contributors to mortality [153]. There is increasing evidence that high intakes of certain nutrients, including vitamin C, vitamin E, and carotenoids (e.g. lycopene, β-carotene, and lutein), may protect against oxidative stress while pro-oxidant factors, including smoking and iron intake increase productions of RONS and accelerate oxidative stress-related cellular damage. However, despite the substantial body of evidence from basic science and animal studies, observational and clinical studies evaluating the effects of individual antioxidant or pro-oxidant factors have shown inconsistent results [134, 135, 154-156].

One potential explanation for this discrepancy is the complex and multi-factorial mechanism by which oxidative stress may affect health. The independent effects of individual exposures may not offer complete insight into their roles in maintaining the overall oxidative balance because of the likely inter-correlations and biological interactions involving multiple pro- and anti-oxidant factors [21]. The concept of an integrated antioxidant network has been proposed, given that antioxidants of differing solubility are residing next to each other in cellular structures and tissues, integrating and regenerating each other [157].

This situation is somewhat similar to the difficulties encountered in nutrition research, in which the pursuit of effects exerted by individual nutrients has been replaced by use of dietary pattern analyses, particularly, in relation to the Mediterranean diet.

Numerous epidemiological studies have examined the health benefits of the Mediterranean diet and evidence consistently shows beneficial effects of the Mediterranean diet pattern on healthier ageing and longevity [158-162]. Recently, we [25-27] and others [143, 163] proposed oxidative balance score (OBS) as a measure of combined pro- and anti-oxidant exposure status, which was used in studies of various chronic diseases. Only one of those studies [143] examined the association between OBS and mortality; however, this study was limited by the relatively small number of score components and by the assumption that the effects of all pro- and anti-oxidants were roughly equal. In this study, we use data from a large national prospective cohort study to examine the relation of OBS comprised of 14 *a priori* selected oxidative stress related exposures to all-cause and cause-specific mortality while exploring alternative methods of weighting the OBS components. We hypothesize that higher OBS, which reflects predominance of anti-oxidant exposures, is associated with a reduction in mortality.

METHODS

Study population and data collection

The Reasons for Geographic and Racial Differences in Stroke (REGARDS) Study is a national, population-based prospective cohort study aimed to examine the reason for variation in stroke incidence and mortality in the United States. Details on recruitment and data collection were reported previously [147]. Briefly, between January 2003 and October 2007, 30,239 black and white individuals aged 45 years or older were randomly selected and recruited through mail and telephone contacts. Of the eligible participants contacted, the participation rate was 49%. The cohort members were recruited from

across the US with oversampling of blacks and persons from the "stroke belt" region of the United States. The "stroke belt" describes the southeastern region of the United States (North Carolina, South Caroline, Georgia, Tennessee, Mississippi, Alabama, Louisiana, and Arkansas) with a high incidence and mortality of stroke [145]. Exclusion criteria were race other than black or white, active treatment for cancer, cognitive impairment as judged by the telephone interviewer, medical conditions preventing long-term participation, residence in or inclusion on a waiting list for a nursing home, or inability to communicate in English.

After obtaining verbal and written informed consent, demographic and medical history data, including information on risk factors, were obtained by computer-assisted telephone interviewing (CATI). Variables included age, race, and sex of the participants, use of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs), cigarette smoking, alcohol intake, and measures of socioeconomic status (education and income). Following the telephone interview, an in-home visit was completed to obtain blood and urine samples and collect information on the presence of risk factors, such as blood pressure, height and weight. Additional information was collected through self-administered questionnaires, including the Block 98 food-frequency questionnaire (FFQ). The Block 98 FFQ is an 8-page form with more than 150 multiple-choice questions based on 107 food items, which was used to assess energy intake, dietary fat, and nutritional intakes. All participants recorded food and nutrient intakes for 1 week before their in-home visits. At every six month interval, each participant was then followed via telephone interview to ascertain development of stroke and other outcomes, including death.

Of the 30,239 participants enrolled in the REGARDS study, 8,603 who did not complete the modified Block 98 FFQ were excluded from the current analysis. In addition, the analytic dataset excluded 456 participants with missing data on at least one OBS component, and 149 participants with missing data on key covariates. After these exclusions, data for 21,031 participants were available for the final analyses. Follow-up was available on

Oxidative Balance Score (Main Exposure Variable)

The oxidative balance score (OBS) was calculated by combining information from a total of 14 *a priori* selected pro- and anti-oxidant factors, including intakes of polyunsaturated fatty acids, iron, vitamin C, lycopene, α-carotene, β-carotene, lutein, β-crypoxanthin, α-tocopherol, selenium, smoking status, alcohol consumption and regular use of aspirin and other NSAIDs (TABLE 2.1). The continuous variables reflecting pro-oxidant (unsaturated fat and iron) and antioxidant (vitamin C, lycopene, α-carotene, β-carotene, lutein, β-cryptoxanthin, α-tocopherol, and selenium) exposures were divided into low, medium, and high categories based on each exposure's tertile values. For anti-oxidants, the first through third tertiles were assigned 0 through 2 points, respectively, whereas the corresponding point assignment for pro-oxidants were carried out in reverse (0 points for the highest tertile and 2 points for the lowest tertile). A similar scoring approach was used for pro- and anti-oxidant categorical variables. Smoking status was categorized as never (2 points), former (1 point), and current (0 points). For aspirin and NSAIDs use, 0 points were assigned to participants with no regular use, 1 point to those with unknown or missing data, and 2 points to those with regular use. For alcohol consumption, non-drinkers, moderate drinkers (1-7 drinks/week for women and 1-14

drinks/week for men), and heavy drinker (>7 drinks/week for women and >14 drinks/week for men) received 2, 1 and 0 points respectively.

OBS Component-Scores Weighting

Each OBS component was included in the overall score using four weighting methods: 1) equal weights; 2) literature-based weights; 3) weights based on the magnitude of association between each component and fluorescent oxidation products (FOP); and 4) weights based on the magnitude of association between each component and F_2-isoprostanes (FIP). The weights for each OBS component-scores are summarized in Table 2.2.

The equal weights approach assumed that all OBS component equally contributed to the overall score. By contrast, the other three methods assigned weights based on the presumed magnitude of the pro- and anti-oxidant effects.

For the literature-based method each OBS component was weighted according to the results of most recent systematic reviews/meta-analysis evaluating the association between this component and mortality. In the absence of a recent systematic review/meta-analysis for any of the OBS components, a *de novo* meta-analysis was conducted. For each OBS component, weights were calculated based on the pooled (i.e., meta-) risk estimates (meta-RR). For each pro-oxidant the weights were equal to meta-RR, while for antioxidants the corresponding weights were calculated as 1/meta-RR.

The two biomarker (FOP and FIP) -based weighting methods used pooled data from two previously completed case-control studies of colorectal adenoma that employed virtually identical protocols. The first study, Markers of Adenomatous Polyps I (MAP I),

recruited cases and controls from gastroenterology practices in Winston-Salem and Charlotte, North Carolina. The second study, Markers of Adenomatous Polyps II (MAP II), was conducted at Consultants in Gastroenterology, Professional Association, a large, private practice in Columbia, South Carolina.

The detailed study methods for MAP I [164, 165] and MAP II [166, 167] have been previously published. In both studies, a modified 153-item Willett Food Frequency Questionnaire was administered to obtain information on dietary intakes and use of nutritional supplements [168, 169]. Additional data included demographics and use of medications, such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs). For both studies, blood was collected, handled, and stored in a manner to allow measurements of FOP and FIP. Plasma FIP concentration specifically measures lipid peroxidation and is considered the most established marker of oxidative stress [149]. The FOP assay measures oxidation products from several sources, including lipids, proteins, and DNA, and thus may serve as a global indicator of oxidative balance; however it remains a relatively novel and untested method [148].

The samples for both MAP I and MAP II were processed and analyzed in a similar manner. After the sample was drawn it was placed on ice and delivered to the laboratory where the blood was processed and centrifuged. Plasma and serum were separated, aliquoted, and frozen at -70 ^{o}C for long term storage. The modified method from Shimasaki [170] was used to measure FOP as described in detail previously [171]. Briefly, 0.2 mL of plasma was extracted with ethanol/ether (3:1 v/v) and vigorously mixed on a vortex mixer. The mixed solution was centrifuged for 10 minutes at 3,000 rpm, and 1 mL of supernatant was added to cuvettes for spectrofluormetric readings. The

fluorescent was determined as relative fluorescence intensity units per milliliter of plasma at 360/430 nm wavelength (excitation/emission) by a spectrofluorometer. Quinine sulfatge diluted in 0.1 N H_2SO_4 was used for calibration. The plasma FIP were measured by gas chromatography-mass spectrometry (GCMS) method [152] at the Molecular Epidemiology and Biomarker Research Laboratory (MEBRL) in the University of Minnesota. This method, measures a well-defined set of FIP isomers. The FIP were extracted from the participant's sample using deuterium (4)-labeled 8-iso-prostaglandin F_2 alpha as an internal standard. Unlabeled, purified FIP was used as a calibration standard.

For both FIP- and FOP-based weighting, we used multivariable logistic regression models to quantify the relation between each OBS component and each of the two markers of oxidative stress. Each model adjusted for other OBS components and for additional confounding factors, including age, race, sex, total energy intake, BMI, plasma cholesterol, hormone replacement therapy (among women), physical activity, fiber, and study. The adjusted odds ratio estimates from these logistic models were used to assign weights.

Outcome Measures

The primary outcome in this study was all-cause mortality. In the REGARDS cohort, a death of a participant was ascertained during telephone monitoring or through Web-based restricted-access database searches (e.g., Lexis-Nexis), and was later confirmed through death certificates. In addition, interviews with next of kin or proxies of deceased participants were conducted to confirm the death and the date of death. Information on the cause of death was also obtained from death certificates. Death cases

and causes of death were reviewed independently by two adjudicators, and disagreements were resolved by committee. Adjudicators used baseline participant clinical characteristics, proxy interviews, death certificates, and if available, medical records from hospitalizations occurring within 30 days of the participant's death to determine the cause of death.

Statistical analysis

Each version of OBS (unweighted, and weighted using literature-, FOP- and FIP-based methods) was divided into quartiles, with the lowest quartile (predominance of pro-oxidants) used as reference. The total follow-up time for each individual was calculated as the time between first visit interview and the date of death, the date of the last study visit, the date of withdrawal or loss to follow-up, or March 1, 2011, whichever came first. The Kaplan-Meier survival curves accompanied by a log-rank test and the corresponding p-value were used to assess the unadjusted association between OBS and all-cause mortality. The adjusted associations between OBS and both all-cause mortality and cause-specific mortality were examined using Cox proportional hazard models, that controlled for age, sex, race, SES, region, BMI, total daily energy intake, and physical activity. Results of multivariable survival analyses were expressed as, adjusted hazard ratios (HR) with corresponding 95% confidence intervals (CI). Since tobacco smoking is a powerful pro-oxidant and a strong risk factor for mortality, we conducted a separate set of analyses by removing smoking from the OBS while controlling for it the model. Proportional hazards assumptions were tested by inspecting –ln(ln) survival curves for each variable in the model. The collinearity was tested using SAS macro. A condition index of 30 or greater, coupled with a variance decomposition proportion of 0.5 or greater

was considered as evidence of collinearity. Stratified analyses were conducted to examine whether the associations between OBS and mortality are modified by each of the covariates. For the purposes of interaction analyses, continuous variables such as age, BMI, and total daily energy intake were dichotomized and -2 log likelihood ratio tests for models with and without interaction terms were used. A two-sided p-value of less than 0.05 was considered as evidence of statistical significance. All statistical analyses was performed with SAS version 9.2 (SAS Institute, Cary, NC) statistical software package.

RESULTS

Baseline characteristics of the study cohort by OBS category are shown in TABLE 2.3. Compared with those in the lowest OBS quartile, participants in the highest quartile were on average three years older (66 vs. 63), included a greater proportion of whites (69.3% vs. 64.9%), and had more females (58.0% vs. 54.3%). Persons in the highest OBS quartile were also more likely to have higher education and income, and reside in the non-stroke belt states. The evaluations of individual OBS components according to OBS quartiles are presented in TABLE 2.4. Contrary to expectation, intakes of daily polyunsaturated fatty acids (PUFA) and iron were higher in higher OBS quartile groups. As expected, the intakes of antioxidants (vitamin C, lycopene, α-carotene, β-carotene, lutein, β-cryptoxanthin, and vitamin E) were significantly increased among cohort members with higher OBS values. Participants in the higher OBS quartiles were also more likely to be never smokers and non-drinkers, take daily selenium supplement, and regularly use NSAIDs and/or aspirin.

Over an average follow-up period of 5.8 years (range 0 – 9.1 years), 2,079 of the 21,031 participants died. FIGURE 2.1 shows that overall survival increased with increasing OBS with a log-rank p-value of <0.001. Higher OBS was associated with statistically significant reduction in all-cause, cancer and non-cancer mortality in the multivariable analyses (TABLE 2.5). After adjusting for age, sex, race, BMI, total daily energy intake, education, exercise, and region of residence, participants in the highest OBS quartile (quartile 4) had a statistically significantly lower risk of all-cause mortality with an HR of 0.70; (95% CI : 0.61 – 0.81) compared to participants in the lowest OBS quartile (p_{trend} <0.001). Excluding smoking from OBS slightly attenuated the hazard ratios, but the association remained statistically significant with evidence of an inverse linear trend (p_{trend} = 0.013). Analysis for 1-point increments in OBS showed a 4% (p < 0.001) and a 2% (p = 0.003) decreases in all-cause mortality for scores with and without smoking, respectively.

Among 1,566 deaths with known cause, about 30% were attributable to cancer. Higher OBS was associated with a 50% (HR = 0.50; 95% CI: 0.37 – 0.67) and 22% (HR = 0.78; 95% CI: 0.67 – 0.91) decreases in risk of death due to cancer and non-cancer, respectively. After smoking was excluded from the OBS, significant association between OBS and cancer mortality remained with quartile 4 showing a 32% decrease in cancer related death when compared to quartile 1 (HR = 0.68; 95% CI: 0.52 – 0.90, p_{trend}=0.009), but the association between OBS and non-cancer mortality was no longer statistically significant. The statistically significant linear trend between OBS and chronic lung disease mortality (p_{trend}=0.024) was also attenuated after removal of smoking from the score (p_{trend}=0.270).

TABLE 2.6 shows the associations between OBS and all-cause**,** cancer, and non-cancer mortality based on different weighting methods. Very similar results were observed across all weighting methods. Comparing equal weights approach (TABLE 5) to the three weighted approaches (TABLE 6), all differences were within 15% and only three estimates differed by more than 10%.

Table 2.7 presents the sensitivity analyses in which the observed results for the original 14-component OBS (treated as a continuous variable) were compared to the corresponding results after each OBS component was removed from the score and included in the model as a covariate. With the exception of smoking, removal of any single OBS component did not produce meaningful changes and the resulting HRs demonstrated departures from the original estimates by no more than two percent. When smoking was removed from the OBS, the association was no longer statistically significant for all-cause and non-cancer mortality (as in TABLE 2.5), but was still statistically significant for cancer mortality.

DISCUSSION

In this population-based large prospective cohort study, we examined a comprehensive oxidative balance score (OBS) as predictor of mortality with an expectation that OBS may better reflect the oxidative balance that any single pro- or anti-oxidant. We found that a higher OBS, which indicates predominance of antioxidant exposures, is associated with significant reduction in all-cause mortality and mortality due to cancer and non-cancer, after adjustment for multiple confounders. This association was only modestly attenuated for all-cause and cancer mortality when smoking was excluded from the OBS, suggesting that the association was not driven by

smoking status. We also examined associations between OBS and mortality using different weighting methods, but the results did not differ substantially from those obtained using equal weights.

Although the idea of combining individual pro- and anti-oxidants into a single score is not new, to our knowledge, ours is the most comprehensive OBS constructed to date and the present study one of the first to evaluate whether OBS is associated with all-cause mortality in the US population. Overall, our results are consistent with other similar studies. Knoops *et al.*[159] investigated the association of a lifestyle score (combined individual scores for Mediterranean dietary pattern, alcohol use, smoking status, and physical activity) with mortality from all causes from 11 European countries. The combination of 4 low risk factors in that study was associated with a 65% lower rate of all-cause mortality. In another cohort study conducted among male smokers in Belgium Van Hoydonck *et al.* combined intakes of two dietary antioxidants (vitamin C and β-carotene) and one pro-oxidant (iron) to develop their oxidative balance score [143]. Men in the highest category OBS, which unlike ours was constructed to reflect a presumably harmful effect, had a statistically significant 44% increase in all-cause mortality and an even greater (62%) increase in cancer mortality compared with men in the lowest OBS group. As in our study, van Hoydonck *et al.* also found no association between OBS and cardiovascular disease mortality.

In the current study, we used different weighting schemes for combining pro- and anti-oxidant exposures into a single score. Previous studies used equal weighting of the OBS components [26, 27, 143, 144], which raised a concern that the resulting score does not represent the true biological contributions of the individual pro- or anti-oxidant

exposures. However, in the present analyses, the associations between OBS and mortality across the different weighting methods were very similar. One limitation of our two biomarker (FOP and FIP) -based weighting methods is use of a relatively small dataset with a lot of uncertainty in the estimates used to create the weights. Similarly, the literature review-based weights were largely similar across OBS components and close to 1.0 because summaries of published studies on the relation of pro- and anti-oxidant components to mortality in most cases (except smoking) show modest departures from the null

Advantages of this study include its prospective design, large size, use of a diverse population, and inclusion of multiple pro- and anti-oxidant components in the OBS. We used 14 pro- and anti-oxidant factors that were selected *a priori* based on previous research [172-182]. We used data-based a priori tertile cut-points for continuous variables to minimize the subjective categorization, which is a general problem with scores attempting to describe complex processes. In this study, the mortality and cause of death were adjudicated by expert clinicians using death certificates, medical records from recent hospitalizations, and interviews with proxies, a methodological feature that helped decrease outcome misclassification.

There are several potential limitations in this study. We used self-reported intakes to assess pro- and anti-oxidant exposure. It has long been acknowledged that the questionnaires may not capture all the possible sources of each nutrient, does not account for bioavailability, and is subject to recall bias [183]. The validity and reliability of the FFQ used in our study has been extensively evaluated [184-186], and if some degree of misclassification exist, we would expect it to be non-differential, although the direction

of bias with complex ordinal variables such as OBS is difficult to estimate. Data on specific causes of death, cancer in particular, was also lacking in this study.

Another limitation of this study is that we did not have participants' genetic information. Genetic factors play an important role in human lifespan [187]. For example, previous genetic studies have shown that common polymorphisms in apolipoprotein E (APOE) influence human mortality, mainly through their association with diseases [188]. Furthermore, the OBS score in our study is limited to dietary / lifestyle exposures and does not include any endogenous factors that influence cellular anti-oxidant defense, DNA damage and its repair, cell growth, and cell death, which all contribute to survival of an individual [12].

In conclusion, this large prospective study demonstrates that higher OBS is associated with lower all-cause and cancer mortality even after controlling for smoking. The observed association for non-cancer mortality and OBS was driven primarily by smoking. These findings confirm results from previous studies and suggest that OBS might be a useful tool for evaluating the roles of oxidative stress-related lifestyle factors, including diet, as determinants of morbidity and mortality.

TABLE 2.1. Oxidative balance score (OBS) assignment scheme

Oxidative Balance Score (OBS) Components	Assignment Scheme†
1. PUFA intake	0 = High (3rd tertile), 1 = Intermediate (2nd tertile), 2 = Low (1st tertile)
2. Total* iron intake	0 = High (3rd tertile), 1 = Intermediate (2nd tertile), 2 = Low (1st tertile)
3. Total vitamin C intake	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
4. Total lycopene level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
5. Total α-carotene level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
6. Total β-carotene level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
7. Total lutein level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
8. Total β-cryptoxanthin level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
9. Total α-tocopherol level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
10. Selenium level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
11. Smoking history	0 = Current smoker, 1 = Former smoker, 2 = Never smoker
12. Regular aspirin use	0 = No regular use, 1 = Unknown (missing data), 2 = Regular Use
13. Regular NSAID use	0 = No regular use, 1 = Unknown (missing data), 2 = Regular Use
14. Alcohol consumption	0 = None, 1 = Moderate, 3 = Heavy

Abbreviations: PUFA = Polyunsaturated fatty acid; NSAID = Non-steroidal anti-inflammatory drug

† Low, intermediate, and high categories correspond to sex-specific tertile values among participants in the REGARDS cohort.

*Total intake = Dietary intake + supplemental intake (when available)

TABLE 2.2. Oxidative balance score (OBS) Weightings

OBS Components	Weightings				
	F_2-isoprostanes		FOP		Lit. Review
	Male	Female	Male	Female	Both sex
1. PUFA intake	0.87	0.91	1.23	1.24	0.98
2. Total iron intake	0.70	0.54	0.88	0.94	1.40
3. Total vitamin C intake	1.81	1.48	1.12	0.85	0.99
4. Total lycopene level	1.17	1.35	0.82	0.89	1.22
5. Total α-carotene level	1.43	1.22	0.85	0.75	1.32
6. Total β-carotene level	1.45	1.31	0.97	0.83	0.95
7. Total lutein level	1.61	1.73	0.97	0.68	1.20
8. Total β-cryptoxanthin level	1.69	1.38	1.16	1.11	1.22
9. Total α-tocopherol level	1.79	1.30	0.98	0.91	0.96
10. Selenium level	1.32	2.18	1.12	1.61	1.11
11. Smoking history	1.74	1.43	1.60	1.80	1.83
12. Regular aspirin use	1.28	1.20	0.87	0.91	1.06
13. Regular NSAID use	1.06	1.04	0.70	0.96	1.00
14. Alcohol consumption	1.07	0.99	1.35	1.23	1.30

Abbreviations: OBS=Oxidative Balance Score PUFA=Polyunsaturated fatty acid; NSAID=Non-steroidal anti-inflammatory drug; FOP=fluorescent oxidation products; Lit.=literature

TABLE 2.3. Selected baseline characteristics of the REGARDS cohort by OBS quartile

Characteristic (Units)†	Q1: OBS 3-11 (n=5,668)	Q2: OBS 12-14 (n=5,593)	Q3: OBS 15-17 (n=5,523)	Q4: OBS 18-26 (n=4,247)
Age, years	63.5 (9.3)	64.7 (9.4)	65.5 (9.1)	66.0 (9.1)
Race				
White	3,680 (64.9%)	3,654 (65.3%)	3,745 (67.8%)	2,944 (69.3%)
Black	1,988 (35.1%)	1,939 (34.7%)	1,778 (32.2%)	1,303 (30.7%)
Sex				
Male	2,589 (45.7%)	2,455 (43.9%)	2,424 (43.9%)	1,785 (42.0%)
Female	3,079 (54.3%)	3,138 (56.1%)	3,099 (56.1%)	2,462 (58.0%)
BMI* (kg/m²)	28.8 (6.0)	29.1 (6.2)	29.1 (6.0)	29.3 (6.1)
Energy (cal)	1,474.6 (604.5)	1,641.6 (669.5)	1,809.9 (726.8)	1972.3 (758.5)
Education				
Less than High School	681 (12.0%)	567 (10.1%)	478 (8.6%)	290 (6.8%)
High School Graduate	1,700 (30.0%)	1,469 (26.3%)	1,286 (23.3%)	9190 (21.6%)
Some College	1,590 (28.0%)	1,559 (27.9%)	1,469 (26.6%)	1,140 (26.9%)
College Graduate and Above	1,697 (30.0%)	1,998 (35.7%)	2,290 (41.5%)	1,898 (44.7%)
Income	1,001 (17.7%)	878 (15.7%)	850 (15.4%)	576 (13.5%)
Less than $20k	1,458 (25.7%)	1,370 (24.5%)	1,253 (22.7%)	1,002 (23.6%)
$20k - $34k	1,680(29.6%)	1,718 (30.7%)	1,806 (32.7%)	1,379 (32.5%)
$35k - $74k	891 (15.7%)	745 (16.9%)	973 (17.6%)	806 (19.0%)
$75k and above	638 (11.3%)	682 (12.2%)	641 (11.6%)	484 (11.4%)
Refused				
Region	2,058 (36.3%)	1,969 (35.2%)	1,821 (33.0%)	1,378 (32.4%)
Stroke Belt	1,306 (23.0%)	1,234 (22.1%)	1,190 (21.5%)	888 (21.0%)
Stroke Buckle	2,304 (40.7%)	2,390 (42.7%)	2,512 (45.5%)	1,981 (46.6%)
Non-belt				
Follow-up Time*, years	5.7 (2.0)	5.8 (2.0)	5.9 (1.9)	5.9 (1.9)

†Values for age, BMI, energy, and follow-up years are mean (±SD) and race, sex, education, income, and region are number (percent).

‡ Based on the ANOVA for continuous variables and chi-square (X^2) test for categorical variables.

TABLE 2.4. Individual components of the score by OBS quartile

Characteristic (Units)	Mean (by OBS Quartile) Q1 (n=5,668)	Q2 (n=5,593)	Q3 (n=5,523)	Q4 (n=4,247)
Daily PUFA from intake (g)				
Men (n=9,253)	18.1 (9.7)	19.7 (10.7)	21.1 (11.0)	21.7 (11.1)
Women (n=11,778)	15.7 (9.1)	16.9 (9.8)	18.2 (10.1)	19.2 (10.4)
Total* iron intake (mg)				
Men	17.7 (13.6)	23.6 (16.0)	27.5 (17.3)	30.9 (19.3)
Women	18.1 (16.5)	23.1 (18.9)	26.7 (19.0)	30.4 (21.2)
Total vitamin C intake (mg)				
Men	121.1 (177.9)	280.9 (356.2)	421.9 (467.2)	644.8(563.2)
Women	148.8 (231.2)	284.7 (349.9)	433.2 (439.2)	621.5 (520.7)
Daily lycopene intake (μg)				
Men	2,918.5 (3,155.0)	4,263.4 (4,646.0)	5,364.0 (5,002.0)	7,431.1 (7,083.0)
Women	2,292.8 (2,493.0)	3,348.4 (3,752.0)	4,313.1 (4,626.0)	5,849.7 (5,785.0)
Daily α-carotene intake (μg)				
Men	327.0 (287.8)	530.8 (494.2)	843.5 (759.9)	1,258.5 (1,120.0)
Women	295.6 (239.3)	517.4 (556.4)	852.8 (901.0)	1,261.0 (1,124.0)
Total β-carotene intake (μg)				
Men	2,161.6 (1,883.0)	3,806.6 (3,639.0)	9,096.7 (5,701.0)	9,110.9 (7,596.0)
Women	2,250.2 (1,563.0)	4,075.5 (3,982.0)	6,395.9 (5,496.0)	9,384.6 (7,433.0)
Daily lutein intake (μg)				
Men	829.2 (602.7)	1,327.1 (1,041.0)	1,958.5 (1,624.0)	2,837.8 (2,452.0)
Women	964.0 (821.3)	1,527.3 (1,304.0)	2,380.4 (2,201.0)	3,358.1 (2,796.0)
Daily β-cryptoxanthin intake (μg)				
Men	63.1 (82.6)	113.9 (120.4)	157.9 (142.7)	209.7 (159.2)
Women	53.6 (71.7)	102.2 (111.0)	142.9 (140.6)	193.1 (157.9)
Total vitamin E intake (α-TE)				
Men	34.2 (87.9)	85.0 (151.1)	130.9 (183.5)	193.5 (193.6)
Women	39.7 (93.5)	87.0 (155.6)	126.4 (171.5)	189.3 (193.9)
Daily selenium supplement (mcg)				
Men	79.4 (35.6)	98.4 (48.6)	117.7 (61.1)	141.5 (75.0)
Women	66.3 (31.7)	81.6 (42.7)	97.4 (50.3)	118.2 (62.6)
Smoking				
Never Smokers	1,775 (31.3%)	2,410 (43.1%)	2,726 (49.4%)	2,615 (61.6%)
Former Smokers	2,429 (42.9%)	2,449 (43.8%)	2,320 (42.0%)	1,445 (34.0%)
Current Smokers	1,464 (25.8%)	734 (13.1%)	477 (8.6%)	187 (4.4%)
Alcohol				
Non-Drinkers	3,030 (53.5%)	3,355 (60.0%)	3,311 (60.0%)	2,858 (67.3%)
Moderate Drinkers	2,198 (38.8%)	2,015 (36.0%)	2,028 (36.7%)	1,310 (30.8%)
Heavy Drinkers	440 (7.7%)	223 (4.0%)	184 (3.3%)	79 (1.9%)
Regular NSAIDs Use	451 (8.0%)	698 (12.5%)	885 (16.1%)	1,114 (26.3%)
Regular Aspirin Use	1,545 (27.3%)	2,327 (41.6%)	2,646 (47.9%)	2,749 (64.8%)
Total OBS	9.2 (1.6)	13.0 (0.8)	16.0 (0.8)	19.4 (1.4)

[1] Values are presented as mean (SD) or number (%). Abbreviations: PUFA = Polyunsaturated fatty acid; NSAID = Non-steroidal anti-inflammatory drug; OBS = Oxidative balance score; SD = Standard deviation

* Total intake = daily intake from food + average daily intake from supplement

TABLE 2.5. Association between all-cause and cause-specific mortality and OBS in the REGARDS cohort: Equal weighing

Interval (OBS Range: 3-26)	Alive (n = 18,952)	Dead (n = 2,079)	With Smoking HR (95% CI)†	Without Smoking HR (95% CI)‡
All Cause-Mortality				
Q 1	5,025	643	1.0	1.0
Q 2	5,047	546	0.81 (0.72 – 0.91)	0.89 (0.79 – 1.00)
Q 3	4,999	524	0.77 (0.68 – 0.87)	0.94 (0.82 – 1.07)
Q 4	3,881	366	0.70 (0.61 – 0.81)	0.87 (0.77 – 0.99)
p-trend*			<0.001	0.013
Cancer Mortality				
Q 1	5,025	163	1.0	1.0
Q 2	5,047	112	0.64 (0.50 – 0.82)	0.84 (0.66 – 1.06)
Q 3	4,999	106	0.60 (0.47 – 0.78)	0.79 (0.59 – 1.05)
Q 4	3,881	69	0.50 (0.37 – 0.67)	0.68 (0.52 – 0.90)
p-trend*			<0.001	0.009
All Non-Cancer Mortality				
Q 1	5,025	480	1.0	1.0
Q 2	5,047	434	0.86 (0.75 – 0.98)	0.91 (0.80 – 1.03)
Q 3	4,999	418	0.84 (0.73 – 0.96)	1.00 (0.86 – 1.16)
Q 4	3,881	297	0.78 (0.67 – 0.91)	0.94 (0.82 – 1.09)
p-trend*			0.002	0.790
Cardiac Mortality				
Q 1	5,025	48	1.0	1.0
Q 2	5,047	36	0.69 (0.45 – 1.08)	0.66 (0.42 – 1.03)
Q 3	4,999	34	0.66 (0.42 – 1.05)	0.78 (0.47 – 1.29)
Q 4	3,881	27	0.69 (0.42 – 1.14)	0.78 (0.49 – 1.25)
p-trend*			0.109	0.402
Heart Failure Mortality				
Q 1	5,025	74	1.0	1.0
Q 2	5,047	59	0.73 (0.51 – 1.04)	0.83 (0.47 – 1.46)
Q 3	4,999	61	0.81 (0.57 – 1.15)	1.20 (0.66 – 2.19)
Q 4	3,881	48	0.84 (0.57 – 1.23)	1.16 (0.65 – 2.07)
p-trend*			0.397	0.388
Chronic Lung Disease Mortality				
Q 1	5,025	24	1.0	1.0
Q 2	5,047	32	1.28 (0.75 – 2.19)	1.52 (0.88 – 2.61)
Q 3	4,999	17	0.66 (0.35 – 1.25)	0.96 (0.48 – 1.92)
Q 4	3,881	11	0.49 (0.22 – 1.05)	0.74 (0.37 – 1.50)
p-trend*			0.024	0.270

Abbreviations: OBS=oxidative balance score; HR=hazards ratio; CI=confidence interval; Q=quartile

† Adjusted for age, sex, race, BMI, and total daily energy, education, exercise, and region

‡ Adjusted for the same variables as above plus smoking

*p-trend assessed by X^2 test for linear trend

OBS ranges for "with smoking": Q1=3-11; Q2=12-14; Q3=15-17; Q4=18-26

OBS ranges for "without smoking" Q1=2-10; Q2=10-13; Q3=13-15.5; Q4=15.5-24

TABLE 2.6. Association between all-cause, cancer, and non-cancer mortality and OBS in the REGARDS cohort: Different weighting approaches

OBS Weighting	All-cause Mortality		Cancer Mortality		Non-cancer Mortality	
	With Smoking HR (95% CI)†	Without Smoking HR (95% CI)‡	With Smoking HR (95% CI)†	Without Smoking HR (95% CI)‡	With Smoking HR (95% CI)†	Without Smoking HR (95% CI)‡
Isoprostane weights						
Q 1	1.0	1.0	1.0	1.0	1.0	1.0
Q 2	0.84 (0.74 – 0.94)	0.91 (0.80 – 1.03)	0.71 (0.56 – 0.92)	0.84 (0.65 – 1.09)	0.88 (0.77 – 1.01)	0.94 (0.82 – 1.08)
Q 3	0.79 (0.70 – 0.90)	0.93 (0.82 – 1.06)	0.62 (0.48 – 0.81)	0.82 (0.63 – 1.07)	0.85 (0.74 – 0.98)	0.98 (0.85 – 1.13)
Q 4	0.67 (0.59 – 0.77)	0.84 (0.73 – 0.96)	0.48 (0.36 – 0.64)	0.66 (0.49 – 0.88)	0.73 (0.63 – 0.85)	0.89 (0.76 – 1.04)
p-trend*	<0.001	0.078	<0.001	0.009	<0.001	0.342
FOP weights						
Q 1	1.0	1.0	1.0	1.0	1.0	1.0
Q 2	0.83 (0.73 – 0.92)	0.92 (0.78 – 1.07)	0.69 (0.54 – 0.87)	0.80 (0.62 – 1.04)	0.88 (0.76 – 1.00)	0.97 (0.84 – 1.11)
Q 3	0.76 (0.66 – 0.84)	1.09 (0.93 – 1.27)	0.52 (0.40 – 0.68)	0.82 (0.63 – 1.07)	0.82 (0.72 – 0.95)	1.04 (0.90 – 1.20)
Q 4	0.66 (0.58 – 0.75)	0.90 (0.76 – 1.06)	0.45 (0.34 – 0.59)	0.67 (0.51 – 0.89)	0.73 (0.63 – 0.85)	0.98 (0.84 – 1.13)
p-trend*	<0.001	0.886	<0.001	0.013	<0.001	0.678
Lit. Review weights						
Q 1	1.0	1.0	1.0	1.0	1.0	1.0
Q 2	0.82 (0.73 – 0.92)	0.94 (0.83 – 1.06)	0.66 (0.52 – 0.85)	0.81 (0.63 – 1.05)	0.88 (0.76 – 1.00)	0.98 (0.85 – 1.13)
Q 3	0.73 (0.65 – 0.83)	0.96 (0.85 – 1.09)	0.56 (0.43 – 0.73)	0.86 (0.66 – 1.11)	0.79 (0.69 – 0.91)	0.99 (0.86 – 1.14)
Q 4	0.65 (0.57 – 0.74)	0.88 (0.77 – 1.00)	0.43 (0.32 – 0.57)	0.66 (0.49 – 0.88)	0.71 (0.61 – 0.82)	0.95 (0.82 – 1.10)
p-trend*	<0.001	0.151	<0.001	0.013	<0.001	0.686

Abbreviations: OBS=oxidative balance score; HR=hazards ratio; CI=confidence interval; FOP=fluorescent oxidation products; Lit.=literature; Q=quartile

† Adjusted for age, sex, race, BMI, and total daily energy, education, exercise, and region

‡ Adjusted for the same variables as above plus smoking

*p-trend assessed by X^2 test for linear trend

TABLE 2.7. Sensitivity analyses to evaluate the impact of individual OBS components on study results

Model	All-cause Mortality	Cancer Mortality	Non-Cancer Mortality
	HR (95% CI)†	HR (95% CI)†	HR (95% CI)†
Original model (Reference)	0.964 (0.953 – 0.976)	0.929 (0.905 – 0.954)	0.973 (0.960 – 0.987)
OBS excluding PUFA controlled for PUFA	0.961 (0.949 – 0.973)	0.928 (0.903 – 0.953)	0.969 (0.956 – 0.983)
OBS excluding iron controlled for iron	0.963 (0.951 – 0.976)	0.933 (0.908 – 0.958)	0.971 (0.957 – 0.985)
OBS excluding vitamin C controlled for vitamin C	0.964 (0.950 – 0.979)	0.928 (0.899 – 0.958)	0.973 (0.957 – 0.989)
OBS excluding lycopene controlled for lycopene	0.958 (0.946 – 0.971)	0.923 (0.897 – 0.949)	0.967 (0.952 – 0.981)
OBS excluding α-carotene controlled for α-carotene	0.961 (0.948 – 0.975)	0.928 (0.900 – 0.958)	0.969 (0.954 – 0.985)
OBS excluding β-carotene controlled for β-carotene	0.954 (0.939 – 0.970)	0.923 (0.891 – 0.956)	0.962 (0.945 – 0.980)
OBS excluding lutein controlled for lutein	0.971 (0.957 – 0.985)	0.924 (0.896 – 0.954)	0.983 (0.967 – 0.999)
OBS excluding β-cryptoxanthin controlled for β-cryptoxanthin	0.964 (0.951 – 0.977)	0.916 (0.889 – 0.943)	0.976 (0.961 – 0.991)
OBS excluding α-tocopherol controlled for αtocopherol	0.967 (0.954 – 0.980)	0.924 (0.897 – 0.952)	0.978 (0.963 – 0.993)
OBS excluding selenium controlled for selenium	0.964 (0.951 – 0.976)	0.926 (0.900 – 0.952)	0.973 (0.959 – 0.987)
OBS excluding smoking controlled for smoking	0.989 (0.977 – 1.002)	0.966 (0.940 – 0.993)	0.995 (0.981 – 1.009)
OBS excluding aspirin controlled for aspirin	0.956 (0.944 – 0.969)	0.928 (0.902 – 0.954)	0.963 (0.949 – 0.977)
OBS excluding NSAID controlled for NSAID	0.965 (0.953 – 0.977)	0.929 (0.904 – 0.954)	0.974 (0.960 – 0.988)
OBS excluding alcohol controlled for alcohol	0.961 (0.949 – 0.973)	0.931 (0.906 – 0.956)	0.968 (0.955 – 0.982)

Abbreviations: OBS=oxidative balance score; HR=hazards ratio; CI=confidence interval; PUFA=polyunsaturated fatty acid; NSAID=non-steroidal anti-inflammatory drug

† HR represents change in hazards for each additional OBS point. All results are adjusted for age, sex, race, BMI, and total daily energy, education, exercise, and region

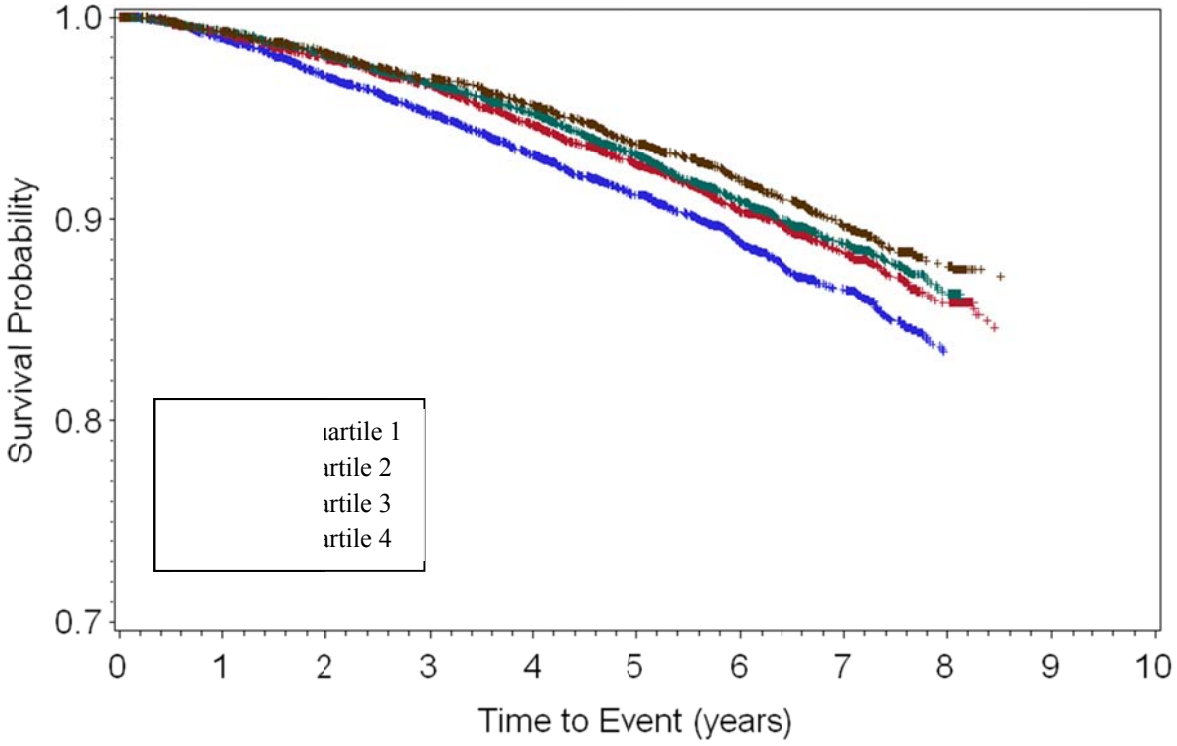

FIGURE 2.1. Kaplan-Meier Survival Curves of All-cause Mortality by OBS quartile

CHAPTER 3. OXIDATIVE BALANCE SCORE TO RISK OF STROKE INCIDENCE AND MORTALITY IN A LARGE NATIONWIDE COHORT

So Yeon J.Kong[1], Roberd M. Bostick[1,2], W. Dana Flanders[1], William McClellan[1], Suzanne Judd[3], Michael Goodman[1,2]

[1] Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA.

[2] Winship Cancer Institute, Emory University, Atlanta, GA.

[3] Department of Biostatistics, University of Alabama at Birmingham, AL

ABSTRACT

Stroke is one of the leading causes of mortality and morbidity worldwide. A considerable body of evidence indicates that oxidative stress is a fundamental mechanism of brain injury in stroke. However, epidemiologic studies that examined the association between individual anti- or pro-oxidant and stroke incidence or mortality had inconsistent results. Recently, we and others proposed oxidative balance score (OBS) as a measure of combined pro- and anti-oxidant exposure status, which was used in studies of various chronic diseases. In this study, we use data from a large national prospective cohort study to examine the relation of OBS comprised of 14 *a priori* selected oxidative stress related exposures to incident stroke while exploring alternative methods of weighting the OBS components.

Data for individual pro- and anti-oxidant exposure were collected at baseline by telephone questionnaire in the Reasons for Geographic and Racial Differences in Stroke (REGARDS) cohort participants, who were enrolled in 2003-2007. The OBS was calculated by combining information from a total of 14 *a priori* selected pro- and anti-oxidant factors, including intakes of polyunsaturated fatty acid, iron, vitamin C, lycopene, α-carotene, β-carotene, lutein, β-crypoxanthin, α-tocopherol, and selenium, smoking status, alcohol, and regular use of aspirin and NSAID. The overall OBS was divided into quartiles with the lowest quartile (predominance of pro-oxidants) as reference. Each OBS component was included in the overall score using four weighting methods: 1) equal weights; 2) literature-based weights; 3) fluorescent oxidation products (FOP)-based weights; and 4) F_2-isoprostanes (FIP)-based weights. The association between OBS and stroke incidence was assessed by Cox proportional hazard regression analysis.

Over a median follow-up period of 5.8 years, 469 of the 19,632 participants had stroke incidence. Higher OBS had no significant effect on incident stroke or stroke mortality in either unadjusted or adjusted analysis. After adjustment for age, sex, race, BMI, systolic blood pressure, cholesterol, diabetes, total energy, education, income, region, and physical activity, the hazard ratios (95% CI) for stroke incidence and stroke mortality for highest quartile were: 0.92 (0.69 – 1.24) and 0.96 (0.48 – 1.91), respectively when compared with those with lowest OBS quartile. Very similar results were observed across all weighting methods. Future researches are warranted to clarify the role of different risk factors for stroke.

INTRODUCTION

Stroke is one of the leading causes of mortality and morbidity worldwide. In the United States stroke is responsible for one of every 19 deaths with approximately 790,000 new or recurrent stroke events reported per year [189]. This makes primary prevention of stroke a major public health priory [190].

A considerable body of evidence indicates that oxidative stress is a fundamental mechanism of brain injury in stroke [124, 191, 192]. Brain tissue is particularly susceptible to free-radical damage because it is very rich in polyunsaturated fatty acids, which are vulnerable to free-radical lipid peroxidation, low in protective antioxidants enzymes, such as glutathione peroxidase, and possesses high levels of iron, which is a potent pro-oxidant [125-128, 190, 193-195]. Reactive oxygen species (ROS) affect both cellular and vascular brain function. Cellular effects of ROS include lipid peroxidation, protein denaturation, DNA modification, damage to the cytoskeletal structure, and chemotaxis [124]. At the vascular level, ROS exert their effects at very low

concentrations, leading to increased vasodilation, platelet aggregation, increased endothelial permeability, altered reactivity to vasodilators, and formation of focal lesions in endothelial cell membrane [129].

Despite biological plausibility, epidemiologic studies that examined the association between individual anti- or pro-oxidant exposures and stroke incidence or mortality produced inconsistent results [196-201]. One potential explanation for this discrepancy is the complex and multi-factorial mechanism by which oxidative stress may affect stroke. The independent effects of individual exposures may not offer complete insight into their roles in maintaining the overall oxidative balance because of the likely inter-correlations and biological interactions involving multiple pro- and anti-oxidant factors [21]. The difficulties of evaluating effects of individual antioxidants may be overcome by employing the concept of an integrated antioxidant network, which takes into account that antioxidants of differing solubility are residing next to each other in cellular structures and tissues, integrating and regenerating each other [157]. This situation is somewhat similar to the difficulties encountered in nutrition research, in which the pursuit of effects exerted by individual nutrients has been replaced by use of dietary pattern analyses, as illustrated by the use of the Mediterranean diet score [202-206].

Recently, we [25-27] and others [143, 163] proposed oxidative balance score (OBS) as a measure of combined pro- and anti-oxidant exposure status. The concept of OBS was used in studies of various chronic diseases; however, none of those studies has examined the association between OBS and stroke. In the present study, we use data from a large national prospective cohort study to examine the relation of OBS comprised

of 14 *a priori* selected oxidative stress related exposures to incident stroke and stroke mortality while exploring alternative methods of weighting the OBS components. We hypothesize that higher OBS, which reflects predominance of anti-oxidant exposures, is associated with a reduction in risks of stroke and stroke-related death.

METHODS

Study population and data collection

The Reasons for Geographic and Racial Differences in Stroke (REGARDS) Study is a national, population-based prospective cohort study aimed to examine the reason for variation in stroke incidence and mortality in the United States. Details on recruitment and data collection were reported previously [147]. Briefly, between January 2003 and October 2007, 30,239 black and white individuals aged 45 years or older were randomly selected and recruited through mail and telephone contacts. The cohort members were recruited from across the US with oversampling of blacks and persons from the "stroke belt" region of the United States. The "stroke belt" describes the southeastern region of the United States (North Carolina, South Caroline, Georgia, Tennessee, Mississippi, Alabama, Louisiana, and Arkansas) with a high incidence and mortality of stroke [145]. Exclusion criteria were race other than black or white, active treatment for cancer, cognitive impairment as judged by the telephone interviewer, medical conditions preventing long-term participation, residence in or inclusion on a waiting list for a nursing home, or inability to communicate in English.

After obtaining verbal and written informed consent, demographic and medical history data, including information on risk factors, were collected by computer-assisted telephone interviewing (CATI). Variables included age, race, and sex of the participants,

use of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs), cigarette smoking, alcohol intake, and measures of socioeconomic status (education and income). Following the telephone interview, an in-home visit was completed to obtain blood and urine samples and collect information on the presence of risk factors, such as blood pressure, height and weight. Additional information was collected through self-administered questionnaires, including the Block 98 food-frequency questionnaire (FFQ). The Block 98 FFQ is an 8-page form with more than 150 multiple-choice questions based on 107 food items, which was used to assess energy intake, dietary fat, and nutritional intakes. At every six month interval, each participant was then followed via telephone interview to ascertain development of stroke and other outcomes.

Of the 30,239 participants enrolled in the REGARDS Study, 8,603 who did not complete the modified Block 98 FFQ were excluded from the current analysis. In addition, the analytic dataset excluded 1,115 participants with prevalent stroke at baseline, 690 participants with missing data on at least one OBS component, and 199 participants with missing data on key covariates. After these exclusions, data for 19,632 participants were available for the final analyses.

Oxidative Balance Score (Main Exposure Variable)

The oxidative balance score (OBS) was calculated by combining information from a total of 14 *a priori* selected pro- and anti-oxidant factors, including intakes of polyunsaturated fatty acids, iron, vitamin C, lycopene, α-carotene, β-carotene, lutein, β-crypoxanthin, α-tocopherol, selenium, smoking status, alcohol consumption and regular use of aspirin and other NSAIDs (TABLE 3.1). The continuous variables reflecting pro-oxidant (unsaturated fat and iron) and antioxidant (vitamin C, lycopene, α-carotene, β-

carotene, lutein, β-cryptoxanthin, α-tocopherol, and selenium) exposures were divided into low, medium, and high categories based on each exposure's tertile values. For anti-oxidants, the first through third tertiles were assigned 0 through 2 points, respectively, whereas the corresponding point assignment for pro-oxidants were carried out in reverse (0 points for the highest tertile and 2 points for the lowest tertile). A similar scoring approach was used for pro- and anti-oxidant categorical variables. Smoking status was categorized as never (2 points), former (1 point), and current (0 points). For aspirin and NSAIDs use, 0 points were assigned to participants with no regular use, 1 point to those with unknown or missing data, and 2 points to those with regular use. For alcohol consumption, non-drinkers, moderate drinkers (1-7 drinks/week for women and 1-14 drinks/week for men), and heavy drinker (>7 drinks/week for women and >14 drinks/week for men) received 2, 1 and 0 points respectively.

OBS Weighting

Each OBS component was included in the overall score using four weighting methods: 1) equal weights; 2) literature-based weights; 3) weights based on the magnitude of association between each component and fluorescent oxidation products (FOP); and 4) weights based on the magnitude of association between each component and F_2-isoprostanes (FIP). The weights for each OBS component are summarized in Table 3.2.

The equal weights approach assumed that all OBS component equally contributed to the overall score. By contrast, the other three methods assigned weights based on the presumed magnitude of the pro- and anti-oxidant effects.

For the literature-based method each OBS component was weighted according to the results of most recent systematic reviews/meta-analysis evaluating the association between this component and incident stroke. In the absence of a recent systematic review/meta-analysis for any of the OBS components, a *de novo* meta-analysis was conducted. For each OBS component, weights were calculated based on the pooled (i.e., meta-) risk estimates (meta-RR). For each pro-oxidant the weights were equal to meta-RR, while for antioxidants the corresponding weights were calculated as 1/meta-RR.

The two biomarker (FOP and FIP)-based weighting methods used pooled data from two previously completed case-control studies of colorectal adenoma (MAP I and MAP II) that employed virtually identical protocols. The detailed study methods for MAP I [164, 165] and MAP II [166, 167] have been previously published. In both studies, a modified 153-item Willett Food Frequency Questionnaire was administered to obtain information on dietary intakes and use of nutritional supplements [168, 169]. For both studies, blood was collected, handled, and stored in a manner to allow measurements of FOP and FIP. For both FIP- and FOP-based weighting, we used adjusted multivariable logistic regression models to quantify the relation between each OBS component and each of the two markers of oxidative stress. The adjusted odds ratio estimates from these logistic models were used to assign weights.

Outcome Measures

The primary outcome in this study was incident stroke. Participants or next-of-kin of the deceased cohort members who reported an incident stroke diagnosis on a follow-up questionnaire were asked permission to review relevant medical records. Two committee members reviewed the records independently using accepted criteria for stroke diagnosis [207, 208] and sub-classified stroke into ischemic, hemorrhagic, or unknown

type. No further action was taken if the two reviewers agreed on the occurrence of stroke and stroke subtype. In cases of disagreement, a third adjudicator reviewed the potential event. We used three different definitions: 1) WHO-defined stroke – focal neurological deficit lasting ≥ 24 hours, confirmed by medical records; 2) Clinical stroke – focal or non-focal neurological deficit with positive imaging that may or may not last 24 hours, and confirmed by medical records; and 3) National Death Index (NDI)-confirmed stroke as underlying cause of death without medical records. Only adjudicated first stroke cases were included in this analysis.

The secondary outcome in this study was stroke mortality. A death of a participant was ascertained during telephone follow up or through Web-based restricted-access database searches (e.g., Lexis-Nexis), and was later confirmed through death certificates. In addition, interviews with next-of-kin or proxies of deceased participants were conducted to confirm the death and the date of death. Information on the cause of death was also obtained from death certificates. Death cases and causes of death were reviewed independently by two adjudicators, and disagreements were resolved by committee. Adjudicators used baseline participant clinical characteristics, proxy interviews, death certificates, and if available, medical records from hospitalizations occurring within 30 days of the participant's death to determine the cause of death.

Statistical analysis

Each version of OBS (unweighted and weighted using literature-, FOP- and FIP-based methods) was divided into quartiles, with the lowest quartile (predominance of pro-oxidant exposures) used as reference. The total follow-up time for each individual was calculated as the time between first visit interview and the date of incident stroke, date of

death, the date of the last study visit, the date of withdrawal or loss to follow-up, or March 1, 2012, whichever came first. The Kaplan-Meier survival curves accompanied by a log-rank test and the corresponding p-value were used to assess the unadjusted association between OBS and incident stroke. The adjusted associations between OBS and both incident stroke and stroke mortality were examined using Cox proportional hazard models, that controlled for age, sex, race, SES, region, BMI, total daily energy intake, systolic blood pressure, diabetes, cholesterol, and physical activity. Results of multivariable survival analyses were expressed as, adjusted hazard ratios (HR) with corresponding 95% confidence intervals (CI). Proportional hazards assumptions were tested by inspecting –ln(ln) survival curves for each variable in the model. A condition index of 30 or greater, coupled with a variance decomposition proportion of 0.5 or greater was considered as evidence of collinearity. Stratified analyses were conducted to examine whether the associations between OBS and incident stroke are modified by race, gender, region, and stroke subtype. We further performed sensitivity analyses to evaluate the effect of individual OBS component on incident stroke. A two-sided p-value of less than 0.05 was considered as evidence of statistical significance. All statistical analyses were performed with SAS version 9.3 (SAS Institute, Cary, NC) statistical software package.

RESULTS

Baseline characteristics of the study cohort by OBS category are shown in TABLE 3. Compared with those in the lowest OBS quartile, participants in the highest quartile were on average three years older (66 vs. 63), included a greater proportion of whites (70.2% vs. 65.3%), and had more females (58.4% vs. 54.8%). There was no

difference in BMI, systolic blood pressure, and diabetes status between highest and lowest OBS quartile groups. However, persons in the highest OBS quartile were also more likely to have higher cholesterol, daily energy intake, education and income, and reside in the non-stroke belt states. The evaluations of individual OBS components according to OBS quartiles are presented in TABLE 4. Contrary to expectation, intakes of daily polyunsaturated fatty acids (PUFA) and iron were higher in higher OBS quartile groups. As expected, the intakes of antioxidants (vitamin C, lycopene, α-carotene, β-carotene, lutein, β-cryptoxanthin, vitamin E, and selenium) were significantly increased among cohort members with higher OBS values. Participants in the higher OBS quartiles were also more likely to be never smokers and non-drinkers, and regularly use aspirin and/or NSAIDs.

During average 5.8 years of follow-up (range 0 – 9.1 years), we ascertained 469 cases of incident stroke, of which 87% were ischemic strokes. There were 72 deaths due to stroke. Higher OBS was not associated with incident stroke or stroke mortality in either unadjusted or adjusted analysis (TABLE 5). TABLE 6 shows the associations between OBS and incident stroke based on different weighting methods. Similar statistically non-significant associations were observed when the analyses were repeated with different weights (TABLE 6). Stratified analysis showed that associations between OBS and incident stroke were not modified by race, gender, region, and stroke subtype (Supplemental Tables).

Table 7 presents the sensitivity analyses in which the observed results for the original 14-component OBS (treated as a continuous variable) were compared to the corresponding results after each OBS component was removed from the score and

included in the model as a covariate. Removal of any single OBS component did not produce meaningful changes.

DISCUSSION

In this population-based large prospective cohort study, we examined a comprehensive oxidative balance score (OBS) as predictor of incident stroke with an expectation that OBS may better reflect the oxidative balance than any single pro- or anti-oxidant. We observed no overall effect of higher OBS on the incidence of stroke. There was also no association between OBS and stroke mortality.

While basic science evidence implicating oxidative stress in stroke pathogenesis is strong, translation of these fundamental concepts into clinical applications has been be challenging and so-far disappointing [209]. The Alpha-Tocopherol Beta-Carotene Prevention (ATBC) Study [195] investigated the effect of β-carotene, vitamin E, or both, on the major cardiac events and lung cancer in a population of middle-aged male cigarette smokers in Finland. The study observed slightly higher rates of stroke incidence and mortality in both the vitamin E and the β-carotene groups compared to the placebo group. Nutrition intervention trial in Linxian, China, which is one of the earliest large-scale randomized trials of vitamins, also examined the effect of multiple vitamins/minerals including antioxidants. In the Linxian study, participants were randomized to one of eight groups which received combinations of four supplements: retinol and zinc (Factor A); riboflavin and niacin (factor B); vitamin C and molybdenum (factor C); and β-carotene, α-tocopherol (vitamin E), and selenium (factor D). Compared to the placebo group, none of the seven randomization groups that received combination of nutritional supplements showed statistically significant reduction in stroke mortality

[210]. The Heart Protection Study (HPS) [211] also showed that antioxidant supplementation has no beneficial effects on the risk of stroke. After 5 years of daily supplementation of high-risk individuals with 600 mg vitamin E, 250 mg vitamin C, and 20 mg β-carotene, no significant differences in incident stroke were observed between treatment and placebo groups (Risk Ratio = 0.99; 95% CI: 0387 – 1.12).

The results of several observational studies evaluating the association of dietary anti-oxidants with stroke were similar to those reported in clinical trials. In a large Danish prospective cohort study, intakes of fruit and vegetables did not significantly reduce the risk of ischemic stroke with a risk ratio of 0.72 (95% CI: 0.47-1.12) when a persons in the top quintile of fruit and vegetable intake compared to the persons in the bottom quintile [212]. In a Dutch cohort study, total and processed fruit and vegetables intakes were not associated with incident stroke [213]. After multivariable adjustments, neither raw fruit and vegetable intake nor processed fruit and vegetable intake was associated with total stroke incidence (HR = 0.70; 95% CI: 0.47 – 1.03 and HR = 1.20; 95% CI: 0.81 – 1.76, respectively) when comparing the highest and the lowest quartiles. Similar results were observed regardless of different stroke subtypes. Yochum *et al.* also examined the association between antioxidant vitamin intakes, both dietary and supplemental, among postmenopausal women in the Iowa Women's Health Study [214]. The study found that total vitamin A, carotenoid, retinol, vitamin C, and vitamin E intakes were not associated with death from stroke after multivariate adjustments.

The null results observed in our and other studies may have several explanations. Studies have shown that different stroke types and locations may have distinct causes and risk factors [215]. Our stratified analysis demonstrated no difference in results for

ischemic and hemorrhagic strokes but this stratification was rather crude and was affected by the small number (n=61) of hemorrhagic strokes. It is also possible that physiologic risk factors, such as hypertension and diabetes, all of which strongly affect oxidative stress, have much greater impact than modifiable exposures included in the OBS. For example, INTERSTROKE, a large international case-control study found that five risk factors – hypertension, current smoking, abdominal obesity, diet, and physical activity – accounted for more than 80% of the global risk of all stroke. [216].

While OBS was not associated with stroke in the present study, in other studies it was found to be associated with several other outcomes including colorectal adenoma, cancer, and all-cause mortality. We others previously reported a substantial decrease in risk associated with a high OBS category for both colorectal adenoma and colorectal cancer [163, 217]. In another study conducted among male smokers in Belgium, Van Hoydonck *et al.* combined intakes of two dietary antioxidants (vitamin C and β-carotene) and one pro-oxidant (iron) to develop their oxidative balance score [143]. Men in the highest category OBS, which unlike ours was constructed to reflect a presumably harmful effect, had a statistically significant 44% increase in all-cause mortality and an even greater (62%) increase in cancer mortality compared with men in the lowest OBS group.

There are several potential limitations in this study. We used self-reported intakes to assess pro- and anti-oxidant exposure. It has long been acknowledged that the questionnaires may not capture all the possible sources of each nutrient, does not account for bioavailability, and is subject to recall bias [183]. Although the validity and reliability of the FFQ used in our study has been extensively evaluated [184-186], it is possible that some degree of misclassification may still exist. We hypothesize that such

bias is non-differential, thus shift our estimates toward the null value. Furthermore, we only used baseline measurements of OBS components. Future studies should consider use of repeated measurements of OBS components to obtain a better assessment of long-term overall anti- and pro-oxidant exposures and to reduce measurement error.

Another limitation of this study is that we did not have participants' genetic information. Twin and family history studies support an important role of genetic factors in stroke risk [218-220]. For example, previous twin studies have shown that stroke prevalence is about five times higher in monozygotic than in dizygotic twins [218]. Furthermore, the OBS score in our study is limited to dietary and lifestyle exposures and does not include any endogenous factors that influence cellular anti-oxidant defense, repair of ROS-induced damage, and other pathophysiologic mechanisms that play a role in stroke [221].

CONCLUSION

This large prospective study shows that higher OBS is not significantly associated with lower stroke incidence or mortality. In 2010, the AHA developed its 2020 impact goal to reduce deaths from cardiovascular disease and stroke by 20% [222]. To help achieve this goal, future researches are warranted to clarify the role of oxidative stress in stroke by focusing on factors that were included in the OBS and by examining different pathophysiologic and anatomic subtypes of stroke.

TABLE 3.1. Oxidative balance score (OBS) assignment scheme

Oxidative Balance Score (OBS) Components	Assignment Scheme†
1. PUFA intake	0 = High (3rd tertile), 1 = Intermediate (2nd tertile), 2 = Low (1st tertile)
2. Total* iron intake	0 = High (3rd tertile), 1 = Intermediate (2nd tertile), 2 = Low (1st tertile)
3. Total vitamin C intake	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
4. Total lycopene level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
5. Total α-carotene level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
6. Total β-carotene level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
7. Total lutein level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
8. Total β-cryptoxanthin level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
9. Total α-tocopherol level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
10. Selenium level	0 = Low (1st tertile), 1 = Intermediate (2nd tertile), 2 = High (3rd tertile)
11. Smoking history	0 = Current smoker, 1 = Former smoker, 2 = Never smoker
12. Regular aspirin use	0 = No regular use, 1 = Unknown (missing data), 2 = Regular Use
13. Regular NSAID use	0 = No regular use, 1 = Unknown (missing data), 2 = Regular Use
14. Alcohol consumption	0 = None, 1 = Moderate, 3 = Heavy

Abbreviations: PUFA = Polyunsaturated fatty acid; NSAID = Non-steroidal anti-inflammatory drug

† Low, intermediate, and high categories correspond to sex-specific tertile values among participants in the REGARDS cohort.

*Total intake = Dietary intake + supplemental intake (when available)

TABLE 3.2. Oxidative balance score (OBS): Weights assigned to individual components

OBS Components	Weightings				
	F_2-isoprostanes		FOP		Lit. Review
	Male	Female	Male	Female	Both sex
1. PUFA intake	0.87	0.91	1.23	1.24	0.91
2. Total iron intake	0.70	0.54	0.88	0.94	1.52
3. Total vitamin C intake	1.81	1.48	1.12	0.85	1.27
4. Total lycopene level	1.17	1.35	0.82	0.89	1.06
5. Total α-carotene level	1.43	1.22	0.85	0.75	1.06
6. Total β-carotene level	1.45	1.31	0.97	0.83	1.11
7. Total lutein level	1.61	1.73	0.97	0.68	1.43
8. Total β-cryptoxanthin level	1.69	1.38	1.16	1.11	1.00
9. Total α-tocopherol level	1.79	1.30	0.98	0.91	0.98
10. Selenium level	1.32	2.18	1.12	1.61	0.98
11. Smoking history	1.74	1.43	1.60	1.80	2.60
12. Regular aspirin use	1.28	1.20	0.87	0.91	1.06
13. Regular NSAID use	1.06	1.04	0.70	0.96	0.64
14. Alcohol consumption	1.07	0.99	1.35	1.23	1.02

Abbreviations: OBS=Oxidative Balance Score PUFA=Polyunsaturated fatty acid; NSAID=Non-steroidal anti-inflammatory drug; FOP=fluorescent oxidation products; Lit.=literature

TABLE 3.3. Selected baseline characteristics of the REGARDS cohort by OBS quartile

Characteristic (Units)†	Q1: OBS 3-11 (n=5,322)	Q2: OBS 12-14 (n=5,211)	Q3: OBS 15-17 (n=5,148)	Q4: OBS 18-26 (n=3,951)
Age, years	63.5 (9.2)	64.5 (9.4)	65.4 (9.1)	65.9 (9.0)
Race				
White	3,475 (65.3)	3,444 (66.1)	3,528 (68.5)	2,774 (70.2)
Black	1,847 (34.7)	1,767 (33.9)	1,620 (31.5)	1,177 (29.8)
Sex				
Male	2,404 (45.2)	2,282 (43.8)	2,250 (43.7)	1,644 (41.6)
Female	2,918 (54.8)	2,929 (56.2)	2,898 (56.3)	2,307 (58.4)
BMI* (kg/m²)	28.8 (6.0)	29.1 (6.1)	29.0 (6.0)	29.3 (6.1)
SBP (mmHg)	126.5 (16.5)	126.6 (15.9)	126.5 (16.1)	126.6 (16.0)
Cholesterol (mg/dL)	188.7 (118.8)	204.9 (125.9)	220.9 (134.3)	229.8 (135.3)
Diabetes				
Yes	980 (18.4)	1,011 (19.4)	983 (19.1)	782 (19.8)
No	4,342 (81.6)	4,200 (80.6)	4,165 (80.9)	3,169 (80.2)
Energy (cal)	1,477.8 (604.5)	1,645.9 (669.1)	1,812.5 (724.7)	1,966.3 (750.8)
Education				
Less than High School	606 (11.4)	501 (9.6)	419 (8.1)	253 (6.4)
High School Graduate	1,587 (29.8)	1,368 (26.3%)	1,167 (22.7)	853 (21.6)
Some College	1,506 (28.3)	1,438 (27.6)	1,360 (26.4)	1,053 (26.6)
College Graduate and Above	1,623 (30.5)	1,904 (36.5)	2,202 (42.8)	1,792 (45.4)
Income	887 (16.7)	786 (15.1)	753 (14.6)	519 (13.1)
Less than $20k	1,344 (25.2)	1,262 (24.2)	1,148 (22.3)	928 (23.5)
$20k - $34k	1,615 (30.3)	1,632 (31.3)	1,714 (33.3)	1,288 (32.6)
$35k - $74k	871 (16.4)	912 (17.5)	935 (18.2)	777 (19.7)
$75k and above	605 (11.4)	619 (11.9)	598 (11.6)	439 (11.1)
Refused				
Region	1,925 (36.2)	1,838 (35.3)	1,699 (33.0)	1,292 (32.7)
Stroke Belt	1,225 (23.0)	1,147 (22.0)	1,126 (21.9)	825 (20.9)
Stroke Buckle	2,172 (40.8)	2,226 (42.7)	2,323 (45.1)	1,834 (46.4)
Non-belt				
Follow-up Time*, years	5.6 (1.9)	5.7 (1.9)	5.8 (1.9)	5.9 (1.9)

Abbreviations: BMI = body mass index; SBP = systolic blood pressure

†Values for age, BMI, energy, and follow-up years are mean (±SD) and race, sex, education, income, and region are number (percent).

‡ Based on the ANOVA for continuous variables and chi-square (X^2) test for categorical variables.

TABLE 3.4. Individual components of the score by OBS quartile

Characteristics (Units)	Mean (by OBS Quartile)			
	Quartile 1 (n=5,322)	Quartile 2 (n=5,211)	Quartile 3 (n=5,148)	Quartile 4 (n=3,951)
Daily PUFA from intake (g)				
Men (n=8,580)	18.3 (9.7)	19.8 (10.8)	21.2 (11.0)	21.7 (11.0)
Women (n=11,052)	15.7 (9.2)	17.0 (6.7)	18.3 (10.2)	19.2 (10.4)
Total* iron intake (mg)				
Men	17.8 (13.5)	23.6 (15.8)	27.8 (17.3)	30.7 (19.1)
Women	18.1 (16.5)	23.1 (18.7)	26.7 (18.9)	30.5 (21.1)
Total vitamin C intake (mg)				
Men	122.6 (178.7)	279.5 (351.2)	427.4 (476.1)	647.9 (559.1)
Women	148.1 (226.9)	285.7 (349.3)	437.1 (442.5)	625.8 (526.9)
Daily lycopene intake (μg)				
Men	2,960.3 (3,177.3)	4,300.4 (4,638.5)	5,397.8 (5,037.8)	7,326.0 (6,898.4)
Women	2324.2 (2,523.6)	3,349.2 (3,733.3)	4,294.0 (4,476.3)	5,796.3 (5,443.0)
Daily α-carotene intake (μg)				
Men	328.7 (291.9)	527.9 (493.2)	857.9 (784.6)	1,239.3 (1,099.9)
Women	298.0 (241.1)	521.3 (561.4)	853.5 (884.3)	1,246.9 (1,087.8)
Total β-carotene intake (μg)				
Men	2,165.9 (1,822.8)	3,783.3 (3,579.1)	6,208.2 (5,797.8)	8,987.3 (7,497.0)
Women	2,264.5 (1,574.1)	4,107.1 (3,999.6)	6,431.5 (5,485.5)	9,402.0 (7,454.6)
Daily lutein intake (μg)				
Men	830.5 (596.7)	1,322.4 (1,011.9)	1,969.1 (1,649.5)	2,827.7 (2,441.4)
Women	976.9 (834.0)	1,545.0 (1,408.8)	2,395.3 (2,176.0)	3,358.7 (2,809.9)
Daily β-cryptoxanthin intake (μg)				
Men	63.3 (83.2)	115.7 (122.2)	156.3 (137.8)	208.1 (161.2)
Women	53.2 (69.7)	102.9 (109.9)	142.4 (141.0)	191.6 (157.0)
Total vitamin E intake (α-TE)				
Men	35.4 (90.3)	83.8 (148.7)	131.9 (184.6)	195.9 (192.5)
Women	38.7 (89.9)	88.6 (156.5)	127.7 (172.2)	191.9 (194.3)
Daily selenium supplement (mcg)				
Men	79.8 (35.4)	98.7 (48.6)	118.5 (61.6)	141.0 (73.9)
Women	66.3 (31.3)	82.0 (42.4)	98.1 (50.8)	118.6 (62.8)
Smoking				
Never smokers	1,708 (32.1)	2,297 (44.1)	2,568 (49.9)	2,248 (61.9)
Former smokers	2,258 (42.4)	2,247 (43.1)	2,147 (41.7)	1,339 (33.9)
Current smokers	1,356 (25.5)	667 (12.8)	433 (8.4)	164 (4.1)
Alcohol intake				
Non-drinkers	2,813 (52.9)	3,087 (59.2)	3,059 (59.4)	2,635 (66.7)
Moderate drinkers	2,089 (39.2)	1,908 (36.6)	1,914 (37.2)	1,239 (31.4)
Heavy drinkers	420 (7.9)	216 (4.2)	175 (3.4)	77 (1.9)
Regular aspirin use	1,414 (26.6)	2,096 (40.2)	2,427 (47.2)	2,537 (64.2)
Regular NSAIDs use	432 (8.1)	659 (12.7)	835 (16.3)	1,055 (26.8)
Total OBS	9.2 (1.6)	13.0 (0.8)	16.0 (0.8)	19.4 (1.4)

[1] Values are presented as mean (SD) or number (%). Abbreviations: PUFA = Polyunsaturated fatty acid; NSAID = Non-steroidal anti-inflammatory drug; OBS = Oxidative balance score; SD = Standard deviation

* Total intake = daily intake from food + average daily intake from supplement

TABLE 3.5. Association between incident stroke and stroke mortality and OBS in the REGARDS cohort: Equal weighting

Interval (OBS Range: 3-26)	Stroke Incidence/ Mortality	No Stroke Incidence/ Mortality	Crude HR (95% CI)	Multivariate HR (95% CI)†
Stroke Incidence	(n = 469)	(n = 19,163)		
Q 1	136	5,186	1.0	1.0
Q 2	115	5,096	0.85 (0.67 – 1.10)	0.80 (0.61 – 1.05)
Q 3	120	5,028	0.89 (0.69 – 1.13)	0.86 (0.65 – 1.12)
Q 4	98	3,853	0.94 (0.72 – 1.21)	0.92 (0.69 – 1.24)
p-trend*			0.84	0.84
Stroke Mortality	(n=72)	(n = 17,814)		
Q 1	21	4,759	1.0	1.0
Q 2	12	4,749	0.57 (0.28 – 1.17)	0.54 (0.26 – 1.12)
Q 3	22	4,682	1.05 (0.58 – 1.90)	0.99 (0.53 – 1.86)
Q 4	17	3,624	1.04 (0.55 – 1.98)	0.96 (0.48 – 1.91)
p-trend*			0.52	0.73

Abbreviations: OBS=oxidative balance score; HR=hazards ratio; CI=confidence interval; Q=quartile

† Adjusted for age, sex, race, BMI, and total daily energy, systolic blood pressure, cholesterol, diabetes, education, income, exercise, and region

*p-trend assessed by X^2 test for linear trend

OBS ranges: Q1=3-11; Q2=12-14; Q3=15-17; Q4=18-26

TABLE 3.6. Association between incident stroke and OBS in the REGARDS cohort: Different weighting approaches

OBS Weighting	FIP	FOP	Lit. Review
	HR (95% CI) †	HR (95% CI)†	HR (95% CI)†
Quartile 1	1.0	1.0	1.0
Quartile 2	0.77 (0.58 – 1.02)	0.81 (0.61 – 1.07)	0.74 (0.56 – 0.98)
Quartile 3	0.86 (0.65 – 1.13)	0.97 (0.74 – 1.27)	0.80 (0.61 – 1.06)
Quartile 4	0.91 (0.68 – 1.22)	0.85 (0.64 – 1.14)	0.82 (0.62 – 1.10)
p-trend*	0.92	0.76	0.49

Abbreviations: OBS=oxidative balance score; FIP=F_2-isoprostanes; FOP=fluorescent oxidation products; Lit.=literature HR=hazards ratio; CI=confidence interval

† Adjusted for age, sex, race, BMI, and total daily energy, systolic blood pressure, cholesterol, diabetes, education, income, exercise, and region

*p-trend assessed by X^2 test for linear trend

TABLE 3.7. Sensitivity analyses to evaluate the impact of individual OBS components on study results

Model	Incident Stroke
	HR (95% CI)†
Original model (Reference)	1.00 (0.97 – 1.02)
OBS excluding PUFA controlled for PUFA	1.00 (0.98 – 1.02)
OBS excluding iron controlled for iron	1.00 (0.97 – 1.02)
OBS excluding vitamin C controlled for vitamin C	1.00 (0.97 – 1.04)
OBS excluding lycopene controlled for lycopene	0.99 (0.96 – 1.02)
OBS excluding α-carotene controlled for α-carotene	0.99 (0.96 – 1.02)
OBS excluding β-carotene controlled for β-carotene	0.99 (0.96 – 1.02)
OBS excluding lutein controlled for lutein	0.99 (0.96 – 1.02)
OBS excluding β-cryptoxanthin controlled for β-cryptoxanthin	1.00 (0.97 – 1.03)
OBS excluding α-tocopherol controlled for αtocopherol	1.00 (0.97 – 1.03)
OBS excluding selenium controlled for selenium	1.00 (0.97 – 1.03)
OBS excluding smoking controlled for smoking	1.01 (0.98 – 1.04)
OBS excluding aspirin controlled for aspirin	0.99 (0.96 – 1.02)
OBS excluding NSAID controlled for NSAID	0.99 (0.97 – 1.02)
OBS excluding alcohol controlled for alcohol	1.01 (0.97 – 1.02)

Abbreviations: OBS=oxidative balance score; HR=hazards ratio; CI=confidence interval; PUFA=polyunsaturated fatty acid; NSAID=non-steroidal anti-inflammatory drug

† HR represents change in hazards for each additional OBS point. All results are adjusted for age, sex, race, BMI, cholesterol, systolic blood pressure, diabetes, total daily energy, education, income, exercise, and region

SUPPLEMENTAL TABLES

TABLE S.3.1. Stratified analysis on sex

OBS Weighting	Sex = Male	Sex = Female
	HR (95% CI) †	HR (95% CI)†
Quartile 1	1.0	1.0
Quartile 2	0.92 (0.63 – 1.32)	0.66 (0.45 – 0.996)
Quartile 3	0.99 (0.69 – 1.43)	0.77 (0.47 – 1.04)
Quartile 4	1.11 (0.75 – 1.67)	0.70 (0.45 – 1.08)
p-trend*	0.45	0.18

TABLE S.3.2. Stratified analysis on race

OBS Weighting	Race = White	Race = Black
	HR (95% CI) †	HR (95% CI)†
Quartile 1	1.0	1.0
Quartile 2	0.71 (0.51 – 0.995)	0.98 (0.62 – 1.55)
Quartile 3	0.76 (0.54 – 1.06)	1.08 (0.68 – 1.71)
Quartile 4	0.90 (0.64 – 1.28)	0.93 (0.54 – 1.61)
p-trend*	0.74	0.94

TABLE S.3.3. Stratified analysis on region

OBS Weighting	Region = Belt	Region = Buckle	Region = Non-belt
	HR (95% CI) †	HR (95% CI)†	HR (95% CI)†
Quartile 1	1.0	1.0	1.0
Quartile 2	0.75 (0.48 – 1.16)	0.81 (0.44 – 1.50)	0.86 (0.57 – 1.29)
Quartile 3	0.80 (0.51 – 1.26)	0.82 (0.45 – 1.52)	0.92 (0.61 – 1.39)
Quartile 4	0.83 (0.51 – 1.37)	1.01 (0.53 – 1.94)	0.93 (0.60 – 1.45)
p-trend*	0.57	0.84	0.96

TABLE S.3.4. Stratified analysis on stroke type

OBS Weighting	Type = Ischemic	Type = Hemorrhagic
	HR (95% CI) †	HR (95% CI)†
Quartile 1	1.0	1.0
Quartile 2	0.48 (0.23 – 1.00)	0.82 (0.61 – 1.09)
Quartile 3	0.74 (0.38 – 1.42)	0.85 (0.64 – 1.14)
Quartile 4	0.75 (0.37 – 1.54)	0.97 (0.71 – 1.32)
p-trend*	0.89	0.94

CHAPTER 4. OXIDATIVE BALANCE SCORE, COLORECTAL ADENOMA, AND MARKERS OF OXIDATIVE STRESS AND INFLAMMATION

So Yeon J.Kong[1], Roberd M. Bostick[1,2], W. Dana Flanders[1], William McClellan[1], Bharat Thyagarajan[3], Myron Gross[3], Suzanne Judd[4], Michael Goodman[1,2]

[1] Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA.

[2] Winship Cancer Institute, Emory University, Atlanta, GA.

[3] Department of Laboratory Medicine and Pathology, University of Minnesota School of Medicine, Minneapolis, MN

[4] Department of Biostatistics, University of Alabama at Birmingham, AL

ABSTRACT

We previously proposed an oxidative balance score (OBS) as a measure of combined pro- and anti-oxidant exposure status and used this approach in studies of incident sporadic colorectal adenoma. We extend the previous analyses by assessing three types of associations: 1) OBS and colorectal adenoma; 2) OBS and biomarkers of oxidative stress (F_2-isoprostanes [FIP] and fluorescent oxidation products [FOP]) or inflammation (C-reactive protein [CRP]); and 3) the same biomarkers and adenoma.

Using a pooled data from two previously conducted colonoscopy-based case-control studies of incident, sporadic colorectal adenoma (n=365), the OBS was constructed and divided into three approximately equal intervals, with the lowest interval used as reference. Biomarker levels were dichotomized as "high" versus "low" based on sex-specific median values among controls. The analyses used multivariable logistic regression models with results expressed as odds ratios (ORs) and 95% confidence intervals (CIs).

For the association between OBS and adenoma the ORs (95% CIs) for the middle and the highest intervals were 0.81 (0.46-1.43) and 0.39 (0.17-0.89), respectively (p for trend = 0.04). The ORs (95% CIs) comparing the highest to the lowest OBS intervals in relation to biomarkers were 0.25 (0.10-0.65), 3.48 (1.51-8.02), and 0.21 (0.09-0.49) for FIP, FOP and CRP, respectively (all p for trend <0.01). All three biomarkers were positively associated with adenoma with ORs (95% CIs) of 1.89 (1.08-3.30) for FIP, 1.82 (1.11-2.99) for FOP, and 1.45 (0.88-2.40) for CRP.

As hypothesized, OBS was inversely associated with colorectal adenoma, plasma FIP, and serum CRP. All three biomarkers (FIP, FOP and CRP) were directly

related to adenoma risk. The direct relation of OBS to FOP is unexpected and is difficult to explain.

INTRODUCTION

Oxidative stress has been described as "a disturbance in the pro-oxidant-antioxidant balance in favor of the former, leading to potential damage[223]." Under normal physiologic conditions, cells respond to oxidative stress by up-regulating antioxidant defense mechanisms and other protective systems to restore the balance [6]. However, when these mechanisms are overwhelmed, oxidative stress can damage DNA, proteins, and lipids and lead to cell injury and death [7, 8]. Oxidative stress has long been thought to play an important role in the development of age-related diseases, including cancer [224, 225]. While a considerable body of evidence from basic science and animal studies supports the role of oxidative stress as both an initiator and promoter of carcinogenesis, epidemiological studies of the associations between individual determinants of oxidative stress and cancer are conflicting [16-20]. One potential explanation for this discrepancy is the complex and multi-factorial nature of mechanisms by which oxidative stress may affect cancer risk. The independent effects of individual oxidant exposures are difficult to ascertain because these effects may be highly correlated and because of the likely biological interactions involving multiple pro- and anti-oxidant factors [21]. Therefore, it was suggested that a combined measure that takes into account a multiple pro- and anti-oxidant exposures might be a more accurate indicator of overall oxidative stress burden of an individual [143, 144]. We previously proposed an oxidative balance score (OBS) as a measure of combined pro- and anti-oxidant exposure status.

We illustrated this approach using data from previously conducted case-control studies of incident sporadic colorectal adenoma [26].

Another methodological issue affecting studies evaluating the relation between oxidative stress and disease outcomes in humans is the relative paucity of validated and reliable biomarkers of oxidation. Currently, F_2-isoprostanes (FIP) are considered to be the best available biomarkers of oxidative stress *in vivo* [226-228]. However, FIP measure only lipid peroxidation and may not reflect oxidative damage of proteins and DNA. Moreover, measuring FIP is expensive and requires careful handling and rapid processing of samples. Thus, the use of FIP may not be practical in very large epidemiological studies and is probably not suitable for the analyses of archived samples that may have been affected by *in vitro* oxidation [148, 229].

A possible alternative to FIP as biomarkers of oxidative stress is plasma fluorescent oxidation products (FOP). FOP measure oxidation products from several sources, including lipids, proteins, and DNA, and thus may serve as a more global indicator of oxidative stress [148, 230]. Previously, FOP have been used in the food industry, in animals, and *in vitro* studies to detect oxidation [170, 231, 232]. At present, however, FOP are gaining increased recognition as potential biomarkers of oxidative stress that can be used in clinical and epidemiologic studies. An additional advantage of FOP is that they are relatively easy to measure, stable, and can be assessed in samples with variable handling and storage protocols [171]. Recently, a nested case-control study [230] and a small prospective study [233] reported that plasma FOP significantly and independently predicted risk of coronary heart disease. Yet, there have been no reported studies that measured plasma FOP in relation to cancer risk. Moreover, no previous

studies assessed the relation of FOP to measures of pro- and antioxidant exposures and the correlation between FOP and FIP.

It is important to emphasize that oxidative stress is closely related to inflammation and these two processes can probably be assessed together. Chronic inflammation is associated with elevated oxidative stress levels, and conversely, oxidative stress has pro-inflammatory effects through activation of nuclear factor-kappa B (NF-kB), a transcription factor that increases expression of cytokines, chemokines, and cell adhesion molecules [234-236]. Thus, inflammation may be seen as both the cause and the consequence of oxidative stress. For all of the above reasons an examination of the relation between oxidative stress-modifying exposures and biomarkers of oxidative stress can also take into consideration the level of inflammation, which is most commonly measured by serum C-reactive protein (CRP). While the link between oxidative stress and inflammation is undisputable [11, 67], the association between markers of oxidative stress and CRP has not been fully evaluated [237].

In this study, we extend our previous analysis of OBS and adenoma [26] by examining whether OBS is associated with FIP and FOP and by assessing the relation of OBS to inflammation as measured by serum CRP. We further explore the relation of these three markers (FIP, FOP and CRP) to the risk of incident sporadic colorectal adenoma.

MATERIALS AND METHODS

Study Population

We used pooled data from two previously conducted colonoscopy-based case-control studies of incident, sporadic colorectal adenoma in 2 different US states by the same principal investigator (RMB). The first study, Markers of Adenomatous Polyps I (MAP I), was conducted in community gastroenterology practices in Winston-Salem and Charlotte, North Carolina. The second study, Markers of Adenomatous Polyps II (MAP II), was identical in design to MAP I and was conducted at Consultants in Gastroenterology, PA, a large, private practice in Columbia, South Carolina. Participants for these two case-control studies included patients who were 30-74 years of age with no prior history of colorectal neoplasms who were scheduled to undergo outpatient, elective colonoscopy at one of the study sites. Assessment of initial participant eligibility was identical in both studies. Cases (n=235) were first incident cases of colon or rectal adenomatous polyps at the time of elective outpatient colonoscopy and controls (n=391) were free of all polyps at colonoscopy. The detailed study methods for MAP I [164, 165] and MAP II [166, 167] have been previously published.

Data Collection

Questionnaire-based Data

In both the MAP I and the MAP II studies, a modified 153-item Willett Food Frequency Questionnaire was administered to obtain information on dietary intakes and use of nutritional supplements [168, 169]. Additional data included demographics and use of medications, such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs). Total intakes for micronutrients (iron, vitamin C, β-carotene, and α-

tocopherol) were calculated based on the sum of total daily dietary intake and total supplementary dose.

Blood Samples

For both studies, blood was collected, handled, and stored in a manner to allow measurements of pro-/anti-oxidants, FIP, FOP, and CRP. The samples were drawn into red-coated, pre-chilled Vacutainer tubes, plunged into ice and shielded from light and immediately delivered to the laboratory where the blood was centrifuged in a refrigerated centrifuge. Plasma and serum were separated; aliquotted into O-ring-capped amber-colored cryopreservation vials; the air in the vials was displaced with inert gas (nitrogen in MAP I and argon in MAP II); and then immediately frozen at -70^{o} C until analysis. Plasma lycopene, α-carotene, β-carotene, lutein, β-cryptoxanthin, and α-tocopherol levels from both studies were measured using high-performance liquid chromatography (HPLC) [150, 151]. The plasma free FIP were measured by a gas chromatography-mass spectrometry (GCMS) method [152] by the Molecular Epidemiology and Biomarker Research Laboratory (MEBRL) at the University of Minnesota (Minneapolis, MN). This method, considered the gold standard for the measurement of FIP, measures a well-defined set of F_2-isoprostane isomers. The FIP were extracted from the participant's sample using deuterium (4)-labeled 8-iso-prostaglandin F_2 alpha as an internal standard. Unlabeled, purified F_2-isoprostane was used as a calibration standard.

The modified method from Shimasaki [170] was used to measure FOP. The procedures have been described in detail previously [171]. Briefly, 0.2 mL of plasma was extracted with ethanol/ether (3:1 v/v) and vigorously mixed on a vortex mixer. The mixed solution was centrifuged for 10 minutes at 3,000 rpm, and 1 mL of supernatant

was added to cuvettes for spectrofluormetric readings. The fluorescent was determined as relative fluorescence intensity units per milliliter of plasma at 360/430 nm wavelength (excitation/emission) by a spectrofluorometer. Quinine sulfate diluted in 0.1 N H_2SO_4 was used for calibration. Due to limited amount of plasma samples available, about 22% of the population's FOP were measured using serum samples instead of plasma; however analyses for subset of patients with both types of samples available indicated that the two sets of values were closely correlated ($r = 0.9$; $p < 0.001$).

Serum CRP was measured by latex-enhanced immunonephelometry, a high-sensitivity method, on the Behring nephelometer II (BN-II) analyzer (inter-assay CV 4%; Behring Diagnostics, San Jose, CA).

Oxidative Balance Score

The oxidative balance score (OBS) was calculated by combining information from *a priori* selected pro- and anti-oxidant factors, which are summarized in Table 4.1. The blood levels of pro-oxidant (iron) and antioxidant (lycopene, α-carotene, β-carotene, lutein, β-cryptoxanthin, and α-tocopherol) nutrients were divided into low, medium, and high categories based on study-specific tertile values among controls. The tertile cutoffs for FFQ-derived variables (polyunsaturated fat, vitamin C, and alcohol) were both study- and sex-specific. The participants with low (1st tertile) pro-oxidant exposures were awarded 2 points, those with medium (2nd tertile) exposures received 1 point, and those with high (3rd tertile) exposures received 0 points. For alcohol consumption, non-drinkers, moderate drinkers (below median), and heavy drinker (above median) received 2, 1 and 0 points respectively For antioxidants, low, medium, and high levels were assigned 0, 1, and 2 points, respectively. A similar scoring approach was used for

categorical variables (selenium supplements, smoking, and use of aspirin and NSAIDs). Smoking status was categorized as never (2 points), former (1 point), and current (0 points). For selenium supplements, aspirin, and NSAID use, 0 points were assigned to participants with no regular use, 1 point to those with unknown or missing data, and 2 points to those with regular use. The overall OBS was then calculated by adding up the points assigned to each participant.

Statistical Analyses

The overall OBS was treated as either a continuous or an ordinal variable with all categories representing an approximately equal interval, with the lowest interval used as reference. The use of equal intervals instead of quantiles (*e.g.,* tertiles or quartiles) allows comparing extremes of the distribution. Logistic regression analyses examined three types of associations. First we examined the relation between the OBS and incident sporadic colorectal adenoma, adjusting for age, race, sex, total energy intake, BMI, plasma cholesterol, hormone replacement therapy (among women), physical activity, fiber, study, and family history of colorectal cancer. Next, we examined the associations between OBS and the markers of oxidative stress (FIP and FOP) and inflammation (CRP), which were dichotomized based on study- and sex-specific median among controls, adjusting for the same potential confounding factors as in the first analysis. Finally, we examined the associations between dichotomized markers of oxidative stress and inflammation and incident sporadic colorectal adenoma. The models for the third analysis included the same covariates as in the analysis of association between OBS and adenoma. The correlation of FIP, FOP, and CRP was also assessed using Pearson correlation coefficients.

We also conducted several sensitivity analyses to evaluate 1) the change in results when quartiles were used instead of equal intervals for OBS; 2) the associations between adenoma and biomarkers using biomarker quartiles and 3) the association between OBS and each biomarker when both former and never smokers were assigned 2 points while current smokers were assigned 0 points to consider the possibility that biomarkers may only be affected by current smoking status.

The results of the logistic regression analyses were expressed as adjusted odds ratios (ORs) with corresponding 95% confidence intervals (CIs). All models were assessed for collinearity and goodness of fit. A two-sided p-value of less than 0.05 was considered to be statistically significant. Statistical analyses were performed with SAS version 9.2 (SAS Institute, Cary, NC) statistical software package.

RESULTS

A total of 150 (64%) cases and 215 (69%) controls in the pooled MAP studies had sufficient information to calculate the OBS. Selected characteristics of cases and controls by study are shown in Table 4.2. Cases and controls did not differ considerably with regard to most risk factors; however, in MAP I, there were more males and current smokers in the case group than in the control group, and controls were more likely to have a history of a first-degree relative with colorectal cancer. In the pooled analysis, mean plasma concentrations of α-carotene and β-cryptoxanthin were statistically significantly higher in controls than in adenoma cases.

The OBS ranged between 2 and 24 points. When the OBS was treated as a continuous variable, a statistically significant inverse association was observed between

incident colorectal adenoma and each additional score point with ORs (95% CIs) of 0.93 (0.88 – 0.99). The results for OBS categorized into three equal intervals, using the lowest interval as reference, are summarized in Table 4.3. The adjusted ORs (95% CIs) for the middle and the highest intervals were 0.81 (0.46 – 1.43) and 0.39 (0.17 – 0.89), respectively (p-trend = 0.04).

The associations of OBS with different markers of oxidative stress and inflammation are shown in Table 4.4. There was significant inverse association between higher OBS and elevated levels of FIP. After adjusting for confounding factors, the ORs (95% CIs) for the middle and the highest OBS intervals (again using first interval as reference) were 0.50 (0.25 – 1.01) and 0.25 (0.10 – 0.65), respectively. The corresponding results for FOP were in the opposite direction. Compared to the reference category, those in the middle and highest OBS intervals had adjusted ORs (95% CIs) of 2.01 (1.13 – 3.75) and 3.48 (1.51 – 8.02), respectively.

The results for CRP were similar to those observed for FIP. Both middle and highest intervals of OBS demonstrated statistically significant inverse associations with elevated levels of CRP (p-trend < 0.01). The middle and highest OBS interval groups were estimated about 40% and 80% reduction in the likelihood of having elevated CRP levels (Table 4.4).

We further examined the associations of markers of oxidative stress and inflammation with incident, sporadic colorectal adenoma (Table 4.5). All three markers (FIP, FOP, and CRP) were positively associated with the risk of colorectal adenoma. For both markers of oxidative stress (FIP and FOP), elevated levels of markers were statistically significantly associated with about 80% increase in the risk of colorectal

adenoma with adjusted ORs of 1.89 (95% CIs 1.08 – 3.30) for FIP and 1.82 (95% CI: 1.11 – 2.99) for FOP. There was no statistically significant association between levels of CRP and risk of colorectal adenoma (OR = 1.45; 95% CI: 0.88 – 2.40).

We also assessed correlations between markers of FIP, FOP, and CRP using Pearson correlation coefficients. None of the markers were correlated with each other with the Pearson correlation coefficients of 0.06, 0.09, and 0.08 between FOP and FIP, FOP and CRP, and FIP and CRP, respectively ($p > 0.05$ for all).

Results of all sensitivity analyses were generally similar to the main findings, although the use of biomarker quartiles resulted in less stable estimates with wide confidence intervals. All sensitivity analyses are presented in supplementary tables.

DISCUSSION

While oxidative stress is thought to play a prominent role in many human diseases, there is no definitive evidence linking pro- and antioxidants to specific human health outcomes [198]. This discrepancy between biological plausibility and lack of established epidemiological associations is likely explained by inadequate methods of assessing oxidative stress in humans.

Previously, we and others used an oxidative balance score (OBS) as a composite measure of combined pro- and anti-oxidant exposure status in relation to risk of several cancers including colorectal, prostate, and lung [21, 25, 27, 143, 144, 182]. The current study extends our previous analyses by assessing associations between OBS and markers of oxidative stress and inflammation and by assessing the relation of various biomarkers to each other and to colorectal adenoma risk. Our analyses demonstrated a rather strong

and statistically significant inverse association between OBS and plasma FIP, and a significant direct association between elevated FIP and colorectal adenoma risk. These associations were in the hypothesized direction and are in agreement with evidence from other studies.

FIP are prostaglandin-like compounds formed non-enzymatically as products of the free radical-mediated lipid peroxidation [226]. Among various markers that are currently available to measure oxidative stress, FIP are considered the "gold-standard" measure in humans [152, 228]. An extensive body of literature also supports the use of FIP in studies of human diseases [149, 226]. Elevated levels of FIP were found to be associated with a wide variety of conditions, including cardiovascular, pulmonary, neurological, and renal diseases [238-241]. Previously reported associations between FIP and individual anti- or pro-oxidant factors have been inconsistent with one another. Block *et al.* examined several physiologic and behavioral factors, including diet, for their individual contribution to oxidative damage, as measured by plasma FIP [242]. While they found significant inverse correlations between plasma FIP and plasma ascorbic acid and several carotenoids ($p < 0.05$ for all), there were no associations with α-tocopherol, alcohol, or smoking. By contrast, Morrow *et al.* found significantly higher circulating plasma FIP in smokers than in the nonsmokers [243].

Upritchard *et al*. conducted a randomized placebo-controlled trial to investigate the effect of a combination of vitamin E and carotenoids on markers of antioxidant status and lipid peroxidation, including plasma FIP [221] in healthy persons. They found that in the group consuming a supplement that provided 111 mg α-tocopherol and 1.24 mg carotenoids daily had a 15% reduction in plasma total $F_{2\alpha}$-isoprostanes concentrations

during the 11 weeks of intervention. In our study, we observed approximately 70% lower levels of plasma FIP in those with highest compared to the lowest OBS, suggesting that the composite measure of OBS is more strongly associated with FIP than are individual anti- or pro-oxidant factors.

Plasma FOP were relatively recently introduced into human population-based studies as biomarkers of oxidative stress, but no reported studies have used FOP in relation to neoplasia outcomes. Measurement of FOP was first developed by Dillard and Tappel [244] in 1971, and was later modified by Shimasaki in 1994 [170]. FOP are thought to measure oxidation products from several sources, including lipids, proteins, and DNA, and thus may be a more global indicator of oxidative balance than are currently available other markers of oxidative balance [230]. The main practical advantage of FOP is that they are relatively unaffected by specimen quality and storage conditions, which makes them particularly useful for epidemiologic field studies.

Two previous studies used plasma FOP as biomarkers of systemic oxidative balance and evaluated whether FOP can predict future risk for cardiovascular [245] or coronary heart diseases [230]. In these studies, high levels of FOP were statistically significantly associated with incidence of both cardiovascular and coronary heart diseases. In another recent study, Wu *et al.* [148] also found levels of FOP statistically significantly, positively associated with variables linked to systemic oxidative balance, including smoking, hypertension, and reduced renal function.

The findings for FOP in this study are unexpected. On the one hand, elevated FOP, like FIP, were associated with higher risk for colorectal adenoma. On the other hand, unlike FIP, higher OBS was significantly associated with higher FOP

concentrations. Moreover, although both FOP and FIP are presumed to reflect oxidative damage there was no correlation between the two markers. Taken together, these results suggest that different components of OBS may be affect 3 different, though related processes. Figure 4.1 depicts a hypothetical directed acyclic graph (DAG) showing possible inter-relation of OBS, FIP, FOP, CRP, and colorectal adenoma. Assume that 3 subsets of the components of OBS each primarily affect a different process: lipid peroxidation, inflammation, and some other process that is independent of lipid peroxidation, but is related to oxidative stress. The degree of lipid peroxidation is reflected by FIP, inflammation by CRP, and non-lipid peroxidation component(s) of oxidative stress by FOP. If we assume that different components of OBS exerts different effects on lipid peroxidation and on other oxidative stress mechanisms then it is plausible that the associations of OBS with FOP and FIP will be in the opposite directions. As shown in supplementary Table S.4.4 this explanation is plausible because several of OBS components exerted opposite effects on FOP and FIP concentrations.

In addition, we included an inflammation marker, CRP, in our oxidative stress-related biomarker analysis. While the oxidative stress pathway is closely related to inflammation, most studies of biomarkers of oxidative stress do not consider inflammatory markers. The role of inflammation as both cause and result of oxidative stress is supported by a considerable body of evidence. Oxidative stress may play a role in inflammation by up-regulating production of pro-inflammatory cytokines such as interleukin (IL)-6 and acute phase proteins such as C-reactive protein (CRP) through activation of redox-sensitive transcription factors such as nuclear factor κB (NF- κB) [246, 247]. In our study, we found statistically significant inverse associations between

OBS and CRP, and these associations were even stronger than the associations between OSB and FIP. Therefore, inclusion of an inflammation marker will strengthen the understanding of biomarkers of oxidative stress as a whole.

One of the main strengths of this study is the use of biomarker-based measurements for most OBS components. Food frequency questionnaires may not capture all sources of each nutrient, do not account for bioavailability, and are subject to recall bias, particularly in case-control studies in which exposure is assessed retrospectively [183]. For these reasons, the use of biochemical indicators is being used with increasing frequency. Unlike questionnaires, biomarkers are independent of recall and social desirability, and because blood levels reflect an individual's absorption and delivery to the circulation, they may be provide better estimates of the relevant tissue doses [248, 249]. On the other hand, biomarker-based measurements represent relatively recent exposures that may not reflect long term patterns.

In addition to the general problems that are applicable to most case-control studies, our study has several limitations that are specific to the current analyses. First, the OBS is limited to dietary/lifestyle exposures and does not include any endogenous measures of antioxidant cell function. Although oxidative balance is affected by modifiable factors, such as those included in the OBS, oxidative balance is also determined by enzymatic mechanisms [250]. Endogenous factors that influence DNA damage, cell growth, and cell death contribute to the development of carcinogenesis through modulating gene expression [12]. Another limitation of the analysis presented herein is that our scoring method assumed that individual pro- and anti-oxidant exposures have equal weights. An equal weighting approach might not represent the real relative

biological contributions of the individual oxidative stress-related exposures. It has been shown, for example, that ascorbate (vitamin C) has a lower redox potential than α-tocopherol and, thus, relative contributions of vitamin C and vitamin E may be different [251]. Therefore, future studies should consider different weighing methods when constructing the OBS. Finally, due to limited amount of plasma samples available, serum was used for FOP measurements instead of plasma in about 22% of total population in the study.

In summary, we found that 1) higher OBS was associated with lower risk of colorectal adenomatous polyps; 2) higher OBS was inversely related to FIP and CRP, but was positively associated with FOP; 3) both oxidative stress markers, FIP and FOP, were significantly associated with risk of colorectal adenoma with very similar magnitude, but were not related to each other; and 4) the associations of OBS with CRP and of CRP with adenoma were in the hypothesized directions, but weaker than the corresponding associations with FIP. The above findings suggest that OBS may serve as a composite measure of oxidative stress- and inflammation-related exposures. It is, however, unclear whether or how these exposures affect FOP, and what biochemical processes are reflected by plasma FOP concentrations. The lack of correlation between FOP and FIP also requires further study.

Table 4.1. Example of Oxidative Balance Score (OBS) Assignment Scheme

Oxidative Balance Score (OBS) Components	Assignment Scheme†
1. PUFA intake	0 = High (3rd tertile), 1 = Medium (2nd tertile), 2 = Low (1st tertile)
2. Serum ferritin	0 = High (3rd tertile), 1 = Medium (2nd tertile), 2 = Low (1st tertile)
3. Total* vitamin C intake	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
4. Plasma lycopene	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
5. Plasma α-carotene	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
6. Plasma β-carotene	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
7. Plasma lutein	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
8. Plasma β-cryptoxanthin	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
9. Plasma α-tocopherol	0 = Low (1st tertile), 1 = Medium (2nd tertile), 2 = High (3rd tertile)
10. Selenium supplements	0 = No supplement, 1 = Unknown (missing data), 2 = Supplement
11. Smoking history	0 = Current smoker, 1 = Former smoker, 2 = Never smoker
12. Regular aspirin use	0 = No regular use, 1 = Unknown, 2 = Regular Use
13. Regular NSAID use	0 = No regular use, 1 = Unknown, 2 = Regular Use
14. Alcohol consumption	0 = Non-drinker 1 = Below median 2 = Above median

Abbreviations: PUFA = polyunsaturated fatty acid; NSAID = non-steroidal anti-inflammatory drug

†Low, intermediate, and high categories correspond to study-specific tertile values among controls

*Total intake = dietary intake + supplemental intake

Table 4.2. Selected Baseline Characteristics of Participants in the MAP I & II Studies of Incident, Sporadic Colorectal Adenomas

Characteristic	MAP I		MAP II		Pooled Analysis	
	Cases (n=106) mean (SD)	Controls (n=106) mean (SD)	Cases (n=33) mean (SD)	Controls (n=92) mean (SD)	Cases (n=139) mean (SD)	Controls (n=201) mean (SD)
Age, years	57.4 (8.9)	56.1 (10.2)	55.4 (7.3)	55.5 (7.9)	56.9 (8.6)	55.9 (9.2)
Male (%)	54.7	32.1[b]	57.6	44.6	55.4	37.8[a]
Body mass index, kg/m^2	27.8 (6.1)	27.1 (5.7)	28.5 (5.2)	28.6 (6.7)	27.9 (5.9)	27.8 (6.2)
Physical activity, MET-hours/week	216.8 (143.4)	196.1 (127.3)	163.7 (116.9)	176.5 (125.2)	204.2 (139.1)	187.1 (126.4)
Family history of colorectal cancer (%)	17.0	33.0[b]	21.1	19.6	18.0	26.9
HRT user (women only) (%)	62.5	54.1	78.6	70.6	66.1	60.8
Regularly take an NSAID (%)	21.2	32.1	33.3	34.8	24.1	33.3
Regularly take aspirin (%)	34.9	35.8	45.5	41.3	37.4	38.3
Current smoker (%)	34.0	20.2[b]	24.2	13.0	31.7	16.9[b]
Alcohol, drinks per week	20.8 (25.6)	14.6 (20.2)	9.4 (9.1)	13.4 (16.3)	16.3 (21.4)	13.9 (17.8)
Dietary intake per day						
Total energy, kcal	2,061.3 (851.8)	2,172.6 (2493.7)	1,831.3 (765.3)	1,648.0 (647.8)	2,006.7 (835.1)	1,932.5 (1902.0)
Total PUFA, gm	14.0 (6.3)	14.4 (14.5)	15.5 (8.9)	14.1 (10.4)	14.3 (7.0)	14.2 (12.8)
Dietary fiber, gm	22.8 (9.4)	25.5 (26.6)	16.6 (6.7)	15.3 (6.7)	21.3 (9.2)	20.9 (20.7)
Total vitamin C, mg	286.6 (388.5)	302.1 (354.6)	237.5 (273.6)	298.9 (369.4)	275.0 (364.2)	300.7 (360.6)
Plasma levels						
Plasma lycopene, µg/dL	26.3 (14.3)	25.8 (13.3)	21.7 (11.4)	24.6 (10.8)	25.2 (13.8)	25.2 (12.2)
Plasma α-carotene, µg/dL	2.7 (2.9)	3.6 (4.8)	2.6 (2.6)	3.5 (3.1)	2.7 (2.8)	3.5 (4.1)[a]
Plasma β-carotene, µg/dL	15.3 (22.5)	16.4 (15.5)	12.6 (11.4)	16.3 (13.0)	14.6 (20.4)	16.4 (14.4)
Plasma lutein, µg/dL	16.8 (7.2)	18.1 (10.3)	17.7 (6.2)	15.7 (6.3)	17.0 (6.9)	17.0 (8.7)
Plasma β-cryptoxanthin, µg/dL	6.0 (4.7)	6.9 (5.8)	6.1 (4.1)	8.1 (7.2)	6.0 (4.5)	7.5 (6.5)[a]
Plasma α-tocopherol, mg/dL	1.2 (0.5)	1.1 (0.5)	1.1 (0.3)	1.2 (0.6)	1.1 (0.5)	1.2 (0.5)
Plasma ferritin, mg/dL	146.1 (135.2)	148.8 (185.9)	144.5 (108.3)	130.8 (127.5)	145.7 (129.0)	140.6 (161.7)
Plasma cholesterol, mg/dL	203.4 (35.8)	206.3 (39.5)	194.8 (32.4)	199.3 (39.5)	201.4 (35.1)	203.1 (39.6)
Biomarker levels						
FIP, pg/mL	94.0 (41.8)	88.8 (38.4)	76.0 (25.0)	78.0 (28.9)	90.1 (39.3)	84.4 (35.1)
FOP, avg. std. ref. adj.‡	0.06 (0.03)	0.05 (0.02)	0.03 (0.01)	0.04 (0.01)	0.05 (0.11)	0.05 (0.13)
CRP, µg/mL	6.1 (6.1)	7.5 (23.8)	3.7 (5.0)	4.6 (6.2)	5.5 (6.0)	6.2 (18.0)

Abbreviations: SD = standard deviation; PUFA = polyunsaturated fatty acid; NSAID = non-steroidal anti-inflammatory drug; FIP = F_2-isoprostanes; FOP = fluorescent oxidation products; CRP = C-reactive protein [a] $P < 0.05$ based on t-test for continuous variable and chi-square test for categorical variables [b] $P < 0.01$ based on t-test for continuous variable and chi-square test for categorical variables

‡unit for FOP measurement is "average standard reference adjusted", which samples were calculated against a 1 ppm fluorescent reference standard quinine in 0.1 N sulfuric acid

Table 4.3. Associations of the OBS with incident, sporadic colorectal adenoma

OBS (Range 2 – 24)	Cases (n)*	Controls (n)*	OR (95% CI)†	p-trend
Interval 1 (OBS 2 – 9)	44	43	1.0	0.04
Interval 2 (OBS 10 – 16)	81	114	0.81 (0.46 – 1.43)	
Interval 3 (OBS 17 – 24)	14	44	0.39 (0.17 – 0.89)	
OBS as continuous variable	139	201	0.93 (0.87 – 0.99)	

Abbreviations: OBS = oxidative balance score; OR = odds ratio; CI = confidence interval

† Adjusted for age, race, BMI, total energy intake, plasma cholesterol, family history of colorectal cancer, physical activity, sex, hormone replacement therapy (among women), study (MAP I or MAP II)

*Total number of subjects in the model is lower due to missing covariate data.

Table 4.4. Associations of OBS with Markers of Oxidative Stress (FIP and FOP) and Inflammation (CRP)

OBS	Biomarkers‡		OR (95% CI)	p-trend
	High	Low		
FIP				
Interval 1 (OBS 2 – 9)	51	20	1.0	< 0.01
Interval 2 (OBS 10 – 16)	91	68	0.50 (0.25 – 1.01)	
Interval 3 (OBS 17 – 24)	17	27	0.25 (0.10 – 0.65)	
Continuous	159	115	0.87 (0.81 – 0.94)	
FOP				
Interval 1 (OBS 2 – 9)	33	45	1.0	< 0.01
Interval 2 (OBS 10 – 16)	107	77	2.01 (1.13 – 3.75)	
Interval 3 (OBS 17 – 24)	36	19	3.48 (1.51 – 8.02)	
Continuous	176	141	1.10 (1.03 – 1.17)	
CRP				
Interval 1 (OBS 2 – 9)	56	31	1.0	< 0.01
Interval 2 (OBS 10 – 16)	108	87	0.57 (0.31 – 1.04)	
Interval 3 (OBS 17 – 24)	19	39	0.21 (0.09 – 0.49)	
Continuous	183	157	0.88 (0.82 – 0.94)	

Abbreviations: OBS = oxidative balance score; OR = odds ratio; CI = confidence interval; FIP = F_2-isoprostanes; FOP = fluorescent oxidation products; CRP = C-reactive protein

†Adjusted for age, race, BMI, total energy intake, plasma cholesterol, and family history of colorectal cancer, sex and hormone replacement therapy (among women), fiber, physical activity, and study (MAP I or MAP II).

*Total number of subjects in the model is lower due to missing covariate data.

‡ Each biomarker was dichotomized into "high" and "low" based on study- and sex-specific median values among controls.

Table 4.5. Associations of Markers of Oxidative Stress (FIP and FOP) and Inflammation (CRP) with incident, sporadic colorectal adenoma

Biomarker‡	Cases	Controls	OR (95% CI)	p-value
FIP				
Low	39	76	1.0	0.03
High	80	79	1.89 (1.08 – 3.30)	
Log (continuous)	119	155	1.38 (0.79 – 2.38)	
FOP				
Low	44	97	1.0	0.02
High	82	94	1.82 (1.11 – 2.99)	
Log (continuous)	126	191	1.32 (0.94 – 1.87)	
CRP				
Low	55	102	1.0	0.14
High	84	99	1.45 (0.88 – 2.40)	
Log (continuous)	139	201	1.14 (0.97 – 1.33)	

Abbreviations: OBS = oxidative balance score; OR = odds ratio; CI = confidence interval; FIP = F_2-isoprostanes;
FOP = fluorescent oxidation products; CRP = C reactive protein
† Adjusted for age, race, BMI, total energy intake, plasma cholesterol, and family history of colorectal cancer, sex and hormone replacement therapy (among women), fiber, study (MAP I or MAP II).
*Total number of subjects in the model is lower due to missing covariate data.
‡ Each biomarker was dichotomized into "high" and "low" based on sex-specific median values among controls.

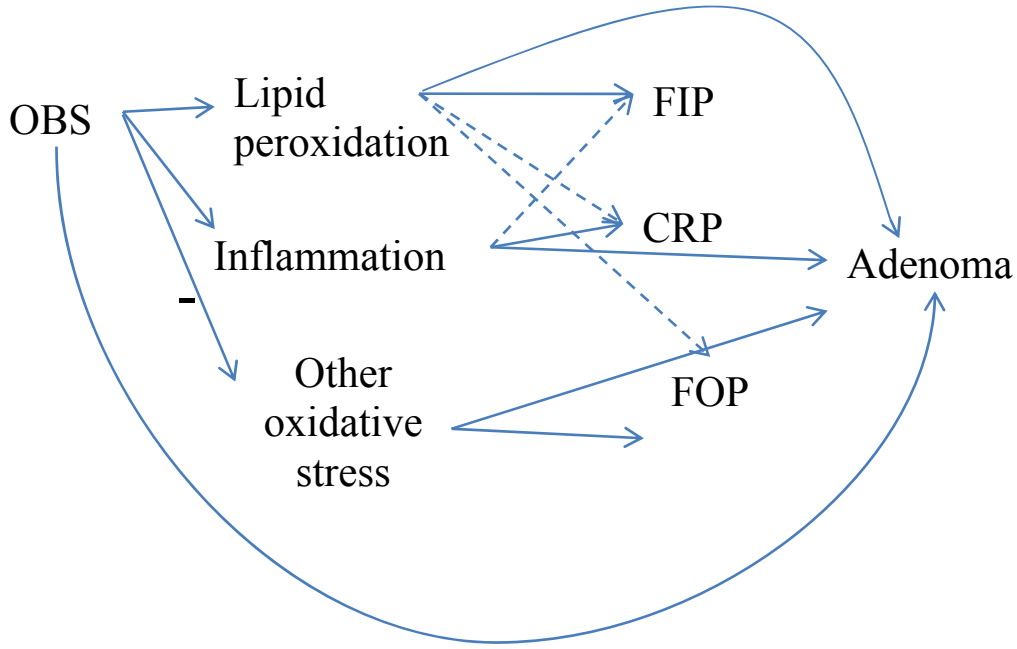

Figure 4.1. A hypothetical directed acyclic graph (DAG) showing possible inter-relation of OBS, biomarkers of oxidative stress (FIP and FOP) and inflammation (CRP), and colorectal adenoma. We first assume that OBS affects three main processes: lipid peroxidation (a component of oxidative stress), inflammation, and some other oxidative stress-related process that is independent of lipid peroxidation. Second, we also assume that each component affects the level of a biomarker: lipid peroxidation is mainly associated with FIP, inflammation with CRP, and non-lipid peroxidation components of oxidative stress with FOP (dotted arrows are weak effects). All effects are positive, except that indicated by a "-".
Abbreviations: FIP = F_2-isoprostanes, FOP = fluorescent oxidation products, CRP= C-reactive protein.

Supplementary Tables

Table S.4.1. Associations of the OBS (in quartiles) with incident, sporadic colorectal adenoma

OBS	Cases (n=139)*	Controls (n=201)*	OR (95% CI)†	p-trend
Quartile 1 (OBS 2 – 9)	44	43	1.0	0.04
Quartile 2 (OBS 10 – 13)	47	64	0.84 (0.46 – 1.56)	
Quartile 3 (OBS 14 – 16)	34	50	0.76 (0.38 – 1.51)	
Quartile 4 (OBS 17-24)	14	44	0.38 (0.17 – 0.88)	

Abbreviations: OBS = oxidative balance score; OR = odds ratio; CI = confidence interval

† Adjusted for age, race, BMI, total energy intake, plasma cholesterol, family history of colorectal cancer, physical activity, sex, hormone replacement therapy (among women), study (MAP I or MAP II)

*Total number of subjects in the model is lower due to missing covariate data.

Table S.4.2. Associations of the biomarkers (divided into quartiles) with incident, sporadic colorectal adenoma

Biomarker‡	Cases	Controls	OR (95% CI)	p-value
FIP				
Quartile 1	23	37	1.0	0.14
Quartile 2	16	40	0.55 (0.24 – 1.27)	
Quartile 3	44	40	1.50 (0.72 – 3.12)	
Quartile 4	36	38	1.34 (0.60 – 3.02)	
FOP				
Quartile 1	25	50	1.0	0.02
Quartile 2	19	47	0.83 (0.39 – 1.75)	
Quartile 3	30	48	1.39 (0.69 – 2.80)	
Quartile 4	52	46	1.96 (1.00 – 3.83)	
CRP				
Quartile 1	22	49	1.0	0.07
Quartile 2	33	53	1.71 (0.84 – 3.50)	
Quartile 3	37	45	1.94 (0.93 – 4.06)	
Quartile 4	47	54	2.06 (0.98 – 4.30)	

Abbreviations: OBS = oxidative balance score; OR = odds ratio; CI = confidence interval; FIP = F_2-isoprostanes; FOP = fluorescent oxidation products; CRP = C-reactive protein

† Adjusted for age, race, BMI, total energy intake, plasma cholesterol, and family history of colorectal cancer, sex and hormone replacement therapy (among women), fiber, study (MAP I or MAP II).

*Total number of subjects in the model is lower due to missing covariate data.

‡ Quartile based on study- and sex-specific median values among controls.

Table S.4.3. Associations of OBS with Markers of Oxidative Stress (FIP and FOP) and Inflammation (CRP) using "former smokers = 2" assignment

OBS	Biomarkers‡		OR (95% CI)	p-trend
	High	Low		
FIP				
Interval 1 (OBS 2 – 9)	51	20	1.0	<0.01
Interval 2 (OBS 10 – 16)	101	68	0.61 (0.30 – 1.25)	
Interval 3 (OBS 17 – 24)	11	27	0.26 (0.09 – 0.72)	
Continuous	159	115	0.87 (0.81 – 0.94)	
FOP				
Interval 1 (OBS 2 – 9)	33	45	1.0	<0.01
Interval 2 (OBS 10 – 16)	107	77	2.06 (1.13 – 3.75)	
Interval 3 (OBS 17 – 24)	36	19	3.48 (1.51 – 8.02)	
Continuous	176	141	1.10 (1.03 – 1.17)	
CRP				
Interval 1 (OBS 2 – 9)	56	31	1.0	< 0.01
Interval 2 (OBS 10 – 16)	108	87	0.57 (0.31 – 1.04)	
Interval 3 (OBS 17 – 24)	19	39	0.21 (0.09 – 0.49)	
Continuous	183	157	0.88 (0.82 – 0.94)	

Abbreviations: OBS = oxidative balance score; OR = odds ratio; CI = confidence interval; FIP = F_2-isoprostanes; FOP = fluorescent oxidation products; CRP = C-reactive protein

†Adjusted for age, race, BMI, total energy intake, plasma cholesterol, and family history of colorectal cancer, sex and hormone replacement therapy (among women), fiber, physical activity, and study (MAP I or MAP II).

*Total number of subjects in the model is lower due to missing covariate data.

‡ Each biomarker was dichotomized into "high" and "low" based on study- and sex-specific median values among controls

TABLE S.4.4. Association of individual OBS components with each biomarker

OBS Components	FIP		FOP		CRP	
	OR (95% CI)		OR (95% CI)		OR (95% CI)	
	Male	Female	Male	Female	Male	Female
1. PUFA intake	1.15 (0.63 – 2.09)	1.35 (0.73 – 2.48)	1.01 (0.60 – 1.72)	0.88 (0.52 – 1.47)	0.73 (0.44 – 1.22)	0.68 (0.39 – 1.19)
2. Serum ferritin	1.13 (0.65 – 1.96)	1.00 (0.63 – 1.60)	1.25 (0.77 – 2.04)	0.91 (0.60 – 1.38)	0.83 (0.50 – 1.37)	0.96 (0.62 – 1.49)
3. Total vitamin C intake	0.52 (0.28 – 0.95)	0.66 (0.40 – 1.10)	1.11 (0.66 – 1.87)	1.09 (0.71 – 1.69)	0.60 (0.36 – 1.01)	0.66 (0.41 – 1.07)
4. Plasma lycopene	0.93 (0.55 – 1.57)	1.08 (0.65 – 1.79)	2.33 (1.40 – 3.88)	3.48 (2.10 – 5.77)	1.17 (0.73 – 1.88)	1.39 (0.88 – 2.21)
5. Plasma α-carotene	0.90 (0.50 – 1.64)	0.82 (0.50 – 1.35)	2.06 (1.19 – 3.54)	1.77 (1.11 – 2.84)	0.74 (0.45 – 1.23)	0.49 (0.30 – 0.81)
6. Plasma β-carotene	0.81 (0.47 – 1.41)	0.61 (0.37 – 1.00)	2.32 (1.34 – 4.02)	1.42 (0.93 – 2.19)	0.67 (0.41 – 1.11)	0.54 (0.33 – 0.86)
7. Plasma lutein	0.98 (0.57 – 1.67)	0.69 (0.43 – 1.09)	1.42 (0.86 – 2.35)	1.34 (0.88 – 2.02)	0.92 (0.57 – 1.49)	0.73 (0.47 – 1.15)
8. Plasma β-cryptoxanthin	0.70 (0.40 – 1.24)	0.76 (0.48 – 1.20)	1.33 (0.81 – 2.17)	1.15 (0.77 – 1.72)	0.69 (0.42 – 1.13)	0.60 (0.39 – 0.93)
9. Plasma α- tocopherol	0.59 (0.31 – 1.09)	0.46 (0.26 – 0.82)	1.45 (0.86 – 2.43)	1.04 (0.64 – 1.70)	1.04 (0.62 – 1.76)	0.72 (0.43 – 1.21)
10. Selenium supplements	0.62 (0.23 – 1.63)	0.80 (0.35 – 1.82)	0.52 (0.23 – 1.17)	1.31 (0.64 – 2.65)	0.31 (0.09 – 1.06)	1.38 (0.64 – 2.95)
11. Smoking history	0.35 (0.18 – 0.69)	0.75 (0.46 – 1.22)	0.71 (0.41 – 1.21)	0.59 (0.39 – 0.90)	0.55 (0.32 – 0.95)	0.88 (0.57 – 1.36)
12. Regular aspirin use	0.82 (0.51 – 1.33)	0.97 (0.63 – 1.49)	1.01 (0.67 – 1.54)	1.09 (0.76 – 1.55)	0.76 (0.49 – 1.17)	0.79 (0.54 – 1.15)
13. Regular NSAID use	0.60 (0.33 – 1.07)	0.99 (0.67 – 1.48)	0.86 (0.54 – 1.36)	0.98 (0.71 – 1.40)	0.93 (0.57 – 1.50)	1.08 (0.76 – 1.54)
14. Alcohol consumption	0.77 (0.47 – 1.26)	0.75 (0.44 – 1.30)	0.68 (0.43 – 1.09)	0.95 (0.59 – 1.51)	0.90 (0.58 – 1.41)	1.07 (0.66 – 1.75)

Abbreviations: OBS=Oxidative Balance Score PUFA=Polyunsaturated fatty acid; NSAID=Non-steroidal anti-inflammatory drug; FIP= F_2-isoprostanes; FOP=fluorescent oxidation products; CRP=C-reactive protein

CHAPTER 5. DISCUSSION AND FUTURE DIRECTIONS

Overview of Findings

The goal of this dissertation was to investigate associations of oxidative balance score (OBS), which is a measure of combined pro- and anti-oxidant exposure status, with selected age-related diseases and mortality, and to further investigate the association between OBS and two markers of oxidative stress, F_2-isoprostanes (FIP) and fluorescent oxidation products (FOP), and one commonly used biomarker of inflammation, C-reactive protein (CRP).

In the first dissertation project, data from a large national prospective cohort study, Reasons for Geographic and Racial Differences in Stroke (REGARDS), were used to examine the relation of OBS to all-cause and cause-specific mortality while exploring alternative methods of weighting the OBS components. Our results suggested that higher OBS, which indicates predominance of anti-oxidant exposures, was significantly associated with reduced risk of all-cause, and in particular cancer-related mortality. Similar significant associations were observed regardless weighting methods used.

In the second dissertation project, associations between OBS and stroke incidence and mortality were examined using the same REGARDS cohort. Contrary to expectations we found no association between OBS and either incident stroke or stroke mortality. Similar non-significant associations were observed regardless of different weighting methods used and the results did not differ by race, sex, geographic region, or type of stroke (ischemic or hemorrhagic).

The third dissertation project used pooled data from two previously conducted colonoscopy-based case-control studies of incident, sporadic colorectal adenoma. We

extended our previous analysis by substituting questionnaire-derived OBS components with the corresponding plasma or serum measures of nutrients, by examining whether OBS is associated with markers of oxidative stress (FIP and FOP) and by assessing the relation of OBS to inflammation as measured by serum CRP. We further explored the relation of these three markers to the risk of incident colorectal adenoma. We confirmed the statistically significant inverse association between incident colorectal adenoma and OBS. There were also significant inverse associations between OBS and levels of FIP and CRP. However, the corresponding results for FOP were unexpectedly in the opposite direction. We further examined the associations of markers of oxidative stress and inflammation with incident, sporadic colorectal adenoma. All three markers were positively associated with the risk of colorectal adenoma.

Overall, the results of this dissertation supported the first hypothesis that higher OBS is associated with a reduction in mortality. However, our findings did not support the second hypothesis that higher OBS is associated with a reduction in stroke incidence or risk of stroke-related death. Furthermore, while we confirmed that higher OBS was associated with lower levels of FIP and CRP, the findings for FOP in this study cannot be readily explained. While elevated FOP, like FIP and CRP, was associated with higher risk for colorectal adenoma; higher OBS was associated with higher FOP concentrations and lower levels of FIP and CRP. Moreover, although both FOP and FIP are presumed to reflect oxidative damage there was no correlation between the two markers. Taken together, these results suggest that different components of OBS may affect different aspects of oxidative stress processes that still need to be understood.

Implications Future Research

Although the idea of combining individual pro- and anti-oxidants into a single score is not new, to our knowledge, the OBS used in this dissertation project is the most comprehensive version of the score constructed to date. Moreover, this dissertation study is one of the first to evaluate whether OBS is associated with all-cause and cause-specific mortality and stroke incidence in the US population.

Although oxidative balance is affected by modifiable factors, such as those included in the OBS, it is also determined by enzymatic mechanisms. Endogenous factors that influence cellular damage, cell function and ROS-induced damage repair to the development of multiple diseases. In addition, genetic factors play an important role in human lifespan. For example, previous genetic studies have shown that common polymorphisms in apolipoprotein E (APOE) influence human mortality, mainly through their association with diseases. Twin and family history studies support an important role of genetic factors in stroke risk. Based on these considerations, future studies should aim to construct OBS with both endogenous and exogenous factors.

In this dissertation project, we used different weighting schemes for combining pro- and anti-oxidant exposures into a single score. Previous studies combined different OBS components using equal weights, which raised a concern that the resulting score did not represent the true biological contributions of the individual pro- or anti-oxidant exposures. Dash *et al*, reported the first attempt of weighting the OBS components by using three novel methods that were based on literature review-, study data, and a Bayesian approach, which combined the first two methods. [217]. Using somewhat different data (only partially overlapping with ours) they also observed a substantial

inverse association between OBS and colorectal adenoma with little discernible difference across the weighting methods. In this dissertation, I further extended the weighting methodology by introducing oxidative stress biomarker-based weights.

It is important to point out that for the biomarker (FIP, FOP, and CRP)-based weighting methods, relatively small dataset was used to create the weights. Similarly, the literature review-based weights were largely similar across OBS components and close to 1.0 because summaries of published studies on the relation of pro- and anti-oxidant components to mortality and stroke incidence in most cases (except smoking) showed modest departures from the null. Thus future studies of OBS, should explore other methods. For example weights could be derived from other biomarkers of oxidative stress such as sulfur-containing amino acids and peptides, notably cysteine and cysteine-containing tripeptide glutathione, which undergo reversible oxidation-reduction (redox) changes under physiologic conditions. The redox states of glutathione/glutathione disulfide (GSH/GSSG) and cysteine/cystine (Cys/CySS) are oxidized in association with several known oxidative stress-related exposures, health conditions, and measures of physiologic function, including age, cigarette smoking, type 2 diabetes, and atherosclerosis [244, 252-254].

In the last dissertation study, I investigated whether OBS is associated with markers of oxidative stress and inflammation, including FOP. Currently, F_2-isoprostanes (FIP) are considered to be the best available biomarkers of oxidative stress *in vivo*. However, FIP measure only lipid peroxidation and may not reflect oxidative damage of proteins and DNA. Moreover, measuring FIP is expensive and requires careful handling and rapid processing of samples. Thus, the use of FIP may not be practical in very large

epidemiological studies and is probably not suitable for the analyses of archived samples that may have been affected by *in vitro* oxidation. Although FOP are stable and could be useful in field research our findings indicate that FOP cannot serve as substitute for FIP because the two markers are not correlated and because the associations of OBS with FIP and FOP appear to be in the opposite directions. Further studies are warranted to examine what biochemical processes are reflected by plasma FOP concentrations using prospective study design and perhaps several measures over time and under different conditions.

REFERENCES

1. Reuter, S., et al., *Oxidative stress, inflammation, and cancer: how are they linked?* Free Radic Biol Med, 2010. **49**(11): p. 1603-16.
2. Fang, J., T. Seki, and H. Maeda, *Therapeutic strategies by modulating oxygen stress in cancer and inflammation.* Adv Drug Deliv Rev, 2009. **61**(4): p. 290-302.
3. Kryston, T.B., et al., *Role of oxidative stress and DNA damage in human carcinogenesis.* Mutat Res, 2011. **711**(1-2): p. 193-201.
4. Ziech, D., et al., *The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development.* Chem Biol Interact, 2010. **188**(2): p. 334-9.
5. Pham-Huy, L.A., H. He, and C. Pahm-Huy, *Free Radicals, Antioxidants in Disease and Health.* International Journal of Biomedical Science, 2008. **4**(2): p. 89-96.
6. Valko, M., et al., *Role of oxygen radicals in DNA damage and cancer incidence.* Mol Cell Biochem, 2004. **266**(1-2): p. 37-56.
7. Halliwell, B., *Free radicals, antioxidants, and human disease: curiosity, cause, or consequence?* Lancet, 1994. **344**(8924): p. 721-4.
8. Jacob, R.A. and B.J. Burri, *Oxidative damage and defense.* Am J Clin Nutr, 1996. **63**(6): p. 985S-990S.
9. Yu, B.P., *Cellular defenses against damage from reactive oxygen species.* Physiol Rev, 1994. **74**(1): p. 139-62.
10. Valko, M., et al., *Free radicals and antioxidants in normal physiological functions and human disease.* Int J Biochem Cell Biol, 2007. **39**(1): p. 44-84.
11. Giustarini, D., et al., *Oxidative stress and human diseases: Origin, link, measurement, mechanisms, and biomarkers.* Crit Rev Clin Lab Sci, 2009. **46**(5-6): p. 241-81.
12. Klaunig, J.E. and L.M. Kamendulis, *The role of oxidative stress in carcinogenesis.* Annu Rev Pharmacol Toxicol, 2004. **44**: p. 239-67.
13. Kuhn, M.A., *Oxygen free radicals and antioxidants.* Am J Nurs, 2003. **103**(4): p. 58-62.

14. Ashok, B.T. and R. Ali, *The aging paradox: free radical theory of aging.* Exp Gerontol, 1999. **34**(3): p. 293-303.
15. Pfeifer, G.P., et al., *Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers.* Oncogene, 2002. **21**(48): p. 7435-51.
16. Kovacic, P. and J.D. Jacintho, *Mechanisms of carcinogenesis: focus on oxidative stress and electron transfer.* Curr Med Chem, 2001. **8**(7): p. 773-96.
17. Poulsen, H.E., H. Prieme, and S. Loft, *Role of oxidative DNA damage in cancer initiation and promotion.* Eur J Cancer Prev, 1998. **7**(1): p. 9-16.
18. Garland, M., et al., *Antioxidant micronutrients and breast cancer.* J Am Coll Nutr, 1993. **12**(4): p. 400-11.
19. Greenberg, E.R., et al., *A clinical trial of antioxidant vitamins to prevent colorectal adenoma. Polyp Prevention Study Group.* N Engl J Med, 1994. **331**(3): p. 141-7.
20. Kearney, J., et al., *Diet, alcohol, and smoking and the occurrence of hyperplastic polyps of the colon and rectum (United States).* Cancer Causes Control, 1995. **6**(1): p. 45-56.
21. Wright, M.E., et al., *Development of a comprehensive dietary antioxidant index and application to lung cancer risk in a cohort of male smokers.* Am J Epidemiol, 2004. **160**(1): p. 68-76.
22. Huijbregts, P., et al., *Dietary pattern and 20 year mortality in elderly men in Finland, Italy, and The Netherlands: longitudinal cohort study.* BMJ, 1997. **315**(7099): p. 13-7.
23. Slattery, M.L., et al., *Eating patterns and risk of colon cancer.* Am J Epidemiol, 1998. **148**(1): p. 4-16.
24. Trichopoulou, A., et al., *Diet and overall survival in elderly people.* BMJ, 1995. **311**(7018): p. 1457-60.
25. Goodman, M., et al., *Hypothesis: oxidative stress score as a combined measure of pro-oxidant and antioxidant exposures.* Ann Epidemiol, 2007. **17**(5): p. 394-9.

26. Goodman, M., et al., *A summary measure of pro- and anti-oxidant exposures and risk of incident, sporadic, colorectal adenomas.* Cancer Causes Control, 2008. **19**(10): p. 1051-64.
27. Goodman, M., et al., *Combined measure of pro- and anti-oxidant exposures in relation to prostate cancer and colorectal adenoma risk: an update.* Ann Epidemiol, 2010. **20**(12): p. 955-7.
28. Harman, D., *Aging: a theory based on free radical and radiation chemistry.* J Gerontol, 1956. **11**(3): p. 298-300.
29. Pelicano, H., D. Carney, and P. Huang, *ROS stress in cancer cells and therapeutic implications.* Drug Resist Updat, 2004. **7**(2): p. 97-110.
30. Halliwell, B., *Biochemistry of oxidative stress.* Biochem Soc Trans, 2007. **35**(Pt 5): p. 1147-50.
31. Valko, M., H. Morris, and M.T. Cronin, *Metals, toxicity and oxidative stress.* Curr Med Chem, 2005. **12**(10): p. 1161-208.
32. Valko, M., et al., *Free radicals, metals and antioxidants in oxidative stress-induced cancer.* Chem Biol Interact, 2006. **160**(1): p. 1-40.
33. Young, I.S. and J.V. Woodside, *Antioxidants in health and disease.* J Clin Pathol, 2001. **54**(3): p. 176-86.
34. Poli, G., et al., *Oxidative stress and cell signalling.* Curr Med Chem, 2004. **11**(9): p. 1163-82.
35. Kregel, K.C. and H.J. Zhang, *An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations.* Am J Physiol Regul Integr Comp Physiol, 2007. **292**(1): p. R18-36.
36. Cheeseman, K.H. and T.F. Slater, *An introduction to free radical biochemistry.* Br Med Bull, 1993. **49**(3): p. 481-93.
37. Riley, P.A., *Free radicals in biology: oxidative stress and the effects of ionizing radiation.* Int J Radiat Biol, 1994. **65**(1): p. 27-33.
38. de Zwart, L.L., et al., *Biomarkers of free radical damage applications in experimental animals and in humans.* Free Radic Biol Med, 1999. **26**(1-2): p. 202-26.

39. Lloyd, R.V., P.M. Hanna, and R.P. Mason, *The origin of the hydroxyl radical oxygen in the Fenton reaction.* Free Radic Biol Med, 1997. **22**(5): p. 885-8.
40. Stohs, S.J. and D. Bagchi, *Oxidative mechanisms in the toxicity of metal ions.* Free Radic Biol Med, 1995. **18**(2): p. 321-36.
41. Leonard, S.S., G.K. Harris, and X. Shi, *Metal-induced oxidative stress and signal transduction.* Free Radic Biol Med, 2004. **37**(12): p. 1921-42.
42. Puntarulo, S., *Iron, oxidative stress and human health.* Mol Aspects Med, 2005. **26**(4-5): p. 299-312.
43. Halliwell, B. and J.M. Gutteridge, *Oxygen toxicity, oxygen radicals, transition metals and disease.* Biochem J, 1984. **219**(1): p. 1-14.
44. Kiechl, S., et al., *Body iron stores and presence of carotid atherosclerosis. Results from the Bruneck Study.* Arterioscler Thromb, 1994. **14**(10): p. 1625-30.
45. Nelson, R.L., et al., *Body iron stores and risk of colonic neoplasia.* J Natl Cancer Inst, 1994. **86**(6): p. 455-60.
46. Selby, J.V. and G.D. Friedman, *Epidemiologic evidence of an association between body iron stores and risk of cancer.* Int J Cancer, 1988. **41**(5): p. 677-82.
47. Stevens, R.G., R.P. Beasley, and B.S. Blumberg, *Iron-binding proteins and risk of cancer in Taiwan.* J Natl Cancer Inst, 1986. **76**(4): p. 605-10.
48. Stevens, R.G., et al., *Body iron stores and the risk of cancer.* N Engl J Med, 1988. **319**(16): p. 1047-52.
49. Stevens, R.G., et al., *Iron-binding proteins, hepatitis B virus, and mortality in the Solomon Islands.* Am J Epidemiol, 1983. **118**(4): p. 550-61.
50. Babbs, C.F., *Free radicals and the etiology of colon cancer.* Free Radic Biol Med, 1990. **8**(2): p. 191-200.
51. Nelson, R.L., *Dietary iron and colorectal cancer risk.* Free Radic Biol Med, 1992. **12**(2): p. 161-8.
52. Jenkinson, A., et al., *Dietary intakes of polyunsaturated fatty acids and indices of oxidative stress in human volunteers.* Eur J Clin Nutr, 1999. **53**(7): p. 523-8.
53. Kremmyda, L.S., et al., *Fatty acids as biocompounds: their role in human metabolism, health and disease - a review. Part 2: Fatty Acid physiological roles*

and applications in human health and disease. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2011. **155**(3): p. 195-218.

54. Schmitz, G. and J. Ecker, *The opposing effects of n-3 and n-6 fatty acids.* Prog Lipid Res, 2008. **47**(2): p. 147-55.
55. Tyagi, A., et al., *Attenuation of colonic inflammation by partial replacement of dietary linoleic acid with alpha-linolenic acid in a rat model of inflammatory bowel disease.* Br J Nutr, 2012: p. 1-11.
56. Calder, P.C., *n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases.* Am J Clin Nutr, 2006. **83**(6 Suppl): p. 1505S-1519S.
57. Csiszar, A., et al., *Oxidative stress and accelerated vascular aging: implications for cigarette smoking.* Front Biosci, 2009. **14**: p. 3128-44.
58. Loft, S., et al., *Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index.* Carcinogenesis, 1992. **13**(12): p. 2241-7.
59. Barreiro, E., et al., *Cigarette smoke-induced oxidative stress: A role in chronic obstructive pulmonary disease skeletal muscle dysfunction.* Am J Respir Crit Care Med, 2010. **182**(4): p. 477-88.
60. Morrow, J.D., et al., *Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage.* N Engl J Med, 1995. **332**(18): p. 1198-203.
61. Loft, S. and H.E. Poulsen, *Cancer risk and oxidative DNA damage in man.* J Mol Med (Berl), 1996. **74**(6): p. 297-312.
62. Nordmann, R., *Alcohol and antioxidant systems.* Alcohol Alcohol, 1994. **29**(5): p. 513-22.
63. Nordmann, R., C. Ribiere, and H. Rouach, *Implication of free radical mechanisms in ethanol-induced cellular injury.* Free Radic Biol Med, 1992. **12**(3): p. 219-40.
64. Das, S.K. and D.M. Vasudevan, *Alcohol-induced oxidative stress.* Life Sci, 2007. **81**(3): p. 177-87.
65. Sergent, O., et al., *[Alcohol and oxidative stress].* Pathol Biol (Paris), 2001. **49**(9): p. 689-95.

66. Wu, D. and A.I. Cederbaum, *Alcohol, oxidative stress, and free radical damage.* Alcohol Res Health, 2003. **27**(4): p. 277-84.
67. Halliwell, B., *Phagocyte-derived reactive species: salvation or suicide?* Trends Biochem Sci, 2006. **31**(9): p. 509-15.
68. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.
69. Costa, A.D. and K.D. Garlid, *Intramitochondrial signaling: interactions among mitoKATP, PKCepsilon, ROS, and MPT.* Am J Physiol Heart Circ Physiol, 2008. **295**(2): p. H874-82.
70. Ryan, K.A., et al., *Reactive oxygen and nitrogen species differentially regulate Toll-like receptor 4-mediated activation of NF-kappa B and interleukin-8 expression.* Infect Immun, 2004. **72**(4): p. 2123-30.
71. Gill, R., A. Tsung, and T. Billiar, *Linking oxidative stress to inflammation: Toll-like receptors.* Free Radic Biol Med, 2010. **48**(9): p. 1121-32.
72. Bohm, F., M. Settergren, and J. Pernow, *Vitamin C blocks vascular dysfunction and release of interleukin-6 induced by endothelin-1 in humans in vivo.* Atherosclerosis, 2007. **190**(2): p. 408-15.
73. Nanri, A., M.A. Moore, and S. Kono, *Impact of C-reactive protein on disease risk and its relation to dietary factors.* Asian Pac J Cancer Prev, 2007. **8**(2): p. 167-77.
74. Wannamethee, S.G., et al., *Associations of vitamin C status, fruit and vegetable intakes, and markers of inflammation and hemostasis.* Am J Clin Nutr, 2006. **83**(3): p. 567-74; quiz 726-7.
75. Chaudiere, J. and R. Ferrari-Iliou, *Intracellular antioxidants: from chemical to biochemical mechanisms.* Food Chem Toxicol, 1999. **37**(9-10): p. 949-62.
76. Mortensen, A., L.H. Skibsted, and T.G. Truscott, *The interaction of dietary carotenoids with radical species.* Arch Biochem Biophys, 2001. **385**(1): p. 13-9.
77. El-Agamey, A., et al., *Carotenoid radical chemistry and antioxidant/pro-oxidant properties.* Arch Biochem Biophys, 2004. **430**(1): p. 37-48.

78. Keys, S.A. and W.F. Zimmerman, *Antioxidant activity of retinol, glutathione, and taurine in bovine photoreceptor cell membranes.* Exp Eye Res, 1999. **68**(6): p. 693-702.
79. Arrigoni, O. and M.C. De Tullio, *Ascorbic acid: much more than just an antioxidant.* Biochim Biophys Acta, 2002. **1569**(1-3): p. 1-9.
80. Carr, A. and B. Frei, *Does vitamin C act as a pro-oxidant under physiological conditions?* FASEB J, 1999. **13**(9): p. 1007-24.
81. Buettner, G.R., *The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate.* Arch Biochem Biophys, 1993. **300**(2): p. 535-43.
82. Li, Y. and H.E. Schellhorn, *New developments and novel therapeutic perspectives for vitamin C.* J Nutr, 2007. **137**(10): p. 2171-84.
83. Naidu, K.A., *Vitamin C in human health and disease is still a mystery? An overview.* Nutr J, 2003. **2**: p. 7.
84. Burton, G.W. and M.G. Traber, *Vitamin E: antioxidant activity, biokinetics, and bioavailability.* Annu Rev Nutr, 1990. **10**: p. 357-82.
85. Mustacich, D.J., R.S. Bruno, and M.G. Traber, *Vitamin E.* Vitam Horm, 2007. **76**: p. 1-21.
86. Kamal-Eldin, A. and L.A. Appelqvist, *The chemistry and antioxidant properties of tocopherols and tocotrienols.* Lipids, 1996. **31**(7): p. 671-701.
87. Mayne, S.T., *Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research.* J Nutr, 2003. **133 Suppl 3**: p. 933S-940S.
88. Mitchinson, M.J., *Macrophages, oxidised lipids and atherosclerosis.* Med Hypotheses, 1983. **12**(2): p. 171-8.
89. Ross, R., *The pathogenesis of atherosclerosis: a perspective for the 1990s.* Nature, 1993. **362**(6423): p. 801-9.
90. Steinberg, D., et al., *Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity.* N Engl J Med, 1989. **320**(14): p. 915-24.

91. Brigelius-Flohe, R., et al., *The European perspective on vitamin E: current knowledge and future research.* Am J Clin Nutr, 2002. **76**(4): p. 703-16.
92. Burk, R.F., *Molecular biology of selenium with implications for its metabolism.* FASEB J, 1991. **5**(9): p. 2274-9.
93. Stadtman, T.C., *Selenium biochemistry.* Annu Rev Biochem, 1990. **59**: p. 111-27.
94. Sunde, R.A., *Molecular biology of selenoproteins.* Annu Rev Nutr, 1990. **10**: p. 451-74.
95. Behne, D., et al., *Two new selenoproteins found in the prostatic glandular epithelium and in the spermatid nuclei.* Biomed Environ Sci, 1997. **10**(2-3): p. 340-5.
96. Boosalis, M.G., *The role of selenium in chronic disease.* Nutr Clin Pract, 2008. **23**(2): p. 152-60.
97. Reddi, A.S. and J.S. Bollineni, *Selenium-deficient diet induces renal oxidative stress and injury via TGF-beta1 in normal and diabetic rats.* Kidney Int, 2001. **59**(4): p. 1342-53.
98. Davies, K.J., *Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems.* IUBMB Life, 2000. **50**(4-5): p. 279-89.
99. Altieri, F., et al., *DNA damage and repair: from molecular mechanisms to health implications.* Antioxid Redox Signal, 2008. **10**(5): p. 891-937.
100. Stark, G., *Functional consequences of oxidative membrane damage.* J Membr Biol, 2005. **205**(1): p. 1-16.
101. Berlett, B.S. and E.R. Stadtman, *Protein oxidation in aging, disease, and oxidative stress.* J Biol Chem, 1997. **272**(33): p. 20313-6.
102. Grune, T., T. Reinheckel, and K.J. Davies, *Degradation of oxidized proteins in mammalian cells.* FASEB J, 1997. **11**(7): p. 526-34.
103. Pacifici, R.E. and K.J. Davies, *Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of aging revisited.* Gerontology, 1991. **37**(1-3): p. 166-80.

104. Ullrich, O., et al., *Poly-ADP ribose polymerase activates nuclear proteasome to degrade oxidatively damaged histones.* Proc Natl Acad Sci U S A, 1999. **96**(11): p. 6223-8.

105. Beckman, J.S., et al., *Oxidative chemistry of peroxynitrite.* Methods Enzymol, 1994. **233**: p. 229-40.

106. Klaunig, J.E., L.M. Kamendulis, and B.A. Hocevar, *Oxidative stress and oxidative damage in carcinogenesis.* Toxicol Pathol, 2010. **38**(1): p. 96-109.

107. Dreher, D. and A.F. Junod, *Role of oxygen free radicals in cancer development.* Eur J Cancer, 1996. **32A**(1): p. 30-8.

108. Halliwell, B. and O.I. Aruoma, *DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems.* FEBS Lett, 1991. **281**(1-2): p. 9-19.

109. Wang, D., D.A. Kreutzer, and J.M. Essigmann, *Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions.* Mutat Res, 1998. **400**(1-2): p. 99-115.

110. Demple, B. and L. Harrison, *Repair of oxidative damage to DNA: enzymology and biology.* Annu Rev Biochem, 1994. **63**: p. 915-48.

111. Dizdaroglu, M., *Oxidative damage to DNA in mammalian chromatin.* Mutat Res, 1992. **275**(3-6): p. 331-42.

112. Lindahl, T., *Instability and decay of the primary structure of DNA.* Nature, 1993. **362**(6422): p. 709-15.

113. Cheng, K.C., et al., *8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions.* J Biol Chem, 1992. **267**(1): p. 166-72.

114. Nyaga, S.G., et al., *Accumulation of oxidatively induced DNA damage in human breast cancer cell lines following treatment with hydrogen peroxide.* Cell Cycle, 2007. **6**(12): p. 1472-8.

115. Kondo, S., et al., *Persistent oxidative stress in human colorectal carcinoma, but not in adenoma.* Free Radic Biol Med, 1999. **27**(3-4): p. 401-10.

116. Lee, B.M., J.J. Jang, and H.S. Kim, *Benzo[a]pyrene diol-epoxide-I-DNA and oxidative DNA adducts associated with gastric adenocarcinoma.* Cancer Lett, 1998. **125**(1-2): p. 61-8.

117. Diakowska, D., et al., *Oxidative DNA damage and total antioxidant status in serum of patients with esophageal squamous cell carcinoma.* Hepatogastroenterology, 2007. **54**(78): p. 1701-4.

118. Miyake, H., et al., *Oxidative DNA damage in patients with prostate cancer and its response to treatment.* J Urol, 2004. **171**(4): p. 1533-6.

119. Tanaka, H., et al., *Hepatic oxidative DNA damage is associated with increased risk for hepatocellular carcinoma in chronic hepatitis C.* Br J Cancer, 2008. **98**(3): p. 580-6.

120. Djuric, Z., et al., *Levels of 5-hydroxymethyl-2'-deoxyuridine in DNA from blood of women scheduled for breast biopsy.* Cancer Epidemiol Biomarkers Prev, 2001. **10**(2): p. 147-9.

121. Yamamoto, T., et al., *Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in women with or without gynecologic cancer.* J Obstet Gynaecol Res, 1996. **22**(4): p. 359-63.

122. Vulimiri, S.V., et al., *Analysis of aromatic DNA adducts and 7,8-dihydro-8-oxo-2'-deoxyguanosine in lymphocyte DNA from a case-control study of lung cancer involving minority populations.* Mol Carcinog, 2000. **27**(1): p. 34-46.

123. Stocker, R. and J.F. Keaney, Jr., *Role of oxidative modifications in atherosclerosis.* Physiol Rev, 2004. **84**(4): p. 1381-478.

124. Allen, C.L. and U. Bayraktutan, *Oxidative stress and its role in the pathogenesis of ischaemic stroke.* Int J Stroke, 2009. **4**(6): p. 461-70.

125. Saeed, S.A., et al., *Some new prospects in the understanding of the molecular basis of the pathogenesis of stroke.* Exp Brain Res, 2007. **182**(1): p. 1-10.

126. Figueroa, S., et al., *Mitochondrial involvement in nitric oxide-induced cellular death in cortical neurons in culture.* J Neurosci Res, 2006. **83**(3): p. 441-9.

127. Herrmann, W., *The importance of hyperhomocysteinemia as a risk factor for diseases: an overview.* Clin Chem Lab Med, 2001. **39**(8): p. 666-74.

128. Hewett, S.J., et al., *Cyclooxygenase-2 contributes to N-methyl-D-aspartate-mediated neuronal cell death in primary cortical cell culture.* J Pharmacol Exp Ther, 2000. **293**(2): p. 417-25.
129. Wei, E.P., H.A. Kontos, and J.S. Beckman, *Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite.* Am J Physiol, 1996. **271**(3 Pt 2): p. H1262-6.
130. Gilca, M., et al., *The oxidative hypothesis of senescence.* J Postgrad Med, 2007. **53**(3): p. 207-13.
131. Khansari, N., Y. Shakiba, and M. Mahmoudi, *Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer.* Recent Pat Inflamm Allergy Drug Discov, 2009. **3**(1): p. 73-80.
132. Harman, D., *Free radical theory of aging.* Mutat Res, 1992. **275**(3-6): p. 257-66.
133. Shigenaga, M.K., T.M. Hagen, and B.N. Ames, *Oxidative damage and mitochondrial decay in aging.* Proc Natl Acad Sci U S A, 1994. **91**(23): p. 10771-8.
134. *The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group.* N Engl J Med, 1994. **330**(15): p. 1029-35.
135. Omenn, G.S., et al., *Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease.* N Engl J Med, 1996. **334**(18): p. 1150-5.
136. Lippman, S.M., et al., *Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT).* JAMA, 2009. **301**(1): p. 39-51.
137. Stampfer, M.J., et al., *Vitamin E consumption and the risk of coronary disease in women.* N Engl J Med, 1993. **328**(20): p. 1444-9.
138. Cook, N.R., et al., *A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: results from the Women's Antioxidant Cardiovascular Study.* Arch Intern Med, 2007. **167**(15): p. 1610-8.

139. Musa-Veloso, K., et al., *Influence of observational study design on the interpretation of cancer risk reduction by carotenoids.* Nutr Rev, 2009. **67**(9): p. 527-45.

140. Ye, Z. and H. Song, *Antioxidant vitamins intake and the risk of coronary heart disease: meta-analysis of cohort studies.* Eur J Cardiovasc Prev Rehabil, 2008. **15**(1): p. 26-34.

141. Brigelius-Flohe, R. and M.G. Traber, *Vitamin E: function and metabolism.* FASEB J, 1999. **13**(10): p. 1145-55.

142. Venkateswaran, V., N.E. Fleshner, and L.H. Klotz, *Synergistic effect of vitamin E and selenium in human prostate cancer cell lines.* Prostate Cancer Prostatic Dis, 2004. **7**(1): p. 54-6.

143. Van Hoydonck, P.G., E.H. Temme, and E.G. Schouten, *A dietary oxidative balance score of vitamin C, beta-carotene and iron intakes and mortality risk in male smoking Belgians.* J Nutr, 2002. **132**(4): p. 756-61.

144. Agalliu, I., et al., *Oxidative balance score and risk of prostate cancer: results from a case-cohort study.* Cancer Epidemiol, 2011. **35**(4): p. 353-61.

145. Alberts, M.J., *The Stroke Belt Consortium.* J Stroke Cerebrovasc Dis, 1996. **6**(1): p. 54-8.

146. Aaron, K.J., et al., *Association of dietary sodium and potassium intakes with albuminuria in normal-weight, overweight, and obese participants in the Reasons for Geographic and Racial Differences in Stroke (REGARDS) Study.* Am J Clin Nutr, 2011. **94**(4): p. 1071-8.

147. Howard, V.J., et al., *The reasons for geographic and racial differences in stroke study: objectives and design.* Neuroepidemiology, 2005. **25**(3): p. 135-43.

148. Wu, T., et al., *Plasma fluorescent oxidation products as potential markers of oxidative stress for epidemiologic studies.* Am J Epidemiol, 2007. **166**(5): p. 552-60.

149. Montuschi, P., P.J. Barnes, and L.J. Roberts, 2nd, *Isoprostanes: markers and mediators of oxidative stress.* FASEB J, 2004. **18**(15): p. 1791-800.

150. De Leenheer, A.P., et al., *Simultaneous determination of retinol and alpha-tocopherol in human serum by high-performance liquid chromatography.* J Chromatogr, 1979. **162**(3): p. 408-13.
151. Bieri, J., E. Brown, and J. Smith, *Determination of individual carotenoids in human plasma by high performance chromatography.* J Liquid Chromatogr, 1985. **8**: p. 473-484.
152. Morrow, J.D. and L.J. Roberts, 2nd, *Mass spectrometric quantification of F2-isoprostanes in biological fluids and tissues as measure of oxidant stress.* Methods Enzymol, 1999. **300**: p. 3-12.
153. Stanner, S.A., et al., *A review of the epidemiological evidence for the 'antioxidant hypothesis'.* Public Health Nutr, 2004. **7**(3): p. 407-22.
154. Clark, L.C., et al., *Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group.* JAMA, 1996. **276**(24): p. 1957-63.
155. Hennekens, C.H., et al., *Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease.* N Engl J Med, 1996. **334**(18): p. 1145-9.
156. Lee, I.M., et al., *Randomised beta-carotene supplementation and incidence of cancer and cardiovascular disease in women: is the association modified by baseline plasma level?* Br J Cancer, 2002. **86**(5): p. 698-701.
157. Eastwood, M.A., *Interaction of dietary antioxidants in vivo: how fruit and vegetables prevent disease?* QJM, 1999. **92**(9): p. 527-30.
158. Buckland, G., et al., *Adherence to the Mediterranean diet reduces mortality in the Spanish cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC-Spain).* Br J Nutr, 2011: p. 1-11.
159. Knoops, K.T., et al., *Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: the HALE project.* JAMA, 2004. **292**(12): p. 1433-9.
160. Lagiou, P., et al., *Mediterranean dietary pattern and mortality among young women: a cohort study in Sweden.* Br J Nutr, 2006. **96**(2): p. 384-92.

161. Tognon, G., et al., *Does the Mediterranean diet predict longevity in the elderly? A Swedish perspective.* Age (Dordr), 2010.
162. Trichopoulou, A., et al., *Adherence to a Mediterranean diet and survival in a Greek population.* N Engl J Med, 2003. **348**(26): p. 2599-608.
163. Slattery, M.L., et al., *Oxidative balance and colon and rectal cancer: Interaction of lifestyle factors and genes.* Mutat Res, 2012. **734**(1-2): p. 30-40.
164. Boyapati, S.M., et al., *Calcium, vitamin D, and risk for colorectal adenoma: dependency on vitamin D receptor BsmI polymorphism and nonsteroidal anti-inflammatory drug use?* Cancer Epidemiol Biomarkers Prev, 2003. **12**(7): p. 631-7.
165. Gong, Y.L., et al., *Vitamin D receptor gene Tru9I polymorphism and risk for incidental sporadic colorectal adenomas.* World J Gastroenterol, 2005. **11**(31): p. 4794-9.
166. Daniel, C.R., et al., *TGF-alpha expression as a potential biomarker of risk within the normal-appearing colorectal mucosa of patients with and without incident sporadic adenoma.* Cancer Epidemiol Biomarkers Prev, 2009. **18**(1): p. 65-73.
167. Sidelnikov, E., et al., *MutL-homolog 1 expression and risk of incident, sporadic colorectal adenoma: search for prospective biomarkers of risk for colorectal cancer.* Cancer Epidemiol Biomarkers Prev, 2009. **18**(5): p. 1599-609.
168. Willett, W.C., et al., *The use of a self-administered questionnaire to assess diet four years in the past.* Am J Epidemiol, 1988. **127**(1): p. 188-99.
169. Willett, W.C., et al., *Reproducibility and validity of a semiquantitative food frequency questionnaire.* Am J Epidemiol, 1985. **122**(1): p. 51-65.
170. Shimasaki, H., *Assay of fluorescent lipid peroxidation products.* Methods Enzymol, 1994. **233**: p. 338-46.
171. Wu, T., et al., *Stability of measurements of biomarkers of oxidative stress in blood over 36 hours.* Cancer Epidemiol Biomarkers Prev, 2004. **13**(8): p. 1399-402.
172. Dagenais, G.R., et al., *Beta-carotene, vitamin C, and vitamin E and cardiovascular diseases.* Curr Cardiol Rep, 2000. **2**(4): p. 293-9.

173. Galli, C. and F. Marangoni, *Recent advances in the biology of n-6 fatty acids.* Nutrition, 1997. **13**(11-12): p. 978-85.
174. Heber, D. and Q.Y. Lu, *Overview of mechanisms of action of lycopene.* Exp Biol Med (Maywood), 2002. **227**(10): p. 920-3.
175. Hercberg, S., et al., *The potential role of antioxidant vitamins in preventing cardiovascular diseases and cancers.* Nutrition, 1998. **14**(6): p. 513-20.
176. Lee, I.M., *Antioxidant vitamins in the prevention of cancer.* Proc Assoc Am Physicians, 1999. **111**(1): p. 10-5.
177. Li, G.X., et al., *delta-tocopherol is more active than alpha - or gamma - tocopherol in inhibiting lung tumorigenesis in vivo.* Cancer Prev Res (Phila), 2011. **4**(3): p. 404-13.
178. Palace, V.P., et al., *Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease.* Free Radic Biol Med, 1999. **26**(5-6): p. 746-61.
179. Pandey, D.K., et al., *Dietary vitamin C and beta-carotene and risk of death in middle-aged men. The Western Electric Study.* Am J Epidemiol, 1995. **142**(12): p. 1269-78.
180. Swain, J.H., et al., *Iron indexes and total antioxidant status in response to soy protein intake in perimenopausal women.* Am J Clin Nutr, 2002. **76**(1): p. 165-71.
181. van der Vaart, H., et al., *Acute effects of cigarette smoke on inflammation and oxidative stress: a review.* Thorax, 2004. **59**(8): p. 713-21.
182. Velthuis-te Wierik, E.J., et al., *Consumption of reduced-fat products: effects on parameters of anti-oxidative capacity.* Eur J Clin Nutr, 1996. **50**(4): p. 214-9.
183. Schatzkin, A. and V. Kipnis, *Could exposure assessment problems give us wrong answers to nutrition and cancer questions?* J Natl Cancer Inst, 2004. **96**(21): p. 1564-5.
184. Boucher, B., et al., *Validity and reliability of the Block98 food-frequency questionnaire in a sample of Canadian women.* Public Health Nutr, 2006. **9**(1): p. 84-93.

185. Caan, B.J., et al., *Comparison of the Block and the Willett self-administered semiquantitative food frequency questionnaires with an interviewer-administered dietary history.* Am J Epidemiol, 1998. **148**(12): p. 1137-47.
186. Subar, A.F., et al., *Comparative validation of the Block, Willett, and National Cancer Institute food frequency questionnaires : the Eating at America's Table Study.* Am J Epidemiol, 2001. **154**(12): p. 1089-99.
187. Christensen, K., T.E. Johnson, and J.W. Vaupel, *The quest for genetic determinants of human longevity: challenges and insights.* Nat Rev Genet, 2006. **7**(6): p. 436-48.
188. Corder, E.H., et al., *Apolipoprotein E genotype determines survival in the oldest old (85 years or older) who have good cognition.* Arch Neurol, 1996. **53**(5): p. 418-22.
189. Go, A.S., et al., *Executive summary: heart disease and stroke statistics--2013 update: a report from the American Heart Association.* Circulation, 2013. **127**(1): p. 143-52.
190. He, F.J., C.A. Nowson, and G.A. MacGregor, *Fruit and vegetable consumption and stroke: meta-analysis of cohort studies.* Lancet, 2006. **367**(9507): p. 320-6.
191. Barnham, K.J., C.L. Masters, and A.I. Bush, *Neurodegenerative diseases and oxidative stress.* Nat Rev Drug Discov, 2004. **3**(3): p. 205-14.
192. Nanetti, L., et al., *Oxidative stress in ischaemic stroke.* Eur J Clin Invest, 2011. **41**(12): p. 1318-22.
193. Allen, C.L. and U. Bayraktutan, *Risk factors for ischaemic stroke.* Int J Stroke, 2008. **3**(2): p. 105-16.
194. McCord, J.M., *Iron, free radicals, and oxidative injury.* J Nutr, 2004. **134**(11): p. 3171S-3172S.
195. Leppala, J.M., et al., *Controlled trial of alpha-tocopherol and beta-carotene supplements on stroke incidence and mortality in male smokers.* Arterioscler Thromb Vasc Biol, 2000. **20**(1): p. 230-5.
196. Gaziano, J.M., *Antioxidants in cardiovascular disease: randomized trials.* Nutrition, 1996. **12**(9): p. 583-8.

197. Hirvonen, T., et al., *Intake of flavonoids, carotenoids, vitamins C and E, and risk of stroke in male smokers.* Stroke, 2000. **31**(10): p. 2301-6.
198. Joshipura, K.J., et al., *Fruit and vegetable intake in relation to risk of ischemic stroke.* JAMA, 1999. **282**(13): p. 1233-9.
199. Keli, S.O., et al., *Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study.* Arch Intern Med, 1996. **156**(6): p. 637-42.
200. Kok, F.J., et al., *Serum selenium, vitamin antioxidants, and cardiovascular mortality: a 9-year follow-up study in the Netherlands.* Am J Clin Nutr, 1987. **45**(2): p. 462-8.
201. Larsson, S.C., J. Virtamo, and A. Wolk, *Total and specific fruit and vegetable consumption and risk of stroke: A prospective study.* Atherosclerosis, 2013. **227**(1): p. 147-52.
202. Estruch, R., et al., *Primary Prevention of Cardiovascular Disease with a Mediterranean Diet.* N Engl J Med, 2013.
203. Gardener, H., et al., *Mediterranean-style diet and risk of ischemic stroke, myocardial infarction, and vascular death: the Northern Manhattan Study.* Am J Clin Nutr, 2011. **94**(6): p. 1458-64.
204. Hoevenaar-Blom, M.P., et al., *Mediterranean style diet and 12-year incidence of cardiovascular diseases: the EPIC-NL cohort study.* PLoS One, 2012. **7**(9): p. e45458.
205. Kastorini, C.M., et al., *Adherence to the mediterranean diet in relation to ischemic stroke nonfatal events in nonhypercholesterolemic and hypercholesterolemic participants: results of a case/case-control study.* Angiology, 2012. **63**(7): p. 509-15.
206. Misirli, G., et al., *Relation of the traditional Mediterranean diet to cerebrovascular disease in a Mediterranean population.* Am J Epidemiol, 2012. **176**(12): p. 1185-92.
207. Adams, H.P., Jr., et al., *Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment.* Stroke, 1993. **24**(1): p. 35-41.

208. Kissela, B., et al., *Greater Cincinnati/Northern Kentucky Stroke Study: volume of first-ever ischemic stroke among blacks in a population-based study.* Stroke, 2001. **32**(6): p. 1285-90.
209. Moskowitz, M.A., E.H. Lo, and C. Iadecola, *The science of stroke: mechanisms in search of treatments.* Neuron, 2010. **67**(2): p. 181-98.
210. Mark, S.D., et al., *Do nutritional supplements lower the risk of stroke or hypertension?* Epidemiology, 1998. **9**(1): p. 9-15.
211. *MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high-risk individuals: a randomised placebo-controlled trial.* Lancet, 2002. **360**(9326): p. 23-33.
212. Johnsen, S.P., et al., *Intake of fruit and vegetables and the risk of ischemic stroke in a cohort of Danish men and women.* Am J Clin Nutr, 2003. **78**(1): p. 57-64.
213. Oude Griep, L.M., et al., *Raw and processed fruit and vegetable consumption and 10-year stroke incidence in a population-based cohort study in the Netherlands.* Eur J Clin Nutr, 2011. **65**(7): p. 791-9.
214. Yochum, L.A., A.R. Folsom, and L.H. Kushi, *Intake of antioxidant vitamins and risk of death from stroke in postmenopausal women.* Am J Clin Nutr, 2000. **72**(2): p. 476-83.
215. Feigin, V.L., et al., *Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review.* Lancet Neurol, 2009. **8**(4): p. 355-69.
216. O'Donnell, M. and S. Yusuf, *Tackling the global burden of stroke: the need for large-scale international studies.* Lancet Neurol, 2009. **8**(4): p. 306-7.
217. Dash, C., et al., *Using pathway-specific comprehensive exposure scores in epidemiology: Application to oxidative balance in a pooled case-control study of incident, sporadic colorectal adenomas.* American Journal of Epidemiology, 2013 (In Press).
218. Brass, L.M., et al., *A study of twins and stroke.* Stroke, 1992. **23**(2): p. 221-3.
219. Jerrard-Dunne, P., et al., *Evaluating the genetic component of ischemic stroke subtypes: a family history study.* Stroke, 2003. **34**(6): p. 1364-9.

220. Wannamethee, S.G., A.G. Shaper, and S. Ebrahim, *History of parental death from stroke or heart trouble and the risk of stroke in middle-aged men.* Stroke, 1996. **27**(9): p. 1492-8.

221. Sims, N.R. and H. Muyderman, *Mitochondria, oxidative metabolism and cell death in stroke.* Biochim Biophys Acta, 2010. **1802**(1): p. 80-91.

222. Lloyd-Jones, D.M., et al., *Defining and setting national goals for cardiovascular health promotion and disease reduction: the American Heart Association's strategic Impact Goal through 2020 and beyond.* Circulation, 2010. **121**(4): p. 586-613.

223. Sies, H., *Oxidative stress: from basic research to clinical application.* Am J Med, 1991. **91**(3C): p. 31S-38S.

224. Ames, B.N., M.K. Shigenaga, and T.M. Hagen, *Oxidants, antioxidants, and the degenerative diseases of aging.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 7915-22.

225. Halliwell, B., J.M. Gutteridge, and C.E. Cross, *Free radicals, antioxidants, and human disease: where are we now?* J Lab Clin Med, 1992. **119**(6): p. 598-620.

226. Milne, G.L., E.S. Musiek, and J.D. Morrow, *F2-isoprostanes as markers of oxidative stress in vivo: an overview.* Biomarkers, 2005. **10 Suppl 1**: p. S10-23.

227. Kadiiska, M.B., et al., *Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning?* Free Radic Biol Med, 2005. **38**(6): p. 698-710.

228. Liu, T., et al., *The isoprostanes: novel prostaglandin-like products of the free radical-catalyzed peroxidation of arachidonic acid.* J Biomed Sci, 1999. **6**(4): p. 226-35.

229. Kitano, S., et al., *Improved method of plasma 8-Isoprostane measurement and association analyses with habitual drinking and smoking.* World J Gastroenterol, 2006. **12**(36): p. 5846-52.

230. Wu, T., et al., *Plasma fluorescent oxidation products: independent predictors of coronary heart disease in men.* Am J Epidemiol, 2007. **166**(5): p. 544-51.

231. Mohanakumar, K.P., et al., *Ferrous-citrate complex and nigral degeneration: evidence for free-radical formation and lipid peroxidation.* Ann N Y Acad Sci, 1994. **738**: p. 392-9.
232. Sziraki, I., et al., *Implications for atypical antioxidative properties of manganese in iron-induced brain lipid peroxidation and copper-dependent low density lipoprotein conjugation.* Neurotoxicology, 1999. **20**(2-3): p. 455-66.
233. Mezzetti, A., et al., *Vitamin E and lipid peroxide plasma levels predict the risk of cardiovascular events in a group of healthy very old people.* J Am Geriatr Soc, 2001. **49**(5): p. 533-7.
234. Chen, X.L., et al., *Angiotensin II induces monocyte chemoattractant protein-1 gene expression in rat vascular smooth muscle cells.* Circ Res, 1998. **83**(9): p. 952-9.
235. Scholz, H., et al., *8-isoprostane increases expression of interleukin-8 in human macrophages through activation of mitogen-activated protein kinases.* Cardiovasc Res, 2003. **59**(4): p. 945-54.
236. Zhang, L., et al., *Diabetes-induced oxidative stress and low-grade inflammation in porcine coronary arteries.* Circulation, 2003. **108**(4): p. 472-8.
237. Abramson, J.L., et al., *Association between novel oxidative stress markers and C-reactive protein among adults without clinical coronary heart disease.* Atherosclerosis, 2005. **178**(1): p. 115-21.
238. Ozkul, Y., H.E. Eroglu, and E. Ok, *Genotoxic potential of Turkish propolis in peripheral blood lymphocytes.* Pharmazie, 2006. **61**(7): p. 638-40.
239. Altmay, E., et al., *Continuous positive airway pressure does not improve lung function after cardiac surgery.* Can J Anaesth, 2006. **53**(9): p. 919-25.
240. Verit, F.F., et al., *Long-term effects of tibolone on ocular functions in postmenopausal women.* Arch Gynecol Obstet, 2007. **275**(4): p. 255-61.
241. Ozdal, B., et al., *A rare cause of hydronephrosis: invasive mole.* Ann Saudi Med, 2006. **26**(4): p. 326-7.
242. Ozkul, A., et al., *Serological evidence of West Nile Virus (WNV) in mammalian species in Turkey.* Epidemiol Infect, 2006. **134**(4): p. 826-9.

243. Nanetti, L., et al., *Effect of consumption of dark chocolate on oxidative stress in lipoproteins and platelets in women and in men.* Appetite, 2012. **58**(1): p. 400-5.
244. Moriarty, S.E., et al., *Oxidation of glutathione and cysteine in human plasma associated with smoking.* Free Radic Biol Med, 2003. **35**(12): p. 1582-8.
245. Mezzetti, A., et al., *Systemic oxidative stress and its relationship with age and illness. Associazione Medica "Sabin".* J Am Geriatr Soc, 1996. **44**(7): p. 823-7.
246. Himmelfarb, J., *Linking oxidative stress and inflammation in kidney disease: which is the chicken and which is the egg?* Semin Dial, 2004. **17**(6): p. 449-54.
247. Rahman, I. and I.M. Adcock, *Oxidative stress and redox regulation of lung inflammation in COPD.* Eur Respir J, 2006. **28**(1): p. 219-42.
248. Kaaks, R., E. Riboli, and R. Sinha, *Biochemical markers of dietary intake.* IARC Sci Publ, 1997(142): p. 103-26.
249. Ingles, S.A., et al., *Plasma tocopherol and prevalence of colorectal adenomas in a multiethnic population.* Cancer Res, 1998. **58**(4): p. 661-6.
250. Bruce, W.R., A. Giacca, and A. Medline, *Possible mechanisms relating diet and risk of colon cancer.* Cancer Epidemiol Biomarkers Prev, 2000. **9**(12): p. 1271-9.
251. Thomas, S.R., et al., *Coantioxidants make alpha-tocopherol an efficient antioxidant for low-density lipoprotein.* Am J Clin Nutr, 1995. **62**(6 Suppl): p. 1357S-1364S.
252. Ashfaq, S., et al., *The relationship between plasma levels of oxidized and reduced thiols and early atherosclerosis in healthy adults.* J Am Coll Cardiol, 2006. **47**(5): p. 1005-11.
253. Jones, D.P., et al., *Redox analysis of human plasma allows separation of pro-oxidant events of aging from decline in antioxidant defenses.* Free Radic Biol Med, 2002. **33**(9): p. 1290-300.
254. Samiec, P.S., et al., *Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes.* Free Radic Biol Med, 1998. **24**(5): p. 699-704.