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Oxidative Stress and Health Outcomes

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# Oxidative Stress and Health Outcomes

By

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B.S., University of Maryland Baltimore County, 2009

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

An abstract of
A dissertation submitted to the Faculty of the
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Abstract

Oxidative Stress and Health Outcomes
By Sindhu Lakkur

Experimental biology evidence demonstrating that antioxidants can reverse the effects of oxidative stress-induced damage is not well supported by human studies. Previous research found that combining individual pro- and anti-oxidant factors into a comprehensive oxidative balance score (OBS) can be more strongly associated with various conditions than any individual factor. In three thematically related studies I investigated the associations of OBS with prostate cancer risk (Study 1), with biomarkers of oxidative stress (Study 2), and with indicators of inflammation and cardiovascular health (Study 3).

**Study 1** used the Cancer Prevention Study-II Nutrition Cohort (n=43,325). The data on OBS were obtained from baseline questionnaires and prostate cancer cases were ascertained via active follow up. Contrary to expectation, there was no inverse association between OBS and prostate cancer risk.

In **Study 2** I investigated the association between OBS and three biomarkers of oxidative stress: F2-isoprostanes (FIP), fluorescent oxidative products (FOP) and mitochondrial DNA (mtDNA) copy number, in the cross-sectional Study of Race, Stress, and Hypertension (n=321). Odds ratios (95% confidence intervals) comparing the highest to lowest OBS tertile for FIP and FOP were 0.10 (0.04 – 0.26; p-trend<0.05), and 3.01 (1.51 – 6.04; p-trend <0.05), respectively.

Using data from the Reasons for Geographic and Racial Differences in Stroke cohort (**Study 3**), I investigated the relation of OBS to indicators of inflammation (C-reactive protein, white blood cell count, albumin) and cardiovascular health (cholesterol, LDL, HDL, and triglycerides). The odds ratios (95% confidence intervals) comparing the highest to the lowest OBS equal interval categories were 0.50 (0.38-0.66) for CRP, 0.50 (0.36-0.71) for WBC, and 0.75 (0.58-0.98) for LDL; all three p-values for trend <0.001. Gender modified the association between OBS and low HDL with significant inverse association observed only among women.

Although OBS was associated with cancer outcomes in previous studies, our results indicate that OBS does not play a role in prostate carcinogenesis. We found that OBS may be associated with some, but not all, biomarkers of inflammation and cardiovascular health. The associations of OBS with FIP and FOP were in the opposite directions; a finding that cannot be readily explained at this time.

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# CHAPTER 1

## INTRODUCTION

### Oxidative Stress

The definition of oxidative stress evolved considerably over the last few decades. In 1985, Helmut Sies proposed to use the term "oxidative stress" to describe an imbalance between prooxidants and antioxidants, in favor of the former [1]. It was believed that this imbalance resulted in excess production of free radicals, molecules with an unpaired electron, capable of causing macromolecular damage. More recently attention began to shift from reactive oxygen species to non-radical oxidants, which were shown to induce oxidative stress by disrupting redox circuitry [2]. This led to the redefinition of oxidative stress, as an imbalance in prooxidants and antioxidants which results in macromolecular damage and disruption of redox signaling and control [3].

*Oxidants*

Free radicals include reactive oxygen species (ROS) such as superoxide anion radical ($O_2^-$) and hydroxyl radical (OH-), and reactive nitrogen species (RNS) such as nitric oxide (NO). Superoxide and nitric oxide can further react to produce the potent oxidant, peroxynitrite anion (ONOO-). The ROS and RNS are produced during normal physiological processes at low levels, [4]. Under pathophysiologic conditions, however these compounds are produced at a greater rate, and become harmful after reaching high concentrations. Immune response can cause macrophages to elicit a free radical burst, further activating downstream immune response and thereby providing a hostile environment for pathogens, but also damaging host cells [5]. The unpaired electrons of

free radicals cause oxidative damage to macromolecules by abstracting electrons from them, in processes known as lipid peroxidation, protein oxidation, and DNA oxidation. The resulting macromolecule radicals can propagate oxidative damage by abstracting electrons from other macromolecules (Figure 1).

Non-radical oxidants such as hydrogen peroxide ($H_2O_2$) are more ubiquitous than free radical oxidants [2]. Under normal physiological conditions, xanthine oxidase produces approximately three times as much $H_2O_2$ as $O_2^-$ [6]. Free radical oxidants can also be converted to non-radical oxidants, for example, superoxide dismutase (SOD) catalyzes the dismutation of $O_2^-$ to $H_2O_2$ [7].

Hydrogen peroxide can promote oxidative stress by oxidizing proteins with thiol components through direct and indirect mechanisms (Figure 2), resulting in the formation of disulfide bonds [8] . Disulfide bond formation affects three key redox systems: cysteine/ cystine (Cys/CySS), glutathione (GHS/GSSG), and thioredoxin-1 (-$SH_2$/-SS-). The redox state of each system can differentially influence cell signaling, transcription, and translation of proteins through numerous discrete pathways [2, 9].

*Pro/Anti-Oxidants*

Pro- and anti-oxidants are generated from both endogenous and exogenous sources. Enzymatic anti-oxidants are endogenously produced proteins that include catalase (CAT), glutathione peroxidase (GPx), and SOD [4]. An important exogenous source many pro-and antioxidants is diet. Many non-enzymatic anti-oxidants such as vitamin A precursor carotenoids, lutien, lycopene, vitamin C, vitamin E, and flavonoids are abundant in fruits and vegetables [10]. Moreover, many enzymatic antioxidants

depend on dietary consumption of its essential components such as selenium, copper, and zinc [11]. An important dietary pro-oxidant is iron, which is present in large quantities along with heme in red meat. Iron, may increase oxidative stress by catalyzing the production of highly reactive hydroxyl radicals via the Haber-Weiss reaction [12]. Some nutrients have both pro-oxidant and anti-oxidant properties. For example omega 3-fatty acids can act as a pro-oxidant by providing a substrate for lipid peroxidation [13, 14]. On the other hand, omega 3-fatty acids, induce electrophile-responsive element (EpRE), which regulates genes responsible for transcription regulation of anti-oxidant enzymes [15-17].

Non-dietary and lifestyle factors also influence anti-oxidant defense. Tobacco smoke acts as a powerful pro-oxidant, since ROS are present in the tar and smoke of cigarettes and smoking further results in the secondary release of ROS from inflammatory cells [18]. Table 1a and b detail extrinsic sources of pro/anti-oxidants and the mechanistic effects on oxidative stress.

**Oxidative Stress and Health**

Oxidative stress is thought to play a critical role in the pathogenesis of numerous health-related processes including carcinogenesis, development of cardiovascular disease, diabetes, and neurological disorders, and more broadly, aging. This can be primarily attributed to: 1) downstream effects of shifting the redox state; 2) the interrelation between oxidative stress and inflammation; and 3) macromolecular damage [4]. For the purpose of this dissertation, I will focus on the role of oxidative stress in prostate cancer and cardiovascular disease.

*Prostate Cancer*

The American Cancer Society estimates that in 2013 in the United States there will be 238,590 new cases of prostate cancer and 29,700 deaths attributed to the disease [19]. In addition to age and family history, the only well accepted risk factor for prostate cancer is race, with African American having the highest incidence rates [19, 20]. Although there is a lack of well-accepted risk factors for prostate cancer, findings from migration studies indicate that environmental factors have a role in prostate carcinogenesis. Cook *et al*. observed that prostate cancer incidence rates among Chinese, Japanese, and Filipino immigrants to the US were half that of their American born children [21]. These observation is likely attributable to differences in lifestyle, most notably diet. One of the proposed mechanistic links between lifestyle and diet-related environmental factors and prostate carcinogenesis is oxidative stress [22].

*Prostate Cancer and Oxidative Stress: Mechanistic Evidence*

Harman's free radical theory of aging postulates that the excessive accumulation of free radicals leads to aging and age related disease [23]. Free radicals can disrupt the integrity of macromolecules, a process that may promote carcinogenesis. ROS/RNS can cause oxidative nuclear and mitochondrial DNA damage which can then lead to mutagenesis [4]. At least 20 forms of DNA base damage have been attributed to oxidative stress [24]. Lipid membranes can also be damaged by free radicals though lipid peroxidation. End products of lipid peroxidation such as malondialdehyde β-gydrixyacrolein (MDA) and 4-hydroxy-2-nonenal (HNE) can also exert harmful effects on cells. MDA can cause mutations in mammalian cells and HNE can interfere with

signal transduction [25]. In proteins, amino acids undergo oxidation as part of normal redox circuitry, but excessive amounts of oxidized protein have been associated with age-related disease [10, 26]. High levels of ROS increase the expression of oncogenic transcription factors such as *c-fos* and *c-jun [27]*. While increased oxidative stress was shown to promote carcinogenesis *in vitro*, decreased levels of oxidative stress may reduce the risk of cancer progression. For example, antioxidants can inhibit NF-kB activity, which controls genes affecting proliferation and angiogenesis [10].

Oxidative stress also influences cell signaling activity, and has been found to play an essential role in prostate carcinogenesis. Higher levels of ROS have been observed in both androgen dependent and independent prostate cancer cell lines, compared to normal cells [28]. Sung *et al*. found that in prostate cancer cells, ROS increased expression of proteins associated with cell survival [29]. Findings from *in-vitro* studies have also demonstrated that high levels of ROS are required for prostate cancer progression. Kumar *et al*. observed that in prostate cancer cells, ROS activate enzymes which damage the extracellular matrix, allowing tumor cells to become more invasive [28].

*Prostate Cancer and Oxidative Stress: Epidemiological Evidence*

Many placebo controlled randomized controlled trials (RCTs) have examined antioxidant supplementation for the prevention of prostate cancer, with largely negative results. In the α -tocopherol and β -carotene (ATBC) clinical trial, 29,133 male smokers received a daily supplement of α –tocopherol (50 mg), β –carotene (20 mg), both, or a placebo. Only α -tocohperol significantly reduced prostate cancer risk by 34% (95% CI,

14%-48%). This reduction in risk was only present during the 5-8 year trial period, and was not sustained post-intervention [30].

Other trials found that antioxidant supplementation was not effective in reducing prostate cancer risk. The Selenium and Vitamin E Cancer Prevention Trial (SELECT) randomized 35,533 men from the US, Canada, and Puerto Rico into groups receiving daily supplements of selenium (200 μg), vitamin E (400 IU), both, or a placebo. During the 7-12 year intervention period, there was no statistically significant reduction in prostate cancer risk [31]. In the Carotene and Retinol Efficacy Trial (CARET) 18,314 smokers received a daily supplement of beta-carotene (30 mg) and retinyl palmitate (25,000 IU). Similar to the ATBC trial, β –carotene supplementation did not decrease prostate cancer incidence [32]. Table 2 details findings from antioxidant supplementation RCT for prostate cancer prevention.

However, observational studies indicate that increased oxidative stress may play a role in prostate cancer. In a Japanese case-control study, it was observed that prostate cancer cases had significantly higher levels of oxidative DNA damage (measured by urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG)) compared to age matched healthy controls [33]. A meta-analysis of 4 cohort studies found that the risk of prostate cancer decreased by 4% (RR=0.96; 95%CI 0.926-0.999) for each 10μg/l increase in serum lycopene [34]. Serum selenium was also found to be inversely associated with prostate cancer risk (RR=0.95; 95% CI 0.98-1.00) per 10 μg/L, in a meta-analysis of 9 cohort studies [34]. Based on this evidence (and despite the inconsistent findings of selenium supplementation RCTs), an expert panel report from the World Cancer Research Fund, lists consumption foods containing lycopene and selenium as probable exposures which

decrease prostate cancer risk [34]. It should be noted that pharmacological doses of anti-oxidants were used in these RCTs, and may not reflect the benefit of consuming anti-oxidants as part of whole foods [34].

*Cardiovascular Disease*

Cardiovascular disease is one of the leading causes of mortality in the U.S, with myocardial infarction (MI) responsible for 141,462 deaths in a single year [35]. Most cases of CVD can be attributed to coronary atherosclerosis, which in turn leads to coronary thrombosis [36]. Currently, the well accepted modifiable risk factors for CVD are hypertension, cigarette smoking, diabetes, obesity, physical inactivity, atherogenic diet, high LDL, and low HDL levels. The non-modifiable risk factors include age, male gender, and family history of CVD [37].

*Cardiovascular Disease and Oxidative Stress: Mechanistic Evidence*

Atherosclerosis occurs when ROS oxidize low density lipoproteins in the intima, recruiting adhesion molecules and inflammatory cytokines to the site. This stimulates monocytes to adhere to the endothelium, migrate to the sub-endothelial space, and differentiate into macrophages. Oxidized low density lipoprotiens (ox-LDLs) are absorbed by macrophages through scavenger receptors, resulting in foam cell development. The combination of foam cells, cellular debris, and vascular smooth muscle cell migration stimulates atherosclerotic plaque formation. The rupture of this arterial plaque can result in MI [36, 38] (Figure 3).

Multiple studies have found oxidized lipids [39], proteins [40], and LDL [41] to be present in arterial plaques. Oxidized LDL (ox-LDL) can stimulate apoptosis [42], which is common in atherosclerotic plaques but scarce in nonatherosclerotic lesions [43]. In addition to forming foam cells, ox-LDL causes inflammation by (1) recruiting inflammatory macrophages to the sub-endothelial space [44], (2) reducing nitric oxide produced in the endothelium, further stimulating inflammatory macrophages [45, 46], and (3) up-regulating the expression of inflammatory genes [47]. This arterial inflammation is believed to further influence the development of atherosclerosis [48].

Inflammation is also believed to have a role in thrombosis. Inflammatory cells can cause apoptosis of endothelial cells, resulting in plaque disruption. Inflammatory cells and ox-LDL also up-regulate the production of matrix metalloproteinases, which reduce the structural integrity of sub-endothelial basement membrane, possibly causing plaque disruption [48].

A study of the probucol, a synthetic cholesterol lowering drug that is also a potent antioxidant [49] , found that rabbits with familial hypercholesterolemia that were treated with probucol had significantly increased LDL resistance to oxidation compared to untreated rabbits [50]. Even when adjusting for the probucol's cholesterol-lowering ability by treating rabbits with both probucol and lavastatin, an inhibitor of 3-hydroxy–3-methylglutaryl coenzyme A reductase, there was a 48% reduction in atherosclerosis [51]. This provides evidence that anti-oxidants can modify the development of cardiovascular disease in general and MI in particular.

*Cardiovascular Disease and Oxidative stress: Epidemiological Evidence*

Multiple clinical studies of individual antioxidants and vascular disease have been conducted [52]. Six large randomized clinical trials tested vitamin E supplementation [53-58], three trials tested beta-carotene [54, 59, 60], one trial examined efficacy of vitamin C [58], and two trials used a combination of those supplements [54, 61]. None of those trials demonstrated an effect of antioxidants in reducing CHD risk. Table 3 provides a detailed description of these anti-oxidant supplementation RCTs and CVD outcomes.

However, findings from observational studies demonstrate that anti-oxidants may play a role in prevention of CVD. Prospective cohort studies examining dietary anti-oxidants suggest that anti-oxidants may reduce the risk of CVD. A meta-analysis of prospective cohort studies found that vitamin E (RR=0.76, 95% CI: 0.63-0.89), beta-carotene (RR=0.78, 95% CI: 0.53-1.04), and vitamin C (RR=0.84, 95% CI: 0.73-0.95) reduce the risk of CHD, when comparing the highest tertile micronutrient intakes to the lowest tertile [62]. When examining whole food sources of anti-oxidants and CVD risk, stronger associations are observed. Dauchet *et al*. found that in a meta-analysis of nine prospective cohort studies, each additional portion of fruit and vegetable intake reduces CHD risk by 4% (RR=0.96, 95% CI: 0.93-0.99) [63] . Although the American Heart Association does not recommend anti-oxidant supplementation for CVD prevention, it recommends a diet high in fruit and vegetable intake [64].

**Biomarkers of Oxidative Stress**

The biomarkers of oxidative stress currently used in population studies include $F_2$-Isoprostanes (FIP), florescent oxidative product (FOP), and mitochondrial DNA (mtDNA) copy number. Each of those biomarkers has its advantages and disadvantages, but a systematic comparison of all three measures using the same samples has not been conducted.

FIP are products of arachadonic acid peroxidation. As previously described elsewhere [65], lipid peroxidation results in production of three arachidonyl radicals, which undergo endocyclization to form four $PGH_2$-like bicyclic endoperoxide intermediate regioisomers, and are then reduced to four F-ring regioisomers, each consisting of eight racemic diastereoisomers. FIP are produced esterified on phospholipids and are then cleaved and released into circulation in free form [65]. Although FIP can be measured in plasma and urine, plasma measurements are preferred because oxidative stress biomarkers in urine are influenced by renal metabolism [66, 67].

The use of FOP as a global measure of oxidation began in the food industry, but is now being proposed for population based human studies of oxidative stress [68]. FOP is a non-specific marker of aldehydes (derived from lipids, proteins, carbohydrates, and DNA oxidation) cross-linked with amino acids [69-72], forming a Schiff base. Examples of these aldehydes include malondaldehyde from lipid peroxidation or glycoaldehyde from carbohydrate autoxidation [72].

Mitochondria are organelles which contain their own circular genome lacking introns and histones. Their primary function is to generate adenine triphosphate (ATP) through cellular respiration, a process that also produces ROS, which can cause oxidative

DNA damage [73]. Unlike nuclear DNA, which is protected by elaborate repair mechanisms [74], mtDNA responds to oxidative damage by increasing the number of its copies. Studies have demonstrated that mtDNA copy number is increased in response to oxidative stress [75-77].

At present, FIP is considered the "gold-standard" biomarker of oxidative stress, but an accurate and reliable analysis of FIP requires careful handling of samples to prevent in vitro oxidation [70]. Wu *et al*. found FOP to be a stable measure, with levels from blood specimens remaining constant over 36 hours, whereas FIP in the same samples increased at each time measured indicating *in vitro* lipid peroxidation [70]. The main disadvantage of FOP and FIP is that both of these biomarkers represent short term oxidative stress levels. By contrast mtDNA copy number is supposed to be a stable biomarker that also indicates (at least in theory) long term cumulative oxidative stress-induced damage.

*Biomarkers of Oxidative Stress in Epidemiological Studies*

Results from several placebo controlled randomized clinical trials (RCTs) of antioxidant supplementation on FIP have been inconsistent. Two studies examined the effects of vitamin C on plasma FIP level [78, 79]. In one of these clinical trials, receiving vitamin C showed no effect in participants with coronary artery disease [79]. In another trial that enrolled current smokers, vitamin C lowered FIP level, but only among participants with high BMI [78]. The same study found that supplementation of a combination of antioxidants (vitamin C, α-lipoic acid, and vitamin E) had no effect on FIP levels [78]. Another clinical trial of vitamin E supplementation in smokers found no

effect on urinary FIP level [80]. FOP and mtDNA copy number have recently been proposed biomarkers of oxidative stress to use in population studies [69], and have not yet been well studied in antioxidant RCTS.

In studies of diets with high levels of anti-oxidants and FIP, the findings are consistent. Studies have found dietary beta-carotene [81, 82], alpha-tocopherol [81], flavonoids[82], and vitamin C [81, 82] to be inversely associated with isoprostane levels. The Mediterranean diet contains a high intake of fruits and vegetables, rich sources of anti-oxidants. Gaskins *et al*. observed that a 1 point increases in the Mediterranean Diet Score (range 0-9 points, with a higher points indicating higher adherence to diet) was associated with 4.5% decrease in FIP (95% CI: -6.32%, -2.65%) [83].

**OBS and Inflammation**

It is important to point out that oxidative stress is closely related to inflammation. Nearly all pro- and anti-oxidants also have pro- or ant-inflammatory properties. For example, vitamin E can inhibit cyclooxygenase-2, an enzyme that is a part of inflammatory pathways and [84] and selenium can inhibit the transcription of genes for inflammatory cytokines [85]. Thus, it is important to examine the association between oxidative stress related exposures and markers of inflammation.

In population and clinical studies, commonly used markers of inflammation include C-reactive protein (CRP), white blood cells (WBC), albumin, and various cytokines. CRP production in the liver is stimulated by increased cytokines. Upon release, CRP can increase the activity of phagocytic cells [86]. CRP quickly responds to reduction in inflammation, with an elimination half-life of 4-9 hours, making it a good

short term marker of inflammatory status[87]. Albumin is synthesized in the liver and released immediately upon production [88]. Inflammation decreases serum albumin by reducing the rate of synthesis [89]. White blood cells (WBC) are involved in the acute phase response [90]. According to the American Heart Association and the Centers for Disease Control and Prevention, WBC count is a potential inflammatory marker that may be associated with cardiovascular events [91]. Since inflammation modulates the levels of these laboratory measures, they can be considered biomarkers of inflammation.

**Oxidative Balance Score (OBS) Concept**

Different oxidative stress-related exposures do not act in isolation and are usually present as part of a diet, which in turn constitutes just one component of a person's lifestyle. For this reason, it has been postulated that a combination of factors may demonstrate a stronger association with disease risk than any pro- or anti-oxidant considered individually [92-94]. The need to consider various pro- and anti-oxidants in combination rather than individually has been proposed as the possible explanation for the discrepancy between mechanistic studies supporting the role of oxidative stress in disease and the lack of epidemiological support for this hypothesis [95].

To address this issue several researchers proposed using an oxidative balance score (OBS), an overall measure of oxidative stress-related exposures based on the summed intake of various pro- and anti- oxidants, with a higher score indicating decreased oxidative stress.

Previous studies found that a higher OBS was associated with decreased risk of colorectal adenoma [94, 96], colorectal cancer [97] , and mortality [98] (Table 4). By

contrast, studies of OBS in relation to prostate cancer risk are conflicting, and at least one previous study found no association between OBS and stroke (Table 4). These observations indicate that the role of oxidative stress in human pathophysiology may be organ-, or disease-specific. Moreover, although OBS has been found to be associated with various health outcomes the mechanisms by which OBS affects these outcomes and the data relating OBS to specific biomarkers are also lacking.

**Study Goals and Specific Aims**

The primary goals of this dissertation are to examine the effects of OBS on prostate cancer incidence, assess the interrelation between OBS and biomarkers of oxidative stress, and evaluate the association between OBS and various biomarkers of inflammation and cardiovascular health. To achieve these goals, we use data from the Cancer Prevention Study-II Nutrition (CPS-II) Cohort, the Study of Race, Stress, and Hypertension (SRSH), and the Reasons for Geographic and Racial Differences in Stroke (REGARDS) cohort. The primary goals of the study are addressed through the following specific aims.

**Aim 1**: Examine the association between Oxidative Balance Score (OBS) and prostate cancer risk in the CPS-II cohort. I hypothesize that OBS is inversely associated with prostate cancer risk.

**Aim 2**: Examine the associations between OBS and three biomarkers (FIP, FOP, and mtDNA) each thought to that reflect different aspects of oxidative stress; and to assess how these biomarkers are related to each other in the SRSH population. I

hypothesize that OBS is inversely associated with all biomarker levels and the biomarkers are inter-correlated.

**Aim 3**: Examine the association between OBS and biomarkers inflammation (CRP, WBC count, and albumin) and cardiovascular health (cholesterol, LDL, HDL, and triglycerides) in the REGARDS cohort. I hypothesize that OBS is inversely associated with CRP, WBC count, cholesterol, LDL, triglycerides, but is positively associated with albumin and HDL.

Each of the above specific aims is addressed in a separate study. The three studies are included in the next sections of this dissertation as complete stand-alone manuscripts.

Figure 1. Lipid peroxidation initiation and propagation [99]

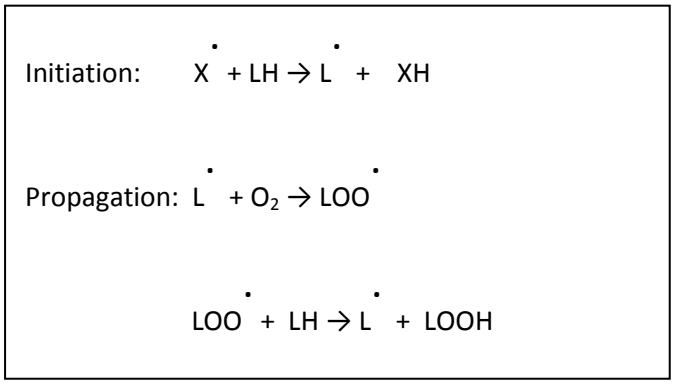
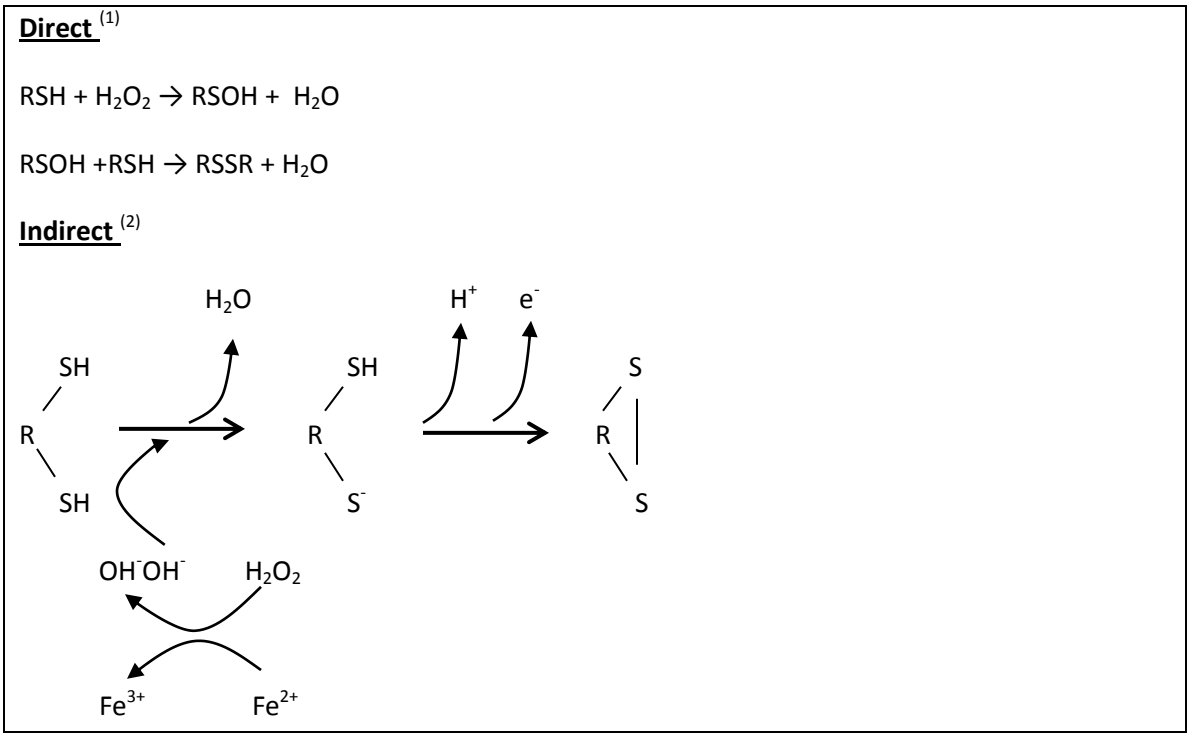



Figure 2. Direct and indirect thiol oxidation by Hydrogen Peroxide




(1) Hydrogen peroxide can combine with thiol to yield sulfenic acid which can go on to form disulfide bond [100].
(2) Hydrogen peroxide forms the hydroxyl radical through the Fenton reaction with iron. The hydroxyl radical reacts with the thiol to create thiyl radicals, which can then form disulfide bonds [8].

Table 1a. Exogenous sources of dietary pro/anti-oxidants and their mechanistic effects on oxidative stress

| Exogenous Pro/Anti-oxidants | Mechanism of Pro/Anti-oxidant Effect |
|---|---|
| ***Dietary anti-oxidants*** | |
| **Carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lutein lycopene)** | · Reduce lipid peroxidation [101]<br>· Scavenge singlet oxygen and peroxyl radical [102]<br>· Activate endogenous antioxidant defense system[103] |
| **Vitamin C** | · Scavenges free radicals<br>· Recycles the α-tocopheroxyl radical [104] |
| **Vitamin E** | · α-tocopherol donates electrons more readily [105]<br>· γ-tocopherol traps RNS more readily [106] |
| **Ω-3 fatty acids** | · Increase antioxidant enzyme activity [107]<br>· Decreases NADPH oxidase activity [108] |
| **Flavonoids** | · Scavenge free radicals<br>· Chelate iron [109] |
| **Glucosinolates** | · Increase GSH<br>· Induce GPx [17] |
| **Selenium** | · Component of antioxidant enzymes (GPx, TrxR) |
| **Zinc** | · Inhibits thiol oxidation of some enzymes<br>· Induces metallothionein antioxidant activity[110] |
| ***Dietary pro-oxidants*** | |
| **Iron** | · Reacts with $H_2O_2$ to generate radicals in the Fenton reaction [111] |
| **Ω-6 fatty acids** | · Undergo lipid peroxidation<br>· Hinder with Ω-3 metabolism |
| **Saturated fat** | · Activates inflammatory response [112] |

[1] Carotenoids vary in efficiency of reducing Thiobarbituric acid-reactive substances (TBAR) formation: lycopene>α-tocopherol>α-carotene>β-cryptoxanthin>β-carotene>lutein [101]

[2]Reduces free radicals, while itself becoming a less reactive radical in the process

Abbreviations: GSH = Glutathione; GPx = Glutathione Peroxidase; TrxR = Thioredoxin Reductase; NSAID = Non-Steroidal Anti-Inflammatory Drug; RNS=Reactive Nitrogen Species; NADPH = nicotinamide adenine dinucleotide phosphate-oxidase

Table 1b. Exogenous sources of non-dietary lifestyle pro/anti-oxidants and their mechanistic effects on oxidative stress

| Exogenous Pro/Anti-oxidants | Mechanism of Pro/Anti-oxidant Effect |
|---|---|
| ***Non-dietary lifestyle anti-oxidants*** | |
| **Physical activity** | · Increases expression of anti-oxidant enzymes [113] |
| **NSAID** | · Inhibits Cyclooxygenase, decreasing inflammation [114] |
| ***Non-dietary lifestyle pro-oxidants*** | |
| **Smoking** | · Tar contains high amounts of ROS [115]<br>· Produces secondary release of ROS caused by inflammation [116] |
| **Alcohol** | · Promotes iron absorption<br>· Depletes GSH in mitochondria<br>· Increases cytochrome P450 activity[117] |
| **Obesity** | · Increased adiposity upregulates NADPH oxidase gene expression<br>· Increased adiposity downregulates antioxidant gene expression [118] |

Abbreviations: GSH = Glutatione; NSAID = Non-Steroidal Anti-Inflammatory Drug; ROS = Reactive Oxygen Species; NADPH = nicotinamide adenine dinucleotide phosphate-oxidase

Table 2. Antioxidant RCTS and prostate cancer outcomes

| Study | Design | Intervention | Risk of Prostate Cancer |
|---|---|---|---|
| **Selenium RCT (1996) [119]** | - RCT<br>- n=1,312 | -Selenium (200 µg) | - **Selenium: RR =0.37 (95%CI: 0.18–0.71)** |
| **ATBC (2003 ) [30]** | - 2x2 RCT<br>- n=27,271 Finnish male smokers | - Vitamin E (50 mg)<br>- Beta carotene (20 mg) | - **Vitamin E: RR=0.64 (95%CI: 0.44–0.94)**<br>- β-carotene: RR=1.23 (95%CI: 0.89–1.70)<br>- β-carotene & Vitamin E: RR=0.84 (95%CI: 0.59–1.20) |
| **SELECT (2009) [31]** | - 2x2 RCT<br>- n=35,533 men from US, Canada, and Puerto Rico | - Selenium (200 µg)<br>- Vitamin E (400 IU) | - Selenium: HR =1.04 (99%CI: 0.87–1.24)<br>- Vitamin E: HR =1.13 (99%CI: 0.95–1.35)<br>- Vitamin E & Selenium: HR =1.05 (99%CI: 0.88–1.25) |
| **CARET (2009) [32]** | -RCT<br>-n=18,314 in US | -Combination of beta-carotene (30 mg), retinyl palmitate (25,000 IU) | - Combination: RR=1.10 (95%CI: 0.81–1.48) |

Abbreviations: RCT= Randomized Controlled Trial, RR=Relative Risk, HR= Hazard Ratio

Figure 3. The development of atherosclerosis caused by LDL oxidation [120]

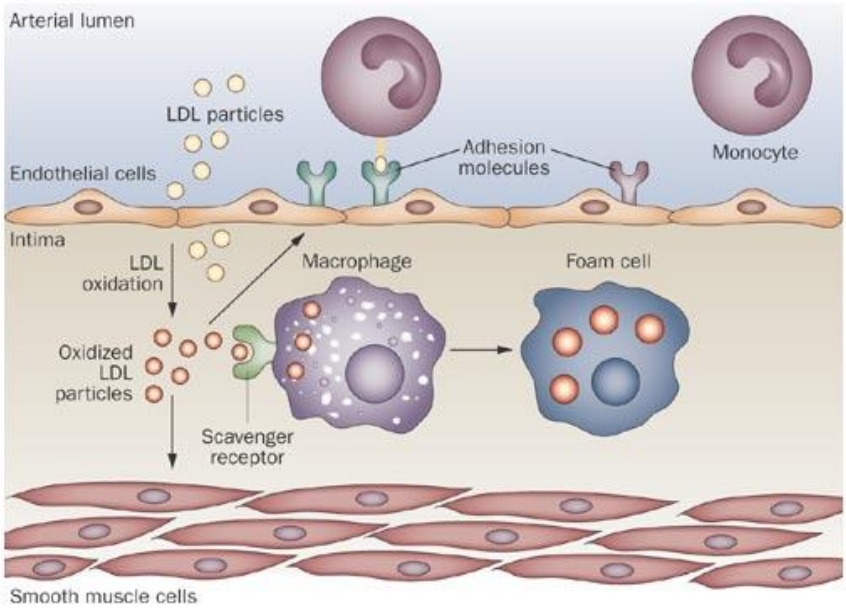


Table 3. Antioxidant Supplementation RCTs and Cardiovascular Outcomes

| Study | Design | Intervention | Risk for Cardiovascular Events RR (95% CI) |
|---|---|---|---|
| **PPP (2001) [53]** | - 2x2 RCT in Italy<br>- n=4,495 | - Vitamin E (300 mg/)<br>- Aspirin | - Vitamin E: 0.94 (0.77–1.16) |
| **ATBC (1998) [54]** | - 2x2 RCT<br>- n=27,271 Finnish male smokers | - Vitamin E (50 mg)<br>- β-carotene (20 mg) | - Vitamin E: 0.98 (0.87–1.10)<br>- β-carotene: 1.03 (0.91–1.16)<br>- β-carotene & Vitamin E : 0.97 (0.86–1.09) |
| **WHS (1999) [55, 60]** | - 2x2x2 RCT in US<br>- n=39,876 | - Vitamin E (600 IU)<br>- β-carotene (20 mg)<br>- Aspirin | - Vitamin E: 0.93 (0.82–1.05)<br>- β-carotene: 1.14 (0.87–1.49) |
| **HOPE (2000) [56]** | - 2x2 RCT in US, Mexico, Europe and South America<br>- n=9,541 | - Vitamin E (400 IU)<br>- Ramipril | -Vitamin E: 1.05 (0.95–1.16) |
| **HOPE-TOO (2005) [57]** | - 2x2 RCT in US, Mexico, Europe and South America<br>- n=7,030 | - Vitamin E (400 IU) | - Vitamin E: 1.04 (0.96–1.14) |
| **PHS II (2008) [58]** | - 2x2 RCT in US<br>- n=14,641 men | - Vitamin E (400 IU )<br>- Vitamin C (500 mg) | - Vitamin E: 1.01 (0.90–1.13)<br>- Vitamin C: 0.99 (0.89–1.11) |
| **PHS (1996) [59]** | - 2x2 RCT in US<br>- n=22,071 men | - β-carotene (50 mg)<br>-Aspirin | -β-carotene: 1.00 (0.91–1.09) |
| **HPS, (2002) [61]** | -RCT in UK<br>- n=20,536 | -Combination of vitamin E (600 mg), vitamin C (250 mg), β-carotene (20 mg) | -Antioxidant combination :1.00 (0.94–1.06) |

Abbreviations: RCT= Randomized Controlled Trial, RR=Relative Risk

Table 4: Findings from Previous Studies of OBS and Health Outcomes

| Study | Outcome | Point Estimate[1] (95% CI) | Study Design |
|---|---|---|---|
| **BIRNH [98]** | Mortality | OR: 1.44 (1.13-1.82) | Prospective cohort |
| **REGARDS [121]** | Mortality | HR: 0.70 (0.61-0.81) | Prospective cohort |
| **MAP [95]** | Colorectal adenoma | OR: 0.45 (0.21-0.99) | Case Control |
| **CPRU [96]** | Colorectal adenoma | OR: 0.55 (0.35-0.89) | Case Control |
| **MAP [122]** [2] | Colorectal adenoma | OR: 0.34 (0.13-0.88) | Case Control |
| **CPS-II** | Colorectal Cancer | RR: 0.65 (0.53-0.79) | Prospective Cohort |
| **REGARDS** | Stroke | HR: 0.92 (0.69-1.24) | Prospective cohort |
| **CSDLH [123]** | Prostate Cancer | OR: 1.01 (0.74-1.36) | Prospective cohort |
| **MPC [95]** | Prostate Cancer | OR: 0.28 (0.10-0.82) | Case-Control |
| **MPC**[2] **[122]** | Prostate Cancer | OR: 0.34 (0.14-0.86) | Case-control |

[1] Point estimates compare the highest OBS category to the lowest OBS category
[2]OBS components measured by FFQ & biomarkers
Abbreviations: BIRNH = Belgian Interuniversity Research on Nutrition and Health; MAP= Markers of Adenomatous Polyps; CPRU = Cancer Prevention Research Unit; MPC = Markers of Prostate Cancer; CSDLH = Canadian Study of Diet, Lifestyle, and Health

# CHAPTER 2

## Oxidative Balance Score and Risk for Incident Prostate Cancer in a Prospective U.S. Cohort Study

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**Abstract**

Oxidative stress is defined as an imbalance between pro-oxidants and anti-oxidants. Although experimental biology evidence demonstrates that antioxidants reduce cell proliferation and oxidative DNA damage in variety of tissues, including prostate epithelium, epidemiological studies relating modifiable factors that affect oxidative stress to prostate cancer risk have been inconsistent. Previous research has shown that including individual pro- and anti-oxidant exposures into a single comprehensive oxidative balance score (OBS) may be associated with various conditions (including prostate cancer) in the absence of associations with individual factors. We investigated an OBS-incident prostate cancer association among 43,325 men in the Cancer Prevention Study-II Nutrition Cohort. From 1999 to 2007, 3,386 incident cases were identified. Twenty different components, used in two ways (unweighted or weighted based on literature reviews), were incorporated into the OBS and the resulting scores were then expressed as three types of variables (continuous, quartiles, or six equal intervals). Multivariable-adjusted rate ratios were calculated using Cox proportional hazards models. We hypothesized that the OBS would be inversely associated with prostate cancer risk; however, the rate ratios (95% confidence intervals) comparing the highest to the lowest OBS categories ranged from 1.15 (1.03-1.30) to 1.41 (0.90-2.21) for all cases, 1.12 (0.85-1.47) to 1.86 (0.74-4.67) for aggressive disease (AJCC stage III/IV or Gleason score 8-10), and 0.91 (0.62-1.35) to 1.18 (0.82-1.71) for non-aggressive disease. Our findings are not consistent with the hypothesis that oxidative stress-related exposures play a role in prostate carcinogenesis.

## Introduction

Prostate cancer is the second leading cause of cancer death among men in the United States [124]. Despite the advances made in diagnosis and treatment for this malignancy, the search for risk factors that can be considered as targets for disease prevention has been largely unsuccessful. The only well accepted risk factors for the disease, besides age, are family history and race [20].

Despite this paucity of established risk factors, evidence from migration studies [125] indicates that environmental factors may play a role in prostate carcinogenesis. One proposed mechanistic link between environmental factors and prostate cancer risk is oxidative stress, which has been shown to play a role in prostate carcinogenesis [22]. Oxidative stress is a multi-factorial condition which occurs when an imbalance between pro-oxidant and anti-oxidant factors results in excessive production of reactive oxygen species (ROS) [126, 127]. ROS interact with macromolecules by causing protein oxidation, lipid peroxidation, and oxidative DNA damage [117], which in turn can lead to mutagenesis and subsequent carcinogenesis [128, 129].

A number of environmental exposures may act as pro-oxidants. ROS are present in cigarette tar and smoke. Smoking also produces a secondary release of ROS from inflammatory cells [18, 130]. Another important pro-oxidant is iron, which is consumed along with heme in large quantities as part of red meat-rich diet. Iron, may increase oxidative stress by catalyzing the production of highly reactive hydroxyl radicals via the Haber-Weiss reaction [131]. Alcohol induces oxidative stress through its metabolism, by inhibiting antioxidant enzymes and causing inflammation [117]. Inflammation acts as both a source and consequence of oxidative stress [132]. Inflammatory cells respond to

stimuli (e.g., microbial agents) by producing superoxide, hydrogen peroxide, singlet oxygen, and nitric oxide. Nitric oxide can react further to form highly reactive peroxynitrite molecules [133].

Many micronutrients are anti-oxidants and can reduce oxidative stress. These anti-oxidant micronutrients can reverse the effects of ROS and oxidative stress-inducing inflammation *in-vitro*, resulting in decreased DNA oxidation [134] and reduced mutagenicity [135-138]. Anti-oxidants, can be classified as enzymatic or non-enzymatic [10]. Non-enzymatic anti-oxidants such as vitamin A precursor carotenoids, lutein, lycopene, vitamin C, vitamin E, and flavonoids which can protect against lipid peroxidation and terminate free radical chain reactions [10]. Enzymatic antioxidants are also nutrient-dependent; for example, selenium and manganese are critical components of the enzymes glutathione peroxidase and superoxide dismutase, which are responsible for intracellular defense against oxidative stress [10]. Other nutrients can also indirectly contribute to a reduction in ROS. Omega-3 fatty acids contribute to oxidative stress through peroxidation [139], but also induce electrophile-responsive element (EpRE), which regulates genes responsible for the transcription of anti-oxidant enzymes [15-17]. Moreover, omega-3 fatty acids have anti-inflammatory properties and therefore indirectly decrease oxidative stress [140].

Although antioxidants have been shown to reduce cell proliferation and oxidative DNA damage *in vivo* and *in vitro*, observational epidemiological studies examining individual anti-oxidants and prostate cancer risk have been inconsistent [20, 34, 129]; whereas clinical trials of anti-oxidants as chemopreventive agents against prostate cancer have produced null results [141]. Other studies of chronic diseases have demonstrated

that nutrients do not act in isolation and a combination of factors can show a stronger association with disease risk than any single nutrient considered individually [93, 94, 142]. This leads us to believe that a combination of pro-oxidant and anti-oxidant exposures incorporated into a composite measure of oxidative balance may be more strongly associated with prostate cancer risk than any one factor considered individually[95, 96, 122].

In the present study, in follow-up to our pilot case-control study in which we found an inverse association between OBS and prostate cancer risk [95], we investigated the association in a large prospective U.S. cohort study. OBS was constructed using information on 15 dietary and 5 lifestyle exposures among 43,325 men enrolled in the Cancer Prevention Study II (CPS-II) Nutrition Cohort.

## Materials and Methods

### *Study Population*

The men in this analysis were participants in the Cancer Prevention Study II (CPS-11) Nutrition Cohort, which was designed to assess the associations of dietary and lifestyle factors with cancer incidence (35). The CPS-II Nutrition Cohort is a subcohort of CPS-II, a prospective study of cancer mortality involving approximately 1.2 million Americans begun in 1982. Participants in the Nutrition Cohort were recruited from CPS-II members who resided in 21 states and were predominantly between the ages of 50 and 74 years. In 1992-93, questionnaires were mailed to CPS-II participants in 21 states -to assess diet, lifestyle, and medical history. A total of 184,194 individuals were enrolled in the CPS-II Nutrition Cohort. Follow-up questionnaires were sent to all living Nutrition Cohort members in 1997 and every two years thereafter to update exposure information

and to ascertain newly diagnosed cancers. The response rates on all of the follow-up questionnaires, among those cohort participants who were mailed surveys, were at least 87%. All aspects of the CPS-II Nutrition Cohort study are approved by the Emory University Institutional Review Board.

For the present study, follow-up was from 1999 until the date of diagnosis of prostate cancer, death, date of the last returned survey, or June 30, 2007. Our analysis began in 1999 because this was the year a more comprehensive 152-item Food Frequency Questionnaire (FFQ) was utilized. Some participants (N=9,518) were excluded because they completed a shorter questionnaire with no dietary information. After excluding participants who were lost to follow-up (N=1,570), had a history of prostate cancer (N=6,100) or cancer other than non-melanoma skin cancer (N=5,468) at baseline, those who had incomplete or improbable FFQ data or did not report data on lifestyle variables required for OBS calculation (N=3,668), and those whose self-reported prostate cancer was not verified (N=82), a total of 43,325 men, were available for analyses.

*Identification of Prostate Cancer Cases*

We identified and verified 3,386 incident cases of prostate cancer between 1999 and June 30, 2007. This included cases verified via medical records (n=2720) or linkage to cancer registry data (n=666).

*OBS components and their assessment*

The OBS is comprised of 20 components that were selected based on *a priori* knowledge about their relation to oxidative stress. The dietary components were derived

from the 152-item semi-quantitative, modified Willet FFQ administered in 1999 [143, 144]. Nutrient values in these data represent the total dietary and supplemental intake for each nutrient. Nutrient contents of various foods were determined using a nutrient database from Harvard University with composition values from the U.S. Department of Agriculture and other sources. Nutrient intakes were calculated by multiplying the reported frequency of consumption by the nutrient composition of the specified portion size of the various foods. All nutrient values derived from the FFQ were energy-adjusted using the residual regression method [145].

The validity and reproducibility of the OBS dietary and lifestyle components measured by the modified Willett FFQ have been documented in other US populations that are similar to the CPS-II nutrition cohort. For example, the correlation coefficients of vitamin C and vitamin E intakes reported on the FFQ with those from a 1-week dietary record were 0.86 and 0.87, respectively [144]; the corresponding correlations between physical activity reported by FFQ and those from 1-week recalls and 7-day diaries were 0.79 and 0.62 [146]. The components of the OBS and how they contribute to this measure are summarized in Table 1.

Continuous dietary variables reflecting anti-oxidant exposures were divided into categories based on their quartile values. Participants whose exposure to a particular dietary anti-oxidant was very low ($1^{st}$ quartile) were assigned zero points, and those whose exposure to the same dietary anti-oxidant was low ($2^{nd}$ quartile), medium ($3^{rd}$ quartile), or high ($4^{th}$ quartile), received one, two, or three points, respectively. The continuous dietary anti-oxidant variables in our study were alpha-carotene, beta-carotene, beta-cryptoxanthin, lutein, lycopene, vitamin C, vitamin E, zinc, omega-3 fatty acids,

flavonoids, and glucosinolates. Measurement of dietary selenium intake by FFQ has been shown to be inaccurate [147-149], and therefore, only supplemental selenium was included in the OBS. As 65% of the study population did not use any selenium supplements selenium intake was categorized as: 0 µg/day (zero points), 1 – 20 µg/day (one point), 21-50 µg/day (two points), and more than 50 µg/day (three points).

Continuous dietary variables reflecting pro-oxidant exposures were also categorized based on their quartile values. Three points were given for very low exposure ($1^{st}$ quartile), two points for low exposure ($2^{nd}$ quartile), one point for medium exposure ($3^{rd}$ quartile), and zero points for high exposure ($4^{th}$ quartile). The continuous dietary pro-oxidants in our study were omega-6 fatty acids, iron, and saturated fat. All continuous nutrient variables were adjusted for total energy intake.

Non-dietary lifestyle variables included in the OBS were assigned 0-3 points to keep them consistent with dietary components. Physical activity (met-hrs) was divided into quartile values with 0 to 3 points assigned to the first to the fourth quartile, respectively. Based on the smoking status each participant was categorized as current smoker (zero points), former smoker for more than 24 years, referring to the mean number of years smoked in formers smokers (one point), former smoker for 24 years or less (two points), and never smoker (three points). Alcohol intake was categorized as <1 drink/week (three points), 1-6 drinks/week (two points), 7 drinks/week (one point), and >7 drinks/week (zero points). Body mass was described using the combined measurement of waist circumference (high: >40 in., medium: 37 in. ≤ waist ≤40 in., low: <37 in.) and BMI (high: 30 ≤BMI<50, medium: 25≤ BMI <30, low: 18.5<BMI<25). Participants with high BMI and high waist measurements were assigned zero points. Those who had either

high BMI and a medium waist measurement, or medium BMI and high waist measurement received one point, and those with any of the following combinations: medium BMI and a medium waist measurement, medium BMI and low waist measurement, or low BMI and a medium waist measurement received two points. Three points were assigned to all subjects that had low BMI and low waist measurement. Duration of non-steroidal anti-inflammatory drug (NSAID) use was categorized based on participants' responses (user or non-user) to the 1992, 1997, and 1999 surveys. Zero points were given to participants who responded that they were not NSAID users in all three surveys. One point was given to participants who reported using NSAID in only one survey, two points were given to participants who reported NSAID use in two surveys (1992 and 1997 or 1997 and 1999), and three points were given to participants who reported NSAID use in all three surveys. A number of participants did not provide any information about NSAID use in at least one of the three surveys. If a participant reported the same NSAID status (a user or non-user) for each of the 2 surveys and the third response was missing, the participant is counted as having 3 surveys with the same response. If the NSAID use status differed in two surveys (e.g., user in 1992 and non-user in 1997) but the third response was missing, the participant is given one point. If a participant was missing information on any two surveys, only the survey with NSAID status information was used with one point assigned for NSAID users and zero points for nonusers. All other non-dietary lifestyle variables were based on self-reports as reflected in the in the 1999 questionnaires. The OBS assignment scheme is listed in Table 1.

The points assigned to each component were summed to create the overall OBS. In the analyses of associations with prostate cancer, the score was used in several ways:

first as a continuous variable, then divided into quartiles, and finally separated into equal interval categories. The cutoffs were determined using the distribution of OBS within the analytical cohort; the specific cutoffs are listed in Tables 2-4. Assessing the OBS using quartiles and equal interval categories allowed for examining particularly low and high scores.

*Weighting*

The OBS components were included in the overall score using two approaches: (1) with equal weights, or (2) weighted according to the reported associations with prostate cancer risk. For each OBS component under study an attempt was made to identify recent comprehensive reviews or meta-analyses that summarized the evidence on the presence and magnitude of their associations with prostate cancer. For components for which no published reviews were found, we conducted our own meta-analysis using previously published studies of nutrients and prostate cancer. The weights for pro-oxidants were the pooled adjusted relative risks (RRs) derived from published studies, whereas weights for the anti-oxidant OBS components were calculated using the inverse RR estimates (Table 1). For example, an antioxidant exposure inversely associated with prostate cancer risk with a pooled RR of 0.8 would receive a weight of 1/0.8, or 1.25 such that 0, 1, 2 and 3 unweighted points would be converted to 0, 1.25, 2.5, and 3.75 weighted points, respectively. Similarly, for a pro-oxidant with a corresponding pooled RR estimate of 1.1 the unweighted points 0-3 after weighting would assume a range of 0-3.3.

*Statistical Analysis*

Multivariable-adjusted rate (hazard) ratios (HRs) and the corresponding 95% confidence intervals (CIs) were calculated using Cox proportional hazards models. All models were tested for proportional hazards assumption violations using the likelihood ratio test. In addition all models were examined for collinearity among independent variables and for interaction between OBS and each covariate. All analyses were conducted using SAS statistical software version 9.2 (SAS institute, Cary North Carolina).

The association between OBS and prostate cancer risk was examined using each version of the OBS after adjusting for the age, total energy intake, total (dietary plus supplemental) calcium intake, total vitamin D intake, and total folate intake, race (white, black, other), education (less than high school, high school graduate, some college, college graduate, missing), family history of prostate cancer in a first degree relative (no, yes), cholesterol lowering drug use (never, former, current, missing), finasteride use (never, former, current, missing), prostate cancer screening (ever, never, unknown). These potential confounders were selected based on evidence in the literature and other *a priori* considerations.

Associations with OBS were also examined separately for aggressive and non-aggressive prostate cancer, after adjusting for the same covariates. Prostate cancer was classified as aggressive based on either an advanced stage (AJCC stage III/IV) or high grade (Gleason score of 8-10).

**Results**

The baseline characteristics of all participants by OBS quartile are presented in Table 2. There were no significant differences at baseline in age, race, family history of prostate cancer in a first degree relative, total energy intake, or finasteride use by OBS quartile. The proportion of participants who were college educated, had undergone PSA screening, and was currently using cholesterol-lowering drugs increased with an increasing OBS score. Total calcium, vitamin D, and folate intake also increased with increasing OBS score.

Table 3 shows the association between prostate cancer risk and OBS with the equally weighted components. After adjusting for covariates, a statistically significant, but modest, positive association was found when OBS was assessed as a continuous variable (HR=1.008 95% CI; 1.002-1.013). The rate of prostate cancer was 17% higher in the highest versus the lowest OBS quartiles (HR=1.17; 95% CI: 1.04-1.32; p-trend = 0.01). A similar analysis using equal interval categories yielded a corresponding HR of 1.39 (95% CI: 0.90-2.15; p-trend = 0.007) (Table 3).

Table 4 shows the corresponding multivariable analyses in which the OBS components were weighted based on their associations with prostate cancer risk, as reported in the literature. The results of the weighted analyses were essentially identical to those obtained without weighting: the HRs were 1.007 (95% CI: 1.002-1.012) for OBS treated as a continuous variable, 1.15 (95% CI: 1.03-1.30) when comparing the lowest to the highest OBS quartile (p-trend = 0.02), and 1.41 (95% CI: 0.90-2.21) when comparing the lowest to the highest category in the equal-interval analyses (p-trend = 0.01).

Table 5 shows the associations of various OBS measures with non-aggressive and aggressive prostate cancer. The associations for aggressive and non-aggressive tumors were very similar to those obtained using all prostate cancer cases combined (as seen in Tables 2 and 3). The only departure from this pattern was the difference between results for aggressive (HR=1.59; 95% CI: 0.57-4.40) and non-aggressive tumors (HR=0.91; 95% CI: 0.62-1.35) in the analyses comparing the highest and the lowest categories when the equally weighted OBS was divided into equal intervals. The weighted results were not substantially different from the unweighted results (data not shown).

**Discussion**

The results from this prospective cohort study provide no evidence that an oxidative balance score is inversely related to prostate cancer risk. In fact the data suggested that persons with a higher OBS may be at increased risk of developing (or perhaps being diagnosed with) prostate cancer. Separating aggressive and non-aggressive prostate cancer cases did not appreciably change the observed associations. These results are not consistent with the hypothesis that a presumably beneficial balance of pro- and anti-oxidant exposures protects against prostate cancer development or progression.

Other studies have examined the association between OBS and prostate cancer with conflicting results. In the Markers of Prostate Cancer (MPC) case-control study, two different methods were used to measure OBS components, a Food Frequency Questionnaire (FFQ)-based method (Goodman *et al.* 2007) [95] and a combined FFQ and biomarker-based method (Goodman *et al.* 2010) [122]. For both methods, a higher OBS was inversely associated with prostate cancer, although a statistically significant trend

was found with the biomarker-based method [95, 122]. In the large Canadian Study of Diet, Lifestyle, and Health cohort study, Agalliu and colleagues (2010) found no association between an OBS and prostate cancer risk with increasing quintiles of OBS [123].

While all three aforementioned studies examined OBS and its association with prostate cancer, our study is different in several aspects. In the two MPC study reports, components of the OBS were dichotomized or divided into three categories using median or tertile cutoffs among controls for FFQ- and biomarker-based analyses, respectively. By contrast, our study divided continuous dietary variables into categories based on their quartile values; whereas Agalliu *et al.* (2010) used quintiles.

Our study examined a more comprehensive list of OBS components than did previous studies, which did not include alpha carotene, zinc, flavonoids, or glucosinolates, all of which may act as antioxidants[10, 17, 150]. In addition, unlike previous studies our OBS also included physical activity and anthropometrics. While strenuous physical activity increases ROS production (at least in the short term), moderate physical activity promotes antioxidant gene expression by activating Nrf2 [151]. Obesity increases ROS production and adipocytokine expression, which induces inflammation, which, in turn, is both a cause and a consequence of oxidative stress [118, 152]. There is a growing body of evidence that suggests that physical activity and obesity are potential modifiable risk factors for prostate cancer [153, 154]. Agalliu *et al.* and Goodman *et al* (2010) studies used all polyunsaturated fats as a pro-oxidant, whereas we examined the constituents of polyunsaturated fatty acids separately. Omega-3 fatty acids were categorized as anti-oxidants as these promote the transcription of anti-oxidant

enzymes, while omega-6 fatty acids were considered pro-oxidants since they serve as precursors to pro-inflammatory eicosanoids [15, 16, 155]. Further, unlike the Agaliu study, our analyses were adjusted for family history of prostate cancer, and in contrast to all of the above-referenced previous studies, we also controlled for statin use.

Perhaps the most important distinguishing feature of this study was the number of ways the OBS was constructed and assessed. None of the previous studies attempted to compare the results of weighted and unweighted OBS in relation to prostate cancer risk. The unweighted model assumes that each component is equally associated with prostate cancer. Although the literature based weights account for the different strengths of association, the range of component weights was quite narrow (0.9-1.2), making the weighted and the unweighted results very similar.

There may be other methods of weighting OBS components. An alternative, perhaps more relevant, approach might be to weight OBS based on the effects of these components on measures of oxidative stress. Such alternative weighting could be based on the associations with biomarkers of oxidation such as isoprostanes, malondialdehyde (MDA), or 8-hydroxy-2' –deoxyguanosine (8-OHdG) [156] or perhaps the degree of disruption of thiol redox circuits as measured by plasma levels glutathione or cysteine redox [9]. Unfortunately, none of these biomarkers are available in the CPS-II study.

In addition to the lack of mechanistic data linking OBS to biochemical measures of oxidative stress, other limitations of the present study also need to be considered. Carcinogenesis is a process that takes years and it may be that OBS at the time of measurement may not be related to cancer risk, and the OBS from an earlier time point would be more relevant. While our OBS is the most comprehensive of those used in the

three previous prostate cancer studies, it included only extrinsic pro- and anti-oxidant factors. Intrinsic factors that affect oxidative stress, such as cellular antioxidant enzymes [10] and DNA damage repair, [157] were not included. Moreover, this cohort included a high proportion of health conscious participants, suggesting there may not have been enough variability in lifestyle to detect an association between OBS and prostate cancer.

A strength of our study was our ability to incorporate a greater number of pro- and antioxidant components into the score than was included in previous studies. The cohort study design used for this analysis is not subject to the recall bias that affects case-control studies. This may partially explain the significant inverse associations found in the case-control, but not the cohort studies. Our study used six different combinations of OBS construction (weighted and unweighted) and assessment (continuous, quartiles, and equal interval categories), providing a comprehensive examination of the association between OBS and prostate cancer.

In summary, despite the study limitations and the apparent inconsistency with mechanistic evidence, our study affirms that when examined at a population level, pro- and antioxidant exposures are unlikely to explain differences in prostate cancer incidence. Prostate cancer is a difficult disease to study, and after much research no modifiable, well-accepted risk factors have been identified. The current study, like many before, underscores the enigmatic nature of this disease.

Table 1a: Oxidative Balance Score Assignment Scheme – Dietary Components

| **OBS Components** | **Weight**[MetaAnalysis] | **Score Assignment** |
|---|---|---|
| Beta Carotene[a] | 0.9[30, 158-172] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Beta Cryptoxanthin[a] | 0.9[158, 162-166, 168-170] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Alpha Carotene[a] | 0.9[158, 160, 162-166, 168-170] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Zinc[a] | 1.1[171, 173-182] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Lutien[a] | 0.9[158, 160, 162-166, 168-170, 183] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Lycopene[a] | 0.9[184] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Vitamin C[a] | 0.9[162, 165, 166, 169-172, 176, 179, 185-190] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Vitamin E[a] | 1.0[162, 166, 169, 170, 172, 174, 176, 187-197] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Ω-3 Fatty Acids[a] | 0.9[198] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Flavanoids | 1.2[199-201] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Glucosinolates | 0.7[202] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Ω -6 Fatty Acids[a] | 0.9[203] | 0 = 4th quartile, 1 = 3rd quartile, 2 = 2nd quartile, 3 = 1st quartile |
| Iron[a] | 1.0[12, 176, 179, 204] | 0 = 4th quartile, 1 = 3rd quartile, 2 = 2nd quartile, 3 = 1st quartile |
| Saturated Fat | 1.0[203] | 0 = 4th quartile, 1 = 3rd quartile, 2 = 2nd quartile, 3 = 1st quartile |
| Selenium, supplements | 1.1[174, 189, 191, 193, 205] | 0 = 0 mcg/day, 1 = 0 to 20 mcg/day, 2 = 20.1 to 50 mcg/day, 3 = 50.1+ mcg/day |

Note: All nutrients were adjusted for energy intake using the residual method

[a]Diet plus supplements

Table 1b: Oxidative Balance Score Assignment Scheme – Lifestyle Components

| **OBS Components** | **Weight**[MetaAnalysis] | **Score Assignment** |
|---|---|---|
| Physical Activity | 0.9[206] | 0 = 1st quartile, 1 = 2nd quartile, 2 = 3rd quartile, 3 = 4th quartile |
| Selenium, supplements | 1.1[174, 189, 191, 193, 205] | 0 = 0 mcg/day, 1 = 0 to 20 mcg/day, 2 = 20.1 to 50 mcg/day, 3 = 50.1+ mcg/day |
| Smoking History | 1.1[207] | 0 = Current smoker<br>1 = Former smoker (>24 years)<br>2 = Former smoker (≤ 24 years)<br>3 = Non-smoker |
| Body Mass[a] | 1.1[208] | 0 = High BMI & High Waist,<br>1 = High BMI & Med. Waist, Med. BMI & High Waist<br>2 = Med BMI & Med waist, Med BMI & Low Waist, Low BMI & Med Waist<br>3 = Low BMI and Low Waist |
| Alcohol | 1.2[209] | 0 =1> drink/day,<br>1 =1 drink/day,<br>2 = 1-6 drinks/week,<br>3 = 0 drinks/day or <1/week |
| NSAID (duration) | 0.9[210] | 0 = Never use,<br>1 =User in only 1 survey,<br>2 =User in 92 &97, or 97&99,<br>3 = User in 92, 97, 99 |

[b]Body mass defined with combined measures of waist circumference (high: >40 in., medium: 37 in. to ≤40 in., low: <37 in.) and BMI (high: 30 to <50 kg/m$^2$, medium: 25 to <30, low: 18.5 to <25

Table 2. Baseline characteristics of CPS-II men by OBS Quartile

| | OBS Quartile 1 | OBS Quartile 2 | OBS Quartile 3 | OBS Quartile 4 |
|---|---|---|---|---|
| Age at 1999 interview, yr, Mean (SD) | 69.84 (5.69) | 70.22 (5.73) | 70.45 (5.45) | 70.45 (5.77) |
| Caucasian N, (%) | 98.15 | 98.06 | 97.74 | 97.57 |
| College education or higher N, (%) | 36.83 | 48.11 | 55.38 | 64.82 |
| Family history of prostate cancer in a first degree relative N,(%) *missing 5680 | 14.06 | 13.56 | 13.77 | 14.59 |
| Current Cholesterol lowering drug use (%) | 23.03 | 27.72 | 30.11 | 32.45 |
| PSA screening (%) | 77.20 | 82.50 | 85.65 | 87.88 |
| Total energy intake, Mean (SD) (kcal/day) | 1,936.11 (604.96) | 1,901.33 (578.14) | 1,892.49 (573.93) | 1,856.77 (551.58) |
| Total calcium intake, Mean (SD) (mg/day) [a] | 752.93 (330.55) | 857.43 (372.79) | 946.68 (408.20) | 1112.69 (473.24) |
| Total vitamin D intake, Mean (SD) (IU/day) [a] | 278.30 (201.74) | 363.89 (227.98) | 427.07 (242.43) | 502.37 (253.17) |
| Total folate intake, Mean (SD) (mcg/day) [a] | 439.36 (203.33) | 567.95 (239.81) | 661.92 (260.78) | 794.47 (280.46) |
| Finasteride use (%) | 2.15 | 2.55 | 2.73 | 3.20 |

Note: All nutrients adjusted for total energy intake.
[a]Diet plus supplements

Table 3. Associations of Oxidative Balance Score comprised of equally weighted OBS components with incident prostate cancer among study participants in the CPS-II Nutrition Cohort (1999-2007)

| **OBS Categories** | **#Cases/Non-cases** | **Age Adjusted HR[a] (95% CI)** | **Multivariable HR[a] (95% CI)** |
|---|---|---|---|
| | | | |
| *OBS-Continuous* | 3386/43325 | 1.008(1.003-1.012) | 1.008 (1.002-1.013) |
| | | | |
| *OBS- Quartiles* | | | |
| 8-25 | 810/11570 | 1.00 | 1.00 |
| 26-31 | 919/11678 | 1.12 (1.02 - 1.23) | 1.12 (1.01-1.24) |
| 32-36 | 954/9442 | 1.12 (1.02 - 1.24) | 1.12 (1.01-1.26) |
| 36-55 | 903/10635 | 1.18 (1.08 - 1.30) | 1.17 (1.04-1.32) |
| $P_{trend}$ [b] | | <0.01 | 0.01 |
| | | | |
| *OBS- Six categories* | | | |
| 8-16 | 92/1303 | 1.00 | 1.00 |
| 17-24 | 595/8586 | 0.97 (0.78-1.21) | 0.98 (0.77-1.23) |
| 25-32 | 1189/15430 | 1.08 (0.87-1.33) | 1.07 (0.85-1.34) |
| 33-40 | 1073/12955 | 1.14 (0.92-1.41) | 1.15 (0.91-1.45) |
| 41-48 | 403/4703 | 1.17 (0.943-1.46) | 1.13 (0.88-1.46) |
| 49-55 | 34/348 | 1.36 (0.91-2.01) | 1.39 (0.90-2.15) |
| $P_{trend}$ [b] | | <0.01 | <0.01 |

[a] Adjusted for age, race, sex, education, family history of prostate cancer in a first degree relative, prostate cancer screening, NSAID/aspirin use, total calcium intake, total vitamin D intake, total energy intake, cholesterol lowering drug use, finasteride use

[b] $P_{trend}$ assessed using category medians

Table 4. Associations of Oxidative Balance Score comprised of OBS components weighted according to literature reviews with incident prostate cancer among study participants in the CPS-II Nutrition Cohort (1999-2007)

| **OBS Categories** | **#Cases/Non-cases** | **Age Adjusted HR[a] (95% CI)** | **Multivariable HR[a] (95% CI)** |
|---|---|---|---|
| *OBS-Continuous* | 3386/43325 | 1.007(1.003-1.011) | 1.007 (1.002-1.012) |
| | | | |
| *OBS- Quartiles* | | | |
| 8.1-27.0 | 758/10831 | 1.00 | 1.00 |
| 27.1-33.2 | 843/10830 | 1.11 (1.00-1.22) | 1.11 (0.99-1.23) |
| 33.3-39.4 | 875/10832 | 1.14 (1.03-1.25) | 1.14 (1.03-1.28) |
| 39.5-60.0 | 910/10832 | 1.17 (1.06-1.29) | 1.15 (1.03-1.30) |
| $P_{trend}$ [b] | | <0.01 | <0.01 |
| | | | |
| *OBS- Six categories* | | | |
| 8.1-16.9 | 65/956 | 1.00 | 1.00 |
| 16.9-25.7 | 536/7924 | 1.00 (0.77-1.29) | 1.03 (0.78-1.36) |
| 25.8-34.5 | 1188/15141 | 1.15 (0.90-1.48) | 1.19 (0.91-1.55) |
| 34.6-43.3 | 1108/13598 | 1.18 (0.92-1.51) | 1.21 (0.92-1.60) |
| 43.3-52.1 | 452/5318 | 1.22 (0.94-1.58) | 1.22 (0.92-1.66) |
| 52.2-60.0 | 37/388 | 1.39 (0.93-2.08) | 1.41 (0.90-2.21) |
| $P_{trend}$ [b] | | <0.01 | 0.01 |

[a] Adjusted for age, race, sex, education, family history of prostate cancer in a first degree relative, prostate cancer screening, NSAID/aspirin use, total calcium intake, total vitamin D intake, total energy intake, physical activity, cholesterol lowering drug use, finasteride use

[b] $P_{trend}$ assessed using category medians

Table 5. Associations of Oxidative Balance Score comprised of equally weighted OBS components with incident aggressive and non-aggressive prostate cancer among study participants in the CPS-II Nutrition Cohort (1999-2007)

| | **Aggressive** | | **Non-Aggressive** | |
|---|---|---|---|---|
| **OBS Categories** | **Cases** | **Multivariate HR[a] (95% CI)** | **Cases** | **Multivariable HR[a] (95% CI)** |
| *OBS-Continuous* | 657/43325 | 1.01 (0.99-1.02) | 2729/43325 | 1.001 (0.995-1.007) |
| | | | | |
| *OBS- Quartiles* | | | | |
| 8-25 | 169/11570 | 1.00 | 649/11570 | 1.00 |
| 26-31 | 170/11678 | 1.06 (0.84-1.35) | 744/11678 | 1.06 (0.94-1.19) |
| 32-36 | 144/9442 | 0.93 (0.72-1.20) | 602/9442 | 1.00 (0.88-1.13) |
| 36-55 | 174/10635 | 1.14 (0.87-1.50) | 734/10635 | 1.02 (1.02-1.04) |
| $P_{trend}$ [b] | | 0.47 | | 0.86 |
| | | | | |
| *OBS- Five categories* | | | | |
| 8-16 | 21/1303 | 1.00 | 71/1303 | 1.00 |
| 17-26 | 169/12116 | 1.00 (0.62-1.61) | 686/12116 | 0.79 (0.61-1.03) |
| 27-36 | 293/19271 | 0.98 (0.61-1.58) | 1238/19271 | 0.84 (0.64-1.09) |
| 37-46 | 164/9801 | 1.12 (0.67-1.86) | 674/9801 | 0.82 (0.62-1.09) |
| 47-55 | 10/834 | 1.59 (0.57-4.40) | 60/834 | 0.91 (0.62-1.35) |
| $P_{trend}$ [b] | | 0.39 | | 0.77 |

[a] Adjusted for age, race, sex, education, family history of prostate cancer in a first degree relative, prostate cancer screening, NSAID/aspirin use, total calcium intake, total vitamin D intake, total energy intake, physical activity, cholesterol lowering drug use, finasteride use

[b] $P_{trend}$ assessed using category medians

aggressive disease = AJCC stage III/IV or Gleason score 8-10

**Oxidative Balance Score and Oxidative Stress Biomarkers in a Study of Whites, African Americans, and African Immigrants**

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**Abstract**

**Background** Oxidative stress is defined as an imbalance between pro-oxidants and anti-oxidants, resulting in macromolecular damage and disruption of redox control. While randomized clinical trials of selected antioxidants did not show consistent effects on markers of oxidative stress, previous studies have found that combining pro- and anti-oxidant exposures into a single oxidative balance score (OBS) is more strongly associated with health outcomes than any individual exposure.

**Methods:** We investigated the association between OBS and three biomarkers of oxidative stress F2-isoprostanes (FIP), fluorescent oxidative products (FOP) and mitochondrial DNA (mtDNA) count, among 321 participants in the cross sectional Study of Race, Stress, and Hypertension (SRSH). Thirteen dietary and lifestyle components derived from biomarkers and questionnaires were incorporated into the OBS and the resulting scores were then expressed in tertiles. Multivariable odds ratios for high biomarker levels were calculated from logistic regression models.

**Results:** Odds ratios (95% confidence intervals) comparing the highest to lowest OBS tertile for FIP and FOP were OR=0.10 (0.04 – 0.26; p-trend<0.05), and OR=3.01 (1.51 – 6.04; p-trend <0.05), respectively. The association with MtDNA was not significantly different from the null. The three markers of oxidative stress were not correlated.

**Conclusions:** While the inverse association between OBS and FIP is consistent with the stated hypothesis, the results for FOP were in opposite direction. Our findings demonstrate that these biomarkers may reflect different biological processes.

## Introduction

Oxidative stress is defined as an imbalance between pro-oxidants and anti-oxidants, resulting in macromolecular damage and disruption of redox signaling and control [3]. Pro-oxidants are factors that help generate reactive oxygen species (ROS), which in turn interact with macromolecules and cause protein oxidation, lipid peroxidation, and DNA damage [117]. By contrast, anti-oxidant factors act by overturning the effects of ROS thereby reducing oxidative stress [4].

Oxidative stress can be affected by intrinsic factors, such as oxidative phosphorylation [73], cellular antioxidant enzyme activity [4], and macromolecular damage [157]. In addition, various extrinsic and presumably modifiable factors such as diet and medications also act as pro- and anti-oxidants. Although experimental biology evidence has demonstrated that antioxidants can slow disease pathogenesis [102, 104, 109], clinical trials of antioxidant supplementation have not shown a clear benefit [211, 212].

Studies of diet and chronic diseases have demonstrated that nutrients do not act in isolation and a combination of factors can show a stronger association with disease risk than any single nutrient considered individually [92-94]. By analogy it appears possible that a combination of oxidative stress-related factors may be more strongly associated health outcomes than any one pro-oxidant and anti-oxidant taken individually.

To test this hypothesis we, and others, proposed using an Oxidative Balance Score (OBS) that combines oxidative stress-related exposures based on the summed intake of various pro- and anti- oxidants, with a higher score indicating decreased oxidative stress. Previous studies found that a higher OBS was associated with decreased risk of colorectal

adenoma [94, 96], colorectal cancer [97], and mortality [98]. By contrast, OBS was not found to be related to prostate cancer [123], indicating that the role of oxidative stress in human pathophysiology may be organ-, or disease-specific.

Many known pro-and anti-oxidants have been shown to act through a variety of mechanisms that may be independent of oxidative stress. Experimental biology evidence demonstrates that lycopene can exert antiproliferative effects on cells [135]. Other carotenoids have been found to regulate gene expression [213] and immune response [214]. These examples illustrate that the associations between OBS and health outcomes may or may not be attributable to changes in oxidative stress. To resolve this uncertainty it is important to assess the relation of OBS to blood levels of various biomarkers of oxidative stress. Several of such biomarkers have been used in population studies.

$F_2$-Isoprostanes (FIP) are products of arachidonic acid peroxidation and a biomarker of oxidative stress. FIP are produced esterified on phospholipids and are then cleaved and released into circulation in free form [65]. Although FIP can be measured in plasma and urine, plasma measurements are preferred because oxidative stress biomarkers in urine are influenced by renal metabolism [66, 67]. High levels of FIP have been associated with cardiovascular disease [215] and Alzheimer's disease [216].

The use of florescent oxidative product (FOP) as a measure of oxidative stress began in the food industry, but is now being proposed for population based human studies [68]. Fluorescent conjugated Schiff bases can be formed when malonaldehyde, a byproduct of lipid peroxidation, reacts with amino groups on proteins, lipids, and DNA [217]. Population based studies have shown that FOP is associated with hypertension [69] and is an independent predictor of coronary heart disease [68].

Studies have demonstrated that mitochondrial DNA (mtDNA) copy number is increased in response to oxidative stress [75-77]. Mitochondria are organelles which contain their own circular genome lacking introns. Their primary function is to generate adenine triphosphate through cellular respiration, a process that also produces ROS, which can cause oxidative DNA damage [73]. Unlike nuclear DNA, which is protected by elaborate repair mechanisms [74], mtDNA responds to oxidative damage by increasing the number of its copies. High levels of mtDNA copy number have been associated with various cancers [76, 218].

The use of each biomarker has distinct advantages and disadvantages. At present, FIP are considered the "gold-standard" biomarker of oxidative stress, but an accurate and reliable analysis of FIP requires careful handling of samples to prevent in vitro oxidation [70]. Wu et al. found FOPs to be a stable measure, with levels from blood specimens remaining constant over 36 hours, whereas FIP in the same samples increased at each time measured [70]. The main disadvantage of FOPs and FIP is that both of these biomarkers represent short term oxidative stress levels [219]. By contrast mtDNA copy number is a stable biomarker that is presumed to indicate long term cumulative oxidative stress-induced damage.

The goals of the present study are to examine the associations between OBS and three biomarkers – FIP, FOP, and mtDNA – each thought to that reflect different aspects of oxidative stress; and to assess how these biomarkers are related to each other. This analysis is based on the cross-sectional Study of Race, Stress, and Hypertension (SRSH), which provided data and samples from a racially and ethnically diverse group of men and women residing in the State of Georgia.

## Methods

*Study Participants*

This analysis was based on the data from the cross sectional Study of Race, Stress, and Hypertension (SRSH), which was designed to assess racial disparities in dietary, lifestyle, and psychosocial exposures in relation to blood pressure. This study includes participants from three groups – US whites, African Americans, and Native Africans. The whites and African Americans were selected among 800 participants in a previously completed study of white and black adults residing in the State of Georgia. This was a feasibility phase of the Georgia Cohort Study (GCS). The Native Africans were recruited *de novo* using previously established ties with the Atlanta churches that include large proportions of West African immigrants. The sample of GCS participants was selected after the recruitment of Native Africans was complete and frequency matched to Native African participants on age and sex. To be included in the study, participants had to be between 25-74 years of age, identify as non-Hispanic Caucasian or African American (for those recruited from GCS), or self-identify as Native African (for those recruited *de novo*), and be permanent Georgia residents. Subjects were excluded if they did not give informed consent.

The current analysis excluded participants for whom no biomarker measurements of interest were recorded (n=14). Of the remaining 321 subjects, the number of participants with measurements for each biomarker varied as follows: $F_2$-IP (n=227), FOP (n=272), and mtDNA copy number (n=182).

*Data and blood sample collection procedures*

Recruitment and data collection occurred after church services for Native African participants and at community events for white and African American participants. Following informed consent, blood was drawn by a phlebotomist into five 10mL vacutainer tubes (2 sodium heparin tubes, 1 EDTA tube, and 2 red top tubes) and immediately placed on ice. Plasma, serum, and buffy coat were separated within 4-8 hours of sample collection by refrigerated (4°C) centrifuge, aliquoted, and frozen at 80°C. The aliquots were then then shipped overnight on dry ice for analysis to the Molecular Epidemiology and Biomarker Research Laboratory (MEBRL) at the University of Minnesota.

The study specific questionnaire was administered to obtain data, medical history, and lifestyle information. Physical activity items were obtained from the validated Paffenberger questionnaire [220]. Other data elements were obtained using instruments adapted from previous studies [221].

*Laboratory Analysis*

Plasma lycopene, α-carotene, β-carotene, β-cryptoxanthin, zeaxanthin, lutein, α – tocopherol, and γ-tocopherol were measured by a high performance liquid chromatography (HPLC) assay as originally described by Bieri *et al.* [222] with several modifications for the analysis of tocopherols, and using calibration methods described by Craft *et al.* [223] The method and its modifications have been described previously by Gross *et al* .[224] Serum ferritin was measured by an antibody-based method. An

antibody specific for ferritin and assay procedures was obtained from Roche Diagnostics. The analysis was performed on a Roche 911 analyzer.

Plasma free FIP were measured by gas chromatography-mass spectrometry (GCMS) as described by Gross [225]. This method, considered the gold standard for the measurement of FIP, measures a distinct set of FIP isomers. The FIP were extracted from the participant's plasma sample using deuterium (4)-labeled 8-iso-prostaglandin $F_2$ alpha as an internal standard. Unlabeled, purified FIP was used as a calibration standard.

The method of measuring FOP was modified from Shimasaki [226]. The procedures have been described in detail previously [70]. Briefly, plasma was extracted with ethanol-ether (3/1, v/v) and mixed on a vortex mixer. The mixed solution was centrifuged for 10 minutes at 3000 rpm, and 1 mL of supernatant was added to cuvettes for spectrofluorometric readings. The measurement is expressed as a relative fluorescence intensity units per milliliter of plasma at 360/430 nm wavelength (excitation/emission) by a spectrofluorometer [70]. The wavelength we used is within the spectrum, but not the same as that used by Wu et al [70]. All samples were calculated against 1 ppm fluorescent reference standard quinine in 0.1 $NH_2SO_4$.

The details of the procedure to measure mtDNA copy number have been described elsewhere [227]. In brief, two pairs of primers were used in the two steps of relative quantification for mtDNA content: one for the amplification of the MT-ND1 gene in mtDNA, and another for the amplification of the single-copy nuclear gene human globulin (HGB). In the first step, the ratio of mtDNA copy number to HGB copy number, which was also referred as mtDNA index, was determined for each sample from standard curves. This ratio was proportional to the mtDNA copy number in each cell and,

for each sample, was normalized to a calibrator DNA. All samples were assayed using 96-well plate with an Applied Biosystems StepOne Plus System. The PCRs for ND-1 and HGB were performed on separate 96-well plates with the same samples in the same well positions to avoid possible position effects. A standard curve of a diluted reference DNA, one negative control, and one calibrator DNA were included in each run. For each standard curve, one reference DNA sample was serially diluted 1:2 to produce a seven-point standard curve between 0.3125 and 20 ng of DNA [227].

Using two different controls, the coefficients of variation (CVs) ranged from 10.29-12.44% for zeaxanthin, 3.27-5.81% for β-cryptoxanthin, 26.38-31.94% for lycopene; 1.11-2.98% for α-carotene, 4.78-9.44% for β-carotene, 0.64-0.76% for α – tocopherol, 0.12-0.16% for γ-tocopherol, 5.4-5.6% for FOP, and 11.9-12.3% for FIP. Using one control, the CVs were 7.2% for ferritin and 5.9% for MtDNA copy number.

*OBS components and their assessment*

The OBS is comprised of 13 components that were selected based on *a priori* knowledge about their relation to oxidative stress (Table 1). The components were derived from plasma micronutrient measurements or questionnaire data. Continuous variables were divided into categories based on tertile values. Participants who had low exposure to a particular dietary anti-oxidant ($1^{st}$ tertile) were assigned zero points, and those whose exposure to the same dietary anti-oxidant is medium ($2^{nd}$ tertile) or high ($3^{rd}$ tertile), received one or two points, respectively. Anti-oxidant OBS components expressed as continuous variables included plasma lycopene, α-carotene, β-carotene, β-cryptoxanthin, zeaxanthin, lutein, α –tocopherol, γ-tocopherol, and physical activity. For serum ferritin,

the only continuous variable reflecting pro-oxidant exposure, two points were given for low exposure (1st tertile), one point for medium exposure (2nd tertile), and zero points for high exposure (3rd tertile). All missing values for continuous components received one point.

Categorical variables were assigned scores from 0 to 2 to maintain consistency with the continuous OBS components. Smoking and alcohol use were categorized as current non-smoker/non-drinker (2 points), missing (1 point), or current smoker/drinker (zero points). For NSAIDs and aspirin, zero points were assigned to participants who reported never using any of these medications, one point to those who did not report usage, and two points to those who reported regular (at least once a week) use. The points assigned to each component were summed to calculate the overall OBS.

*Statistical Analysis*

In the present analysis OBS was divided into tertiles, and blood levels of oxidative stress biomarkers were dichotomized as high versus low using median values as the cutoffs. High FOP, FIP, and mtDNA were defined as $\geq$0.04 (average standard reference adjusted units), $\geq$ 48.37 (pg/mL), and $\geq$3.05 (relative copy number) respectively. Covariates in this analysis were age, sex, BMI, and racial/ethnic group (defined as white, African American, or Native African).

Participants with high and low levels of FIP, FOP and mtDNA were compared with respect to various OBS components demographic characteristics using chi-square tests for categorical variables and t-tests for continuous variables. Correlation

coefficients were calculated for oxidative stress biomarkers and for plasma OBS components.

Multivariable linear regression models were constructed to examine the association between OBS and each biomarker. The results of linear regression models were expressed as regression coefficients and their corresponding 95% confidence intervals (CIs) adjusted for age, sex, BMI, and race/origin. The biomarker measurements were not normally distributed, and for this reason they were log transformed when used in linear regression.

Multivariable logistic regression models were used to examine the association between OBS and high biomarker levels after controlling for age, sex, BMI, and race/origin confounders. The results of logistic regression models were expressed as adjusted odds ratios (ORs) and corresponding 95% confidence intervals (CIs). The potential confounders were selected based on evidence in the literature and other *a priori* considerations. All models were examined for collinearity among independent variables and for interaction between the OBS and each covariate. Sensitivity analyses to examine the impact of individual OBS components were conducted by removing each component from the score and controlling for it as a covariate. Additional sensitivity analyses evaluated the impact of excluding missing values. Adjusted ORs and 95% CIs were also calculated for individual OBS components. All analyses were conducted using SAS statistical software version 9.2 (SAS institute, Cary North Carolina).

## Results

The non-dietary characteristics of SRSH participants are shown by high and low biomarker levels in Table 2. In the high FOP category, there was a higher proportion of Native Africans. Participants with higher FIP levels had a higher BMI, and had a greater proportion of Caucasians, African Americans, and non-drinkers. In the high mtDNA copy number category, there was a lower proportion of males, and a higher proportion of Native Africans.

Table 3 examines dietary characteristics of SRSH participants by high and low biomarker levels. Plasma levels of zeaxanthin, cryptoxanthin, lycopene, α-carotene, and β-carotene were greater in the high FOP group compared to the low FOP group. By contrast, plasma levels of these nutrients were lower in the high FIP group, compared to the low FIP group. Serum ferritin levels were greater in all three high biomarker groups, compared to the low biomarker groups.

Table 4 examines the association between OBS and oxidative stress biomarkers. For FOP, the adjusted ORs (95% CIs) comparing the second and third tertiles to the first OBS tertile were 1.85 (0.96 **–** 3.59**)** and 3.01 **(**1.51 **–** 6.04**)** respectively (p-trend <0.05). The same analysis for FIP demonstrated a statistically significant inverse trend (p-trend <0.01) with corresponding ORs of 0.30 (95% CI: 0.13 – 0.70) for the second tertile and 0.10 (95% CI: 0.04 – 0.26) for the third tertile. None of the tertile-specific ORs for high mtDNA copy number were statistically significantly different from the null and there was no evidence of a dose-response (p-trend=0.14).

The two-way correlations involving individual dietary OBS components and biomarkers of oxidative stress are presented in Table 5. The strongest positive correlation

was observed between alpha carotene and beta carotene (r=0.88) and both these carotenoids were negatively correlated with gamma-tocopherol (r=-0.35 and -0.30, respectively). There was no evidence of a positive correlation for biomarkers of oxidative stress with Pearson coefficients ranging from -0.17 to 0.00.

Table 6 assesses the associations between individual OBS components and each oxidative stress biomarker of interest. For a number of individual components, the associations with FOP and FIP were in the opposite directions. The differences were particularly pronounced for lycopene, α-carotene, and β-carotene.

Tables 7-8 present the results of sensitivity analyses. In Table 7 the associations between high levels of biomarkers and the 13-component OBS (examined as a continuous variable) are compared to alternative models, where each component is removed from the score and included in the model as a covariate. For all alternative models, removing an OBS component resulted in few meaningful differences in ORs compared to the original model. The OR estimates in alternative models were within 12% of the OR from the original model. In a separate sensitivity analysis that excluded participants with missing OBS components, the results for FOP and FIP were similar to the original findings, but the association changed for mtDNA copy number (Table 8).

Table 9 examines the association between OBS and each of the biomarkers using linear regression. The results are similar to the findings from logistic regression for all biomarkers. The regression coefficients for FIP and FOP were statistically significant ($p<0.01$), but the regression coefficient for MtDNA copy number bordered on statistical significance.

**Discussion**

In this cross sectional study, we examined the association between OBS and biomarkers of oxidative stress (FIP, FOP, and mtDNA copy number), with the hypothesis that a high OBS would be inversely associated with all biomarker levels. This was confirmed for FIP, but not for mtDNA copy number and the association was opposite of the hypothesized direction for FOP.

Other studies have examined the association between OBS and these biomarkers in different populations. *Dash et al.* observed a significant inverse association between questionnaire-derived OBS an FIP in a case-control study of colorectal adenoma [228]. In the same population, *Kong et al.* performed a separate analysis using an OBS comprised of components measured by both FFQ and blood markers [121]. As in our study, Kong and colleagues found that relative to the lowest interval category of OBS, the highest interval scores had a significant inverse association with FIP but were directly associated with FOP [121].

FIP biomarker is considered the gold-standard measure of oxidative stress in population based studies [229]. Results from several placebo controlled randomized clinical trials (RCTs) of antioxidant supplementation on FIP have been inconsistent [78-80]. However, when nutrients were examined in combination as a dietary score (similarly to OBS), stronger associations with FIP have been observed [230]. In the Coronary Artery Risk Development in Young Adults (CARDIA) study, a diet quality score was determined by assigning higher points to frequent consumption of foods beneficial to health, and lower points to frequent consumption of foods believed to be

detrimental to health (all determined *a priori*) [230]. A significant inverse association was observed between dietary score and plasma FIP [230].

Higher OBS in our study was associated with higher FOP. This observation is unexpected and appears counterintuitive; however it is in agreement with the results previously reported by *Kong et al.* [121]. Moreover, our analysis found FOP to be uncorrelated with FIP. In addition, the associations between many individual antioxidants and FIP were in the opposite direction compared to the corresponding association observed for FOP. Since FOP is a non-specific measure of global oxidative stress, it may also be comprised of non-oxidative products [69]. Considering that FOP has been previously associated with coronary heart disease, hypertension, and adenoma [68, 69, 121] , future studies should be conducted to understand the specific components of FOP and their role in human pathophysiology.

Our data indicated no statistically significant association between OBS and mtDNA copy number. We also observed that mtDNA copy number was not significantly correlated with other biomarkers of oxidative stress. *Liu et al.* observed mtDNA copy number to be correlated with thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation [231]. In an *in vitro* study, when human lung fibroblasts were exposed to oxidative stress, mtDNA copy number was increased [75]. However, as shown in mouse models oxidative stress may not be solely responsible for increasing mtDNA copy number [232].

One of the strengths of this study is the diverse population. The design of this study dictated recruitment of similar numbers of African American, Native African, and

white participants. This allowed us to better examine possible interaction between OBS and race/ethnicity, although none was observed in this analysis. We also used plasma measures of dietary OBS components, which most accurately represent current intake and availability of nutrient for metabolism without the recall bias which affects FFQ-derived measures [233].

One of the major limitations of this study was the substantial proportion of participants with missing information (shown in Table 2 and Table 3). In the score, participants with missing data for specific OBS components were assigned one point, which may result in misclassification. In a sensitivity analysis conducted to examine the impact of missing information the results were similar for FIP and FOP, but the direction of the association was reversed for mtDNA copy number. Thus the results for FIP and FOP appear to be reasonably robust, but the interpretation of the findings for mtDNA copies is difficult at this time

In conclusion, we found that the three biomarkers thought to reflect oxidative stress are not inter-correlated and thus are unlikely to measure the same or similar biological processes. Moreover higher OBS, a composite measure that reflects predominantly antioxidant exposure was inversely associated with FIP, but positively associated with FOP. These results, which confirm the findings from a previous study [121] present a paradox that cannot be readily explained at this time.

Table 1. Oxidative balance score (OBS) assignment scheme with missing

| **OBS components** | **Score Assignment Scheme** |
|---|---|
| Zeaxanthin | 0 = low (1st tertile), 1 = medium (2nd tertile) & missing; 2 = high (3rd tertile) |
| Cryptoxanthin | 0 = low (1st tertile), 1 = medium (2nd tertile) & missing; 2 = high (3rd tertile) |
| Lycopene | 0 = low (1st tertile), 1 = medium (2nd tertile) & missing; 2 = high (3rd tertile) |
| Alpha carotene | 0 = low (1st tertile), 1 = medium (2nd tertile) & missing; 2 = high (3rd tertile) |
| Beta carotene | 0 = low (1st tertile), 1 = medium (2nd tertile) & missing; 2 = high (3rd tertile) |
| Alpha tocopherol | 0 = low (1st tertile), 1 = medium (2nd tertile) & missing; 2 = high (3rd tertile) |
| Gamma tocopherol | 0 = low (1st tertile), 1 = medium (2nd tertile) & missing; 2 = high (3rd tertile) |
| Ferritin | 0 = high (3rd tertile), 1 = medium (2nd tertile) & missing, 2 = low (1st tertile) |
| Physical Activity | 0 = high (3rd tertile), 1 = medium (2nd tertile) & missing, 2 = low (1st tertile) |
| Smoking history | 0 = current smoker, 1 = missing, 2 = never smoker |
| Aspirin | 0 = never, 1 = missing, 2 = regular user |
| Other NSAID | 0 = never, 1 = missing, 2 = regular user |
| Alcohol | 0 = current drinker, 1 = missing, 2 = never drinker |

Abbreviations: NSAID = non-steroidal anti-inflammatory drug (not including aspirin); PUFA = polyunsaturated fatty acids

Table 2: Selected non-dietary characteristics of SRSH participants

| | FOP[a] | | FIP[b] | | MtDNA[c] | |
|---|---|---|---|---|---|---|
| | **Low (n=132)** | **High (n=140)** | **Low (n=113)** | **High (n=114)** | **Low (n=92)** | **High (n=90)** |
| **Age yr, Mean (SD)** | 45.5 (11.7) | 47.3 (12.2) | 45.2 (12.7) | 48.2 (12.5) | 47.7 (12.2) | 45.2 (11.3) |
| **Missing (%)** | 3 (2.3) | 2 (1.4) | 0 (0.0) | 5 (4.4) | 5 (5.4) | 5 (5.6) |
| **Male, (%)** | 48 (36.4) | 53 (37.9) | 43 (38.1) | 42 (36.8) | 54 (58.7) | 35 (38.9)[d] |
| **Missing (%)** | 5 (3.8) | 5 (3.6) | 5 (4.4) | 5 (4.4) | 6 (6.5) | 7 (7.8) |
| **Race** | | | | | | |
| **Caucasian (%)** | 65 (49.3) | 50 (35.7)[d] | 13 (11.5) | 71 (62.3)[d] | 15 (16.3) | 14 (15.6)[d] |
| **African American (%)** | 44 (33.3) | 32 (22.9) | 24 (21.3) | 32 (28.1) | 38 (41.3) | 20 (22.2) |
| **Native African (%)** | 21 (15.9) | 58 (41.4) | 72 (63.7) | 7 (6.1) | 37 (40.2) | 55 (61.1) |
| **Missing (%)** | 2 (1.5) | 0 (0.0) | 4 (3.5) | 4 (3.5) | 2 (2.2) | 1 (1.1) |
| **BMI (kg/m$^2$), Mean (SD)** | 30.4 (7.2) | 29.8 (5.5) | 28.6 (5.4) | 31.3 (7.0)[d] | 31.1 (6.9) | 30.8 (6.5) |
| **Missing (%)** | 11 (8.33) | 26 (18.6) | 24 (21.2) | 10 (8.8) | 15 (16.3) | 23 (25.6) |
| **Physical Activity, MET-mins/week** | 3,045.0 (2943.5) | 3,665.2 (3,798.2) | 3,889.8 (3,508.6) | 2,979.0 (3,013.4) | 3,149.0 (3,178.1) | 4,019.8 (3,582.1) |
| **Missing (%)** | 48 (36.4) | 40 (28.6) | 27 (23.9) | 44 (38.6) | 42 (45.7) | 26 (28.9) |
| **Smoking history** | | | | | | |
| **Current (%)** | 5 (3.8) | 5 (3.6) | 2 (1.8) | 7 (6.1) | 2 (2.2) | 3 (3.4) |
| **Non-smoker (%)** | 83 (62.9) | 108 (77.1) | 93 (82.3) | 68 (59.7) | 57 (62.0) | 65 (72.2) |
| **Missing (%)** | 44 (33.3) | 27 (19.9) | 18 (15.9) | 39 (34.2) | 33 (35.8) | 22 (24.4) |
| **Aspirin** | | | | | | |
| **Current (%)** | 17 (12.9) | 30 (21.4) | 14 (12.4) | 20 (17.6) | 15 (16.3) | 14 (15.6) |
| **Never (%)** | 72 (54.5) | 84 (60.0) | 82 (72.6) | 56 (49.1) | 45 (48.9) | 57 (63.3) |
| **Missing (%)** | 43 (32.6) | 26 (18.6) | 17 (15.0) | 38 (33.3) | 32 (34.8) | 19 (21.1) |
| **Other NSAID** | | | | | | |
| **Current (%)** | 28 (21.2) | 25 (17.8) | 17 (15.0) | 25 (21.9)[d] | 15 (16.3) | 12 (13.3) |
| **Never (%)** | 61 (46.2) | 89 (63.6) | 81 (71.7) | 51 (44.7) | 45 (48.9) | 59 (65.6) |
| **Missing (%)** | 43 (32.6) | 26 (18.6) | 15 (13.3) | 38 (33.3) | 32 (34.8) | 19 (21.1) |
| **Alcohol** | | | | | | |
| **Current (%)** | 41 (31.0) | 49 (35.0) | 31 (27.4) | 43 (37.7)[d] | 15 (16.3) | 25 (27.8) |
| **Non-drinker (%)** | 45 (34.1) | 62 (44.3) | 63 (55.8) | 31 (27.2) | 45 (48.9) | 41 (45.6) |
| **Missing (%)** | 46 (34.9) | 29 (20.7) | 19 (16.8) | 40 (35.1) | 32 (34.8) | 24 (26.7) |

[a] FOP cutoffs: Low FOP, <0.04 average standard reference adjusted (n=132); High FOP, ≥0.04 average standard reference adjusted (n=140)
[b] FIP cutoffs: Low FIP, < 48.37 pg/mL (n=113); High FIP, ≥ 48.37 pg/mL (n=114)
[c] MtDNA count: Low MtDNA count, <3.05 relative copy number (n=92); High MtDNA count, ≥3.05 relative copy number (n=90)
[d] P<0.05 based on t-test for continuous variables and chi-square test for categorical variables

Table 3: Selected dietary characteristics of SRSH participants

| | FOP | | FIP | | MtDNA | |
|---|---|---|---|---|---|---|
| | **Low (n=132)** | **High (n=140)** | **Low (n=113)** | **High (n=114)** | **Low (n=92)** | **High (n=90)** |
| **Zeaxanthin** | 17.66 (10.31) | 22.90 (10.30)[d] | 22.87 (9.86) | 19.88 (11.42)[d] | 23.70 (9.94) | 22.06 (10.15) |
| **Missing (%)** | 14 (10.6) | 11 (7.9) | 10 (8.9) | 7 (6.1) | 19 (20.7) | 8 (8.9) |
| **Cryptoxanthin** | 6.54 (5.13) | 7.36 (4.58) | 9.96 (1.35) | 5.79 (4.01)[d] | 9.85 (16.06) | 7.05 (4.39) |
| **Missing (%)** | 14 (10.6) | 11 (7.9) | 10 (8.9) | 7 (6.1) | 19 (20.7) | 8 (8.9) |
| **Lycopene** | 32.58 (17.27) | 54.92 (26.09)[d] | 57.77 (27.79) | 36.18 (17.68)[d] | 50.95 (26.54) | 56.87 (27.11) |
| **Missing (%)** | 14 (10.6) | 11 (7.9) | 10 (8.9) | 7 (6.1) | 19 (20.7) | 8 (8.9) |
| **Alpha carotene** | 6.02 (10.65) | 13.06 (16.50)[d] | 18.18 (17.91) | 4.05 (7.16)[d] | 13.75 (14.43) | 18.90 (19.60) |
| **Missing (%)** | 14 (10.6) | 11 (7.9) | 10 (8.9) | 7 (6.1) | 19 (20.7) | 8 (8.9) |
| **Beta carotene** | 13.74 (14.42) | 27.50 (26.61)[d] | 33.90 (28.24) | 12.74 (11.69)[d] | 25.97 (19.64) | 31.49 (30.17) |
| **Missing (%)** | 14 (10.6) | 11 (7.9) | 10 (8.9) | 7 (6.1) | 19 (20.7) | 8 (8.9) |
| **Alpha tocopherol** | 0.91 (0.30) | 1.00 (0.27)[d] | 0.94 (0.29) | 1.00 (0.29) | 0.94 (0.26) | 0.94 (0.28) |
| **Missing (%)** | 14 (10.6) | 11 (7.9) | 10 (8.9) | 7 (6.1) | 19 (20.7) | 8 (8.9) |
| **Gamma tocopherol** | 0.21 (0.09) | 0.19 (0.08)[d] | 0.16 (0.06) | 0.32 (0.10)[d] | 0.19 (0.09) | 0.17 (0.08) |
| **Missing (%)** | 14 (10.6) | 11 (7.9) | 10 (8.9) | 7 (6.1) | 19 (20.7) | 8 (8.9) |
| **Ferritin** | 108.80 (105.70) | 138.20 (309.80) | 127.50 (343.90) | 133.4 0(125.80)[d] | 143.00 (111.20) | 158.40 (379.50) |
| **Missing (%)** | 10 (7.6) | 6 (4.3) | 12 (10.6) | 6 (5.3) | 12 (13.0) | 7 (7.8) |

[a] FOP cutoffs: Low FOP, <0.04 average standard reference adjusted (n=132); High FOP, ≥0.04 average standard reference adjusted (n=140)
[b] FIP cutoffs: Low FIP, < 48.37 pg/mL (n=113); High FIP, ≥ 48.37 pg/mL (n=114)
[c]MtDNA count: Low MtDNA count, <3.05 relative copy number (n=92); High MtDNA count, ≥3.05 relative copy number (n=90)
[d]P<0.05 based on t-test for continuous variables and chi-square test for categorical variables
Abbreviations: FOP =Florescent Oxidative Product; FIP=F2-Isoprostane; MtDNA =Mitochondrial DNA count

Table 4. Association between OBS and biomarkers of oxidative stress (based on OBS table 1)

| Biomarker | Low Biomarker Levels | High Biomarker Levels | OR (95% CI)[d] | p-trend[e] |
|---|---|---|---|---|
| **FOP** | | | | |
| **OBS Tertile** | | | | |
| **4-12** | 56 | 29 | 1.0 | <0.01 |
| **13-15** | 47 | 48 | 1.85 (0.96 – 3.59**)** | |
| **16--23** | 29 | 63 | 3.01 **(**1.51 – 6.04**)** | |
| **FIP** | | | | |
| **OBS Tertile** | | | | |
| **4-11** | 17 | 52 | 1.0 | <0.01 |
| **12-14** | 43 | 41 | 0.30 (0.13 – 0.70) | |
| **15--22** | 53 | 21 | 0.10 (0.04 – 0.26) | |
| **MtDNA count** | | | | |
| **OBS Tertile** | | | | |
| **5-11** | 26 | 31 | 1.0 | 0.14 |
| **12-13** | 33 | 23 | 0.46 (0.18 – 1.20**)** | |
| **14-22** | 33 | 36 | 0.50 **(**0.19 - 1.31**)** | |

[a] FOP cutoffs: Low FOP, <0.04 average standard reference adjusted (n=132); High FOP, ≥0.04 average standard reference adjusted (n=140)

[b] FIP cutoffs: Low FIP, < 48.37 pg/mL (n=113); High FIP, ≥ 48.37 pg/mL (n=114)

[c]MtDNA count: Low MtDNA count, <3.05 relative copy number (n=92); High MtDNA count, ≥3.05 relative copy number (n=90)

[d] Adjusted for age, sex, origin, and BMI

[e] $X^2$ test for linear trend

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval FOP =Florescent Oxidative Product; FIP=F2-Isoprostane; MtDNA count =Mitochondrial DNA count

Table 5. Correlations among individual biomarkers

| | Correlation Coefficients | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Zeaxanthin | Cryptoxanthin | Lycopene | α-carotene | β-Carotene | α-tocopherol | γ-tocopherol | Ferritin | FOP | FIP | MtDNA |
| **Zeaxanthin** | - | 0.24[a] | 0.23[a] | 0.21[a] | 0.28[a] | 0.13[a] | 0.08 | 0.00 | 0.20[a] | -0.25[a] | -0.10 |
| **Cryptoxanthin** | | - | 0.05 | 0.11 | 0.22[a] | 0.09 | 0.17[a] | -0.04 | 0.17[a] | -0.19[a] | -0.09 |
| **Lycopene** | | | - | 0.60[a] | 0.59[a] | 0.04 | -0.21[a] | -0.04 | 0.40[a] | -0.34[a] | 0.04 |
| **α-carotene** | | | | - | 0.88[a] | -0.11 | -0.35[a] | -0.04 | 0.24[a] | -0.39[a] | 0.12 |
| **β-carotene** | | | | | - | 0.04 | -0.30[a] | -0.07 | 0.29[a] | -0.38[a] | 0.13 |
| **α-tocopherol** | | | | | | - | 0.21[a] | 0.12[a] | 0.17[a] | 0.09 | -0.07 |
| **γ-tocopherol** | | | | | | | - | 0.03 | -0.15[a] | 0.35[a] | -0.16[a] |
| **Ferritin** | | | | | | | | - | 0.03 | -0.01 | -0.06 |
| **FOP** | | | | | | | | | - | -0.17[a] | 0.00 |
| **FIP** | | | | | | | | | | - | -0.14 |
| **MtDNA** | | | | | | | | | | | - |

Abbreviations: OBS=Oxidative balance score; FOP =Florescent Oxidative Product; FIP=F2-Isoprostane; MtDNA =Mitochondrial DNA count

[a]P<.05

Table 6. Associations between individual OBS components and biomarkers

| Variables | | FOP OR (95% CI) [a] | FIP OR (95% CI) [a] | MtDNA OR (95% CI) [a] |
|---|---|---|---|---|
| **Zeaxanthin** | 1 point relative to 0 | 1.93 (0.99-3.75) | 0.30 (0.13-0.68) | 0.43 (0.17-1.11) |
| | 2 points relative to 0 | 3.06 (1.51-6.21) | 0.24 (0.10-0.55) | 0.44 (0.16-1.23) |
| **Cryptoxanthin** | 1 point relative to 0 | 1.70 (0.89-3.26) | 0.21 (0.09-0.49) | 0.62 (0.26-1.53) |
| | 2 points relative to 0 | 1.70 (0.84-3.45) | 0.15 (0.06-0.38) | 0.71 (0.26-1.92) |
| **Lycopene** | 1 point relative to 0 | 2.65 (1.35-5.23) | 0.45 (0.21-0.99) | 0.68 (0.27-1.68) |
| | 2 points relative to 0 | 11.15 (4.62-26.96) | 11.15 (4.62-26.96) | 1.06 (0.34-3.28) |
| **α-carotene** | 1 point relative to 0 | 2.84 (1.47-5.48) | 0.30 (0.13-0.68) | 0.57 (0.23-1.43) |
| | 2 points relative to 0 | 8.72 (3.45-22.03) | 0.05 (0.01-0.15) | 0.60 (0.17-2.05) |
| **β-carotene** | 1 point relative to 0 | 2.95 (1.52-5.73) | 0.21 (0.09-0.51) | 0.55 (0.23-1.34) |
| | 2 points relative to 0 | 5.08 (2.26-11.42) | 0.07 (0.02-0.20) | 0.93 (0.31-2.81) |
| **α-tocopherol** | 1 point relative to 0 | 2.19 (1.09-4.39) | 0.79 (0.35-1.78) | 0.45 (0.18-1.13) |
| | 2 points relative to 0 | 3.03 (1.41-6.53) | 1.26 (0.52-3.07) | 1.01 (0.35-2.96) |
| **γ-tocopherol** | 1 point relative to 0 | 1.73 (0.86-3.46) | 0.75 (0.33-1.59) | 0.62 (0.24-1.65) |
| | 2 points relative to 0 | 0.87 (0.41-1.85) | 3.16 (1.27-7.86) | 0.96 (0.32-2.88) |
| **Ferritin** | 1 point relative to 0 | 0.84 (0.43-1.63) | 0.68 (0.30-1.51) | 0.67 (0.28-1.64) |
| | 2 points relative to 0 | 0.92 (0.43-1.96) | 0.54 (0.21-1.38) | 0.57 (0.18-1.83) |
| **Smoking** | 1 point relative to 0 | 0.70 (0.70-2.74) | 0.22 (0.02-2.13) | 0.54 (0.07-4.19) |
| | 2 points relative to 0 | 1.18 (0.32-4.32) | 0.16 (0.02-1.39) | 0.48 (0.07-3.52) |
| **Alcohol** | 1 point relative to 0 | 0.55 (0.28-1.10) | 0.79 (0.34-1.84) | 0.73 (0.27-1.98) |
| | 2 points relative to 0 | 0.88 (0.46-1.68) | 0.29 (0.13-0.65) | 0.39 (0.15-1.05) |
| **Aspirin** | 1 point relative to 0 | 0.68 (0.35-1.34) | 1.27 (0.58-2.80) | 0.85 (0.33-2.17) |
| | 2 points relative to 0 | 1.78 (0.78-4.17) | 1.49 (0.54-4.15) | 0.78 (0.25-2.27) |
| **Other NSAID** | 1 point relative to 0 | 0.49 (0.25-0.95) | 1.57 (0.70-3.52) | 0.76 (0.30-1.94) |
| | 2 points relative to 0 | 0.52 (0.25-1.06) | 1.45 (0.63-3.37) | 0.49 (0.17-1.39) |
| **Physical activity** | 1 point relative to 0 | 1.10 (0.57-2.14) | 0.87 (0.40-1.92) | 1.27 (0.49-3.25) |
| | 2 points relative to 0 | 1.68 (0.73-3.89) | 0.61 (0.23-1.59) | 2.17 (0.62-7.68) |

Abbreviations: OBS=oxidative balance score; OR= odds ratio; CI=confidence interval; NSAID=non-steroidal anti-inflammatory drug

[a]All results are adjusted for age, sex, origin, and BMI

Table 7. Sensitivity Analysis to evaluate the impact of individual OBS components on biomarkers

| **Model**[a] | **OR (95%CI)**[b] | | |
|---|---|---|---|
| | Elevated FOP | Elevated FIP | Elevated MtDNA |
| **Original (reference)** | 1.21 (1.11-1.32) | 0.75 (0.67-0.84) | 0.92 (0.79-1.07) |
| **OBS excluding Zeaxanthin** | 1.21 (1.08-1.35) | 0.75 (0.65-0.87) | 0.87 (0.74-1.02) |
| **OBS excluding Cryptoxanthin** | 1.28 (1.15-1.44) | 0.79 (0.68-0.90) | 0.97 (0.93-1.02) |
| **OBS excluding Lycopene** | 1.09 (0.98-1.21) | 0.77 (0.68-0.88) | 0.87 (0.75-1.00) |
| **OBS excluding Alpha carotene** | 1.09 (0.98-1.23) | 0.85 (0.74-0.98) | 0.88 (0.76-1.03) |
| **OBS excluding Beta carotene** | 1.14 (1.02-1.28) | 0.83 (0.73-0.96) | 0.85 (0.72-0.99) |
| **OBS excluding Alpha tocopherol** | 1.19 (1.09-1.30) | 0.73 (0.64-0.82) | 0.88 (0.78-1.00) |
| **OBS excluding Gamma tocopherol** | 1.22 (1.11-1.33) | 0.73 (0.64-0.82) | 0.89 (0.79-1.01) |
| **OBS excluding Ferritin** | 1.23 (1.12-1.34) | 0.75 (0.67-0.85) | 0.90 (0.80-1.02) |
| **OBS excluding Smoking history** | 1.21 (1.11-1.32) | 0.75 (0.67-0.85) | 0.90 (0.79-1.02) |
| **OBS excluding Alcohol** | 1.25 (1.14-1.37) | 0.77 (0.68-0.87) | 0.92 (0.81-1.05) |
| **OBS excluding Aspirin** | 1.21 (1.11-1.32) | 0.74 (0.66-0.84) | 0.90 (0.79-1.01) |
| **OBS excluding Other NSAID** | 1.22 (1.12-1.34) | 0.74 (0.66-0.84) | 0.90 (0.80-1.01) |
| **OBS excluding Physical activity** | 1.23 (1.13-1.35) | 0.74 (0.66-0.84) | 0.92 (0.81-1.03) |

[a]Model controlled for excluded component

[b]OR represents change in odds for each additional OBS point. All results are adjusted for age, sex, origin, and BMI

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval; FOP =Florescent Oxidative Product; FIP=F2-Isoprostane; MtDNA =Mitochondrial DNA count

Table 8. Association between OBS and biomarkers of oxidative stress after excluding participants with missing information on OBS components

| Biomarker | Low Biomarker Levels | High Biomarker Levels | OR (95% CI)[d] | p-trend[e] |
|---|---|---|---|---|
| **FOP** | | | | |
| **OBS Tertile** | | | | |
| **4-12** | 44 | 18 | 1.0 | <0.01 |
| **13-15** | 16 | 21 | 2.06 (0.78 – 5.44) | |
| **16--23** | 14 | 46 | 5.64 (2.35 – 13.54) | |
| **FIP** | | | | |
| **OBS Tertile** | | | | |
| **4-11** | 11 | 38 | 1.0 | <0.01 |
| **12-14** | 23 | 21 | 0.34 (0.11 – 1.08) | |
| **15--22** | 33 | 7 | 0.04 (0.01 – 0.17) | |
| **MtDNA count** | | | | |
| **OBS Tertile** | | | | |
| **5-10** | 19 | 14 | 1.0 | 0.44 |
| **11-15** | 18 | 14 | 1.60 (0.39 – 6.65) | |
| **16-21** | 8 | 17 | 6.09 (1.09 – 34.02) | |

[a] FOP cutoffs: Low FOP, <0.04 average standard reference adjusted (n=74); High FOP, ≥0.04 average standard reference adjusted (n=85)

[b] FIP cutoffs: Low FIP, < 46.44 pg/mL (n=67); High FIP, ≥ 48.34 pg/mL (n=66)

[c] MtDNA count: Low MtDNA count, >=3.19 (n=45); High MtDNA count, <3.19 (n=45)

[d] Adjusted for age, sex, origin, and BMI

[e] $X^2$ test for linear trend

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval; FOP =Florescent Oxidative Product; FIP=F2-Isoprostane; MtDNA count =Mitochondrial DNA count

Table 9. Association between OBS and Biomarkers (continuous) in the SRSH population using linear regression

| Biomarker | Regression Coefficient[a] | Confidence Interval | P-value |
|---|---|---|---|
| **Ln (FIP)** | -0.02514 | **(-0.04310, -0.00719)** | **<0.01** |
| **Ln (FOP)** | 0.03038 | **(0.01642, 0.04433)** | **<.0.01** |
| **Ln (mtDNA)** | -0.01462 | **(-0.02918, -0.00006)** | **0.049** |

[a]Adjusted for age, sex, race/ethnicity, and BMI

**Oxidative Stress, Inflammation, and Markers of Cardiovascular Health**

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**Abstract**

**Background:** Experimental biology evidence demonstrating that antioxidants can reverse the effects of oxidative stress-induced damage is not supported by human studies evaluating the association between intakes of antioxidant nutrients disease risk. Previous research has shown that including individual pro- and anti-oxidant factors into a single comprehensive oxidative balance score (OBS) may reveal associations with various conditions in the absence of similar associations for individual score components.

**Methods:** We investigated the relation between OBS and biomarkers of inflammation (C-reactive protein, white blood cell count, albumin) and cardiovascular health (cholesterol, LDL, HDL, and triglycerides) among 19,825 participants in a nationwide prospective cohort study. Fourteen dietary and lifestyle components were incorporated into the OBS and the resulting score was then expressed as a continuous variable or divided into equal intervals. Multivariable-adjusted odds ratios (ORs) for abnormal biomarker levels and 95% confidence intervals (CIs) were calculated using logistic regression models.

**Results:** The ORs (95% CIs) comparing the highest to the lowest OBS categories were 0.50 (0.38-0.66) for CRP, 0.50 (0.36-0.71) for WBC, and 0.75 (0.58-0.98) for LDL; all three p-values for trend <0.001. Gender modified the association between OBS and low HDL with significant inverse association observed only among women. OBS was not associated with hypoalbuminemia or elevated serum triglycerides.

**Conclusions:** Our findings demonstrate that OBS may be associated with some, but not all, biomarkers of inflammation and cardiovascular health.

## Introduction

Oxidative stress is defined as an imbalance in prooxidants and antioxidants, which results in macromolecular damage and disruption of redox signaling and control [3]. It is a complex physiological process, closely interrelated with inflammation [234]. Macrophages respond to immunogenic stimuli by eliciting a burst of reactive oxygen species (ROS), and this further activates the downstream immune response and provides a hostile environment for pathogens. [5]. The active inflammatory state is normally resolved when the pathogen is eliminated [235]. The relationship between oxidative stress and inflammation is still not well understood. Whereas macrophages act as a source of ROS, increased production of ROS in turn may stimulate inflammation by activating cytokine transcription factors, such as nuclear factor kappa B (NFkB) and activator protein-1 (AP-1). An increase in cytokines further stimulates ROS production, resulting in a continuous cycle of oxidative stress and inflammation [236].

A number of exogenous factors may act as pro-oxidants and also possess pro inflammatory properties. ROS are present in the tar and smoke of cigarettes, and smoking also produces secondary release of ROS from inflammatory cells [18, 130]. Another important pro-oxidant is iron, which is consumed along with heme in large quantities as part of red meat-rich diet. Iron, may increase oxidative stress by catalyzing the production of highly reactive hydroxyl radicals via the Haber-Weiss reaction [131]. Alcohol induces oxidative stress through its metabolism, by inhibiting antioxidant enzymes, and by causing inflammation [117].

*In-vitro* evidence indicates that the effects of ROS and oxidative stress-induced inflammation can be reversed by certain anti-oxidant nutrients. [102, 104, 109]. Antioxidants can be classified as enzymatic or non-enzymatic [10]. Non-enzymatic anti-

oxidants such as vitamin A precursor carotenoids, lutein, lycopene, vitamin C, vitamin E, and flavonoids which can protect against lipid peroxidation and terminate free radical chain reactions [10]. Enzymatic antioxidants are also nutrient-dependent; for example, selenium and manganese are critical components of the enzymes glutathione peroxidase and superoxide dismutase, which are responsible for intracellular defense against oxidative stress [10]. Other nutrients can also indirectly contribute to a reduction in ROS. Omega-3 fatty acids contribute to oxidative stress through peroxidation [139], but also induce electrophile-responsive element (EpRE), which regulates genes responsible for transcription of anti-oxidant enzymes [15-17]. Moreover, omega-3 fatty acids have anti-inflammatory properties and therefore indirectly decrease oxidative stress [140].

Although oxidative stress and inflammation are implicated in the pathogenesis of numerous diseases [234, 237] and anti-oxidants have been shown to slow down these processes *in-vitro* [102, 104, 109], clinical trials of antioxidants as disease prevention agents have produced null results [211, 212]. Other studies of chronic diseases have demonstrated that nutrients do not act in isolation and a combination of factors can show a stronger association with disease risk than any single nutrient considered individually [93, 94, 142]. This leads us to believe that a combination of pro-oxidant and anti-oxidant exposures incorporated into a composite measure of oxidative balance may be more strongly associated with health outcomes more than any one factor considered individually [95, 96, 122].

To address this issue we, and others, proposed using an oxidative balance score (OBS), an overall measure of oxidative stress-related exposures based on the summed intakes of various pro- and anti- oxidants, with a higher score indicating decreased

oxidative stress [95, 96, 98, 121-123, 228]. Previous studies found that a higher OBS was associated with decreased risk of colorectal adenoma [94, 96], colorectal cancer [97], and mortality [98]. By contrast, OBS was not found to be related to prostate cancer [123] and stroke [121], indicating that the role of oxidative stress in human pathophysiology may be organ-, or disease-specific. To better understand the specific roles of oxidative stress-modifying exposures in various health outcomes, the mechanistic effects of OBS should be examined. Such an examination may rely on biomarkers, which can act as upstream indicators of future health events, and may be part of the causal disease pathway [238].

The present study examines the association between OBS and biomarkers of inflammation (C-reactive protein, albumin, white blood cell count) and general cardiovascular health (cholesterol, LDL, HDL, and triglycerides). In this analysis we use data from a large nationwide cohort that serves as the basis for the Reasons for Geographic and Racial Differences in Stroke (REGARDS) study.

## Materials and Methods

### *Study Population*

The REGARDS study is designed to examine the causes of racial and geographic disparities in stroke, but offers an opportunity for ancillary research projects such as ours. REGARDS is a cohort of 30,239 black and white individuals of both sexes, ages 45 or older, enrolled from January 2003 to October 2007. Participants were recruited by telephone or mail, from 1842 (59%) of 3140 US counties, with an oversampling of blacks and residents of the Stroke Belt (non-coastal regions of Alabama, Arkansas, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Tennessee) and Stroke

Buckle (coastal plain regions of North Carolina, South Carolina, and Georgia) [239]. Upon consent, interviews were conducted by telephone to obtain demographic and risk factor information, and blood samples and physical measurements (anthropometric and blood pressure data) were obtained during in-home visits. Follow up information was obtained by telephone interviews at 6 month intervals and by consulting medical records of hospitalizations from stroke. Details of the study design can be found elsewhere [240].

Among all REGARDS participants 21,636 (72%) returned a completed food frequency questionnaire (FFQ). Individuals were excluded from the present analysis if no biomarker measurements were recorded (n =770), OBS components were missing (n = 433), or if they had a body mass index (BMI) <18.5 $kg/m^2$ (n = 336), or incomplete covariate information (n = 272). This resulted in an analytical cohort of 19,825 participants who had measurements for at least one biomarker. Of these, the number of participants with measurements for each biomarker varied as follows: CRP (n=19,531), cholesterol (n= 19,790), HDL (n=19,685), LDL (n=19,416), triglycerides (n= 19,782), albumin (n= 14,475), WBC count (n=13,716).

*Laboratory Analyses*

After a 10- to 12-hour overnight fast, blood samples were drawn, centrifuged, and then shipped the University of Vermont central laboratory for reprocessing and analysis. CRP was measured using particle-enhanced immunonephelometry (N High-Sensitivity CRP assay; Dade Behring, Inc., Deerfield, Illinois) [241]. WBC count was measured using an automated analyzer (Beckman Coulter, Inc., Fullerton, California) [242]. Total cholesterol, HDL, triglyceride, and albumin were measured by colorimetric reflectance

spectrophotometry using the Ortho Vitros Clinical Chemistry System 950IRC instrument (Johnson & Johnson Clinical Diagnostics). LDL concentrations were determined using the Friedewald equation [243]. Two of the biomarkers (albumin and WBC count) were collected only in a subset of participants (n = 21,658) who were enrolled in an ancillary study [242].

*Definitions*

Elevated CRP was defined as >3 mg/L [91]. Hypoalbuminemia was defined as <3.5g/dL [244]. Cutoffs for lipid biomarkers were defined using the National Cholesterol Education Program's (NCEP) Adult Treatment Panel III Guidelines as (elevated total cholesterol: $\geq$ 200 mg/dL, elevated LDL: >100 mg/dL, elevated triglycerides: $\geq$ 150 mg/dL, and low HDL for men and women: <40 mg/dL) [245]. Elevated WBC count was defined as being above the $75^{th}$ percentile (>6.86 x $10^9$ cells/L) [246].

Covariates in this analysis were age, sex, total energy intake, BMI, self-reported race (black or white), educational level, region, and physical activity. Educational level was categorized as college graduate or higher, some college, high school graduate, or less than high school. Region of residence was reported as stroke buckle (North Carolina, South Carolina, and Georgia), rest of the stroke belt, or other. Frequency of reported intense physical activity was classified as 4 or more times per week, 1-3 times per week, or none.

*OBS components and their assessment*

The OBS is comprised of 14 components that were selected based on *a priori* knowledge about their relation to oxidative stress. The dietary components were derived from the self-administered 98-item Block FFQ [247], which participants were instructed to complete at the end of their home visit. Nutrient contents of various foods were determined using the Block nutrient database with composition values from the U.S. Department of Agriculture and other sources [248]. The nutrient intakes were calculated by multiplying the reported frequency of consumption by the nutrient composition of the specified portion size for each food item. The Block 98 FFQ has not yet been validated in the REGARDS study population but has been validated in other studies for most nutrients [239, 243]. Nutrient values in the present analysis represent the total dietary and supplemental intake for each nutrient.

The components of the OBS and how they contribute to this measure are summarized in Table 1. Continuous dietary variables reflecting anti-oxidant exposures were divided into categories based on their sex-specific tertile values. Participants whose exposure to a particular dietary anti-oxidant was low ($1^{st}$ tertile) were assigned zero points, and those whose exposure to the same dietary anti-oxidant was medium ($2^{nd}$ tertile), or high ($3^{rd}$ tertile), received one or two points, respectively. The continuous dietary anti-oxidant variables in our study were alpha-carotene, beta-carotene, beta-cryptoxanthin, lutein, lycopene, vitamin C, vitamin E, and selenium. Continuous dietary variables reflecting pro-oxidant exposures were also categorized based on their tertile values. Two points were given for low exposure ($1^{st}$ tertile), one point for medium

exposure (2rd quartile), and zero points for high exposure (3rd tertile). The continuous dietary pro-oxidants in our study were polyunsaturated fatty acids and saturated fat.

Non-dietary lifestyle variables included in the OBS were assigned 0-2 points to keep them consistent with dietary components. Smoking status was categorized as never (2 points), former (1 point), or current (zero points). For NSAIDs and aspirin, zero points were assigned to participants who reported no regular use, one point for missing responses, and two points to those who reported regular use. Sex specific cutoffs were used for alcohol intake. For women alcohol intake was categorized as none (two points), 1-7 drinks/week (one point), and >7 drinks/week (zero points). For men alcohol intake was categorized as none (two points), 1-14 drinks/week (one point), and >14 drinks/week (zero points).

The points assigned to each component were summed to create the overall OBS. In the analyses of association with biomarkers, the score was used separated into equal interval categories. The cutoffs were determined using the distribution of OBS within the analytical cohort and specific cutoffs are listed in Table 2. The assessment of OBS equal interval categories allowed for examination of particularly low and high scores.

*Statistical Analysis*

In descriptive analyses, the means, standard deviations, and frequencies were calculated for covariates and biomarker measurements within each OBS interval. To assess differences in the distributions parameters across OBS intervals, the chi-square test was used for categorical variables and the analysis of variance (ANOVA) was used for continuous variables. With the exception of serum albumin, the biomarker measurements

were not normally distributed, and for this reason they were log transformed when used in linear regression. Multivariable linear regression models were constructed to examine the association between OBS and each biomarker. The results of linear regression models were expressed as regression coefficients and their corresponding 95% confidence intervals (CIs) adjusted for age, sex, total energy intake, BMI, self-reported race, educational level, region, and physical activity.

Multivariable logistic regression models were used to examine the association between OBS and abnormal biomarker levels. The results of logistic regression models were expressed as odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) adjusted for age, sex, total energy intake, BMI, self-reported race, educational level, region, and physical activity. The potential confounders were selected based on evidence in the literature and other *a priori* considerations. All models were examined for collinearity among independent variables and for interaction between OBS and each covariate. When a statistically significant interaction was found, stratified analysis was conducted to determine whether the association between the OBS and the biomarker was appreciably modified by the covariate. Sensitivity analyses to examine the impact of individual OBS components were conducted by removing each OBS component from the score and controlling for it as a covariate. Additional sensitivity analyses evaluated the impact of different OBS categorization approaches and different outcome definitions on the study results. Adjusted ORs and 95% CIs were also calculated for individual OBS components. All analyses were conducted using SAS statistical software version 9.2 (SAS institute, Cary North Carolina).

**Results**

The characteristics of all REGARDS participants are presented by equal interval OBS categories in Table 2. Compared to the lowest OBS interval, participants in the highest OBS interval were older and had a higher energy intake. The proportion of participants who were female, Caucasian, college educated, exercised four or more times per week, and were residents of the non-stroke belt region increased with increasing OBS. There was no significant difference in BMI across OBS intervals.

Table 3 examines individual OBS components by OBS categories. The intake of the antioxidants β-carotene, β-cryptoxanthin, α-carotene, lutein, lycopene, vitamin, vitamin C, and selenium increased with an increasing OBS interval. The pro-oxidant iron also increased with an increasing OBS interval. Compared to the lowest OBS interval, participants in the highest interval had a higher proportion of never smokers, non-drinkers, and regular NSAID users. There was no clear pattern of PUFA intake and regular aspirin use by OBS intervals.

The association between OBS and biomarkers of inflammation is shown in Table 4. After adjusting for covariates, the odds of elevated CRP and WBC were both lower in the highest versus the lowest OBS interval with ORs (95% CIs) of 0.50 (0.38-0.66) and 0.50 (0.36-0.71), respectively. In both analyses p-values for trend were < 0.01. None of the OBS interval-specific ORs for low albumin were statistically significant and there was no evidence of a dose-response (p-trend=0.65).

Table 5 examines the association between OBS and biomarkers of cardiovascular health. For high total cholesterol, the ORs (95% CIs) comparing the second through fifth intervals to the lowest (first) interval were 0.89 (0.77-1.03), 0.85 (0.74 – 0.99), 0.84 (0.72

– 0.99) and 0.85 (0.66 – 1.10), respectively (p-trend=0.05). The same analyses for elevated LDL demonstrated a statistically significant inverse trend ($p<0.01$) with the adjusted OR for the highest versus lowest OBS interval of 0.75 (95% CI: 0.58-0.98). None of the OBS interval-specific ORs for elevated triglycerides were statistically significant and there was no evidence of a dose-response (p-trend=0.17).

In the analyses for low HDL there was a statistically significant interaction between OBS and gender ($p<0.01$), and for this reason all analyses are presented separately for men and women (Table 6) Using the NCEP definition, the odds of low HDL were 63% higher among men (OR=1.63; 95% CI: 1.09-2.45; p-trend = 0.05) but 52% lower among women (OR=0.48; 95% CI: 0.28-0.83; p-trend = 0.08) in the highest versus the lowest OBS category.

The correlations among individual nutritional components of the OBS are presented in Table 7. The strongest correlation was observed between vitamin C and beta carotene (r=0.49). Conversely, the weakest correlations were observed between PUFA and selenium (r=0.03) and between beta cryptoxanthin and vitamin E (r=0.03).

Tables 8-10 present sensitivity analyses where associations between abnormal levels of biomarkers and the 14-component OBS (treated as a continuous variable) are compared to alternative models, where one OBS component was removed from the score and included in the model as a covariate. For all alternative models, removing an OBS component resulted in OR estimates within 5% of the original model result.

Table 11 further explores the apparent modification of the effect of OBS on HDL by gender by assessing the associations between individual score components and low HDL separately in men and women. Lutein and vitamin E intake both had a statistically

significant association with low HDL among men and women. Regular aspirin use relative to non-use was also significantly associated with low HDL, but only among women. The association between smoking and low HDL was stronger in women than in men but, but all ORs were in the hypothesized direction. The association between alcohol consumption and low HDL was opposite to the *a priori* expectation, but the direction was the same for men and women.

Table 12 examines the association between OBS and each of the biomarkers using linear regression. The results are similar to the findings from logistic regression for all biomarkers. Only the associations for CRP, cholesterol, LDL, triglycerides, serum albumin, WBC, and HDL among females were statistically significant ($p<0.01$).

Additional sensitivity analyses were conducted to examine the results using multiple categorizations of OBS (5 equal intervals, 4 equal intervals, or quartiles). The results using quartiles or 4 equal interval categories of OBS were essentially the same for all biomarkers, except in the case of HDL, where the interaction for sex was no longer present. More robust results for albumin were observed using four equal interval categories due to the low number of participants with hypoalbuminemia, but 5 equal intervals were used for consistency. Analyses were also conducted to examine the results for low HDL using the American Heart Association's guidelines for CVD prevention (<50mg/dL in women and <40 mg/dL in men) . Under the AHA guidelines [91], the association between OBS and low HDL (<50 mg/dL) among women was also inverse and most pronounced when comparing the highest to the lowest interval (OR=0.65; 95% CI: 0.46 – 0.92; p-trend < 0.01).

**Discussion**

In this large prospective cohort, we examined the association between OBS and biomarkers of inflammation (CRP, WBC, albumin) and cardiovascular health (cholesterol, triglycerides, LDL, and HDL), expecting to find that a presumably beneficial balance of pro/anti-oxidants will be inversely associated with abnormal biomarker levels. Among inflammatory biomarkers, this expectation was confirmed for high CRP and WBC, but not for low albumin. For biomarkers of cardiovascular health, an inverse association was present with high LDL, less so for cholesterol and not at all for triglycerides. Gender modified the association between OBS and low HDL; among women there was an inverse association, but among men the association was in the opposite direction.

The idea that scores may serve as better predictors of health related outcomes compared to individual factors is not new, and has been particularly well accepted in nutrition research [249]. In a cross-sectional ATTICA study, a Mediterranean diet score was calculated by assigning higher points to frequent consumption of food items adhering to the pattern and lower points to items not adhering to the diet. Participants in the highest tertile of total adherence had 20% lower CRP levels and 14% lower white blood cell counts, relative to those in the lowest tertile of adherence [250]. A separate analysis from the ATTICA study also found a significant inverse association between adherence to the Mediterranean diet and total serum cholesterol [251]. The recent successes of large clinical trials of Mediterranean [252] and DASH [253] diets in preventing cardiovascular disease and its risk factors, further support the usefulness of examining dietary factors together rather than individually.

Other studies have examined the association between OBS and biomarkers of oxidative stress and inflammation. Our findings in the REGARDS population are similar to those of *Kong et al.*, where OBS was comprised of components measured by both FFQ and blood markers. It was found that relative to the lowest interval category of OBS, the highest interval scores had a significant inverse association with F2-isoprostanes and CRP, but a direct association with florescent oxidative products (a relatively novel biomarker of oxidative damage) [121]. Using an OBS score with components measured only by FFQ in the same study population, *Dash et al.* also found a significant inverse association between OBS an F2-isoprostanes [228].

In the present study, we found that gender modified association between OBS and low HDL, an observation that finds support elsewhere in the literature. On average, women have higher HDL levels than men [245]. Environmental factors may explain some, but not all, of the sex difference [254]. Several studies have found that association between smoking and HDL level is greater among women than among men [254-256]. In an analysis of a population from six different countries *Davis et al.* observed that smoking was associated with lower HDL level among females (-0.15 mmol/L) than males (-.05 mmol/L) [254]. Similarly, in the Framingham Offspring Study it was reported that compared to non-smokers of the same sex, female smokers had a significantly lower HDL level than male smokers [257]. We also found that the magnitude of the association between smoking and low HDL was greater among females than males. However, analyses assessing the relation between each individual component and low HDL indicated that no single factor could explain the opposite direction of the association with OBS in men and women

One of the strengths of the present study was our ability to incorporate both dietary and lifestyle components into the score, allowing a more comprehensive view of various determinants of oxidative stress [258]. An additional strength of this analysis is the large and diverse study population. Besides being racially and geographically diverse, there was substantial variability in the intake of dietary components. For example, in our population the mean daily intake of total vitamin C among males in the lowest OBS equal interval was 64.6 mg, a level below the RDA, while the mean intake in the highest interval was 795.3 mg, nine times the RDA [259]. This broad range in consumption of OBS components allowed us to compare extremes of the OBS score.

One of the limitations of this study was the lack of information about genotype status which may influence metabolism of OBS components. For example, polymorphisms in alcohol dehydrogenase type 3 change the rate of ethanol oxidation, and were found to modify the association between alcohol consumption and HDL level [260]. We also relied on FFQ to measure OBS components, a method that is subject to recall bias [261]. In the examination of the association between OBS and CRP *Kong et al.* observed a stronger inverse association using a combination of FFQ and biomarker components than did our analysis, and for this reason a logical next step is to develop OBS based on circulating levels of nutrients in the same study population[121]. The advantages and disadvantages of FFQs and biomarkers have been extensively discussed in the literature. The primary reasons for using biomarkers include 1) no need to rely on recall and reporting ; 2) better ability to capture the variability in the nutritional content of foods due to processing and storage, and 3) more accurate reflection of the amount of the nutrient available for metabolism after intake and absorption [233]. On the other

hand, the use of FFQs is characterized by lower costs and the ability to examine long-term nutritional intakes [233, 262].

In summary, our cross-sectional study found that OBS was significantly associated with several, although not all, makers of inflammation and cardiovascular health. The association between OBS and HDL was modified by gender, an observation that requires confirmation, and if confirmed exploration of the underlying mechanisms This analysis provides further support for studying oxidative stress-related dietary and lifestyle factors in combination, rather than as individual exposures.

Table 1. Oxidative balance score (OBS) point assignment scheme

| OBS components | Score Assignment Scheme |
|---|---|
| Total Vitamin C[a] | 0 = low (1st tertile), 1 = medium (2nd tertile); 2 = high (3rd tertile) |
| α-Carotene | 0 = low (1st tertile), 1 = medium (2nd tertile); 2 = high (3rd tertile) |
| Total β–Carotene[a] | 0 = low (1st tertile), 1 = medium (2nd tertile); 2 = high (3rd tertile) |
| β-Crytoxanthin | 0 = low (1st tertile), 1 = medium (2nd tertile); 2 = high (3rd tertile) |
| Total Vitamin E[a] | 0 = low (1st tertile), 1 = medium (2nd tertile); 2 = high (3rd tertile) |
| Lutein | 0 = low (1st tertile), 1 = medium (2nd tertile); 2 = high (3rd tertile) |
| Lycopene | 0 = low (1st tertile), 1 = medium (2nd tertile); 2 = high (3rd tertile) |
| Total Selenium[a] | 0 = low (1st tertile), 1 = medium (2nd tertile); 2 = high (3rd tertile) |
| PUFA | 0 = high (3rd tertile), 1 = medium (2nd tertile), 2 = low (1st tertile) |
| Total Iron[a] | 0 = high (3rd tertile), 1 = medium (2nd tertile), 2 = low (1st tertile) |
| Smoking history | 0 = current smoker, 1 = former smoker, 2 = never smoker |
| Aspirin | 0 = never, 1 = missing, 2 = regular user |
| Other NSAID | 0 = never, 1 = missing, 2 = regular user |
| Alcohol | Male: 0 = >14 drinks/week, 1 = 1-14 drinks/week, 2 = none<br>Female: 0 = >7 drinks/week, 1 = 1-7 drinks/week, 2 = none |

Abbreviations: NSAID = non-steroidal anti-inflammatory drug (not including aspirin); PUFA = polyunsaturated fatty acid

[a] Total intake=dietary intake plus supplement

Table 2: REGARDS cohort characteristics and biomarker measurements by OBS categories

| Covariates | Equal Interval OBS Categories | | | | | |
|---|---|---|---|---|---|---|
| | 3-7 (n=861) | 8-12 (n=6,050) | 13-17 (n=8,862) | 18-21 (n=3,682) | 22-26 (n=370) | P-value |
| **Age yr, Mean (SD)** | 61.78 (8.90) | 63.98 (9.23) | 65.13 (9.21) | 65.85 (9.08) | 66.92 (8.56) | <0.01 |
| **Male, (%)** | 416 (48.32) | 2,789 (46.10) | 3,901 (44.02) | 1,581 (42.94) | 142 (38.38) | <0.01 |
| **Caucasian, (%)** | 555 (64.46) | 3937 (65.07) | 5,957 (67.22) | 2,543 (69.07) | 271 (73.24) | <0.01 |
| **Education** | | | | | | <0.01 |
| **College education or higher, (%)** | 212 (24.62) | 1,942 (32.10) | 3,489 (39.37) | 1,647 (44.73) | 178 (48.11) | |
| **Some college, (%)** | 269 (31.24) | 1,682 (27.80) | 2,402 (27.10) | 989 (26.86) | 92 (24.86) | |
| **High School Graduate, (%)** | 275 (31.94) | 1,731 (28.61) | 2,187 (24.68) | 787 (21.37) | 82 (22.16) | |
| **Less than High School (%)** | 105 (12.20) | 695 (11.49) | 784 (8.85) | 259 (7.03) | 18 (4.86) | |
| **Region** | | | | | | <0.01 |
| **Stroke Belt (%)** | 327 (37.98) | 2,187 (36.15) | 3,014 (34.01) | 1,199 (32.56) | 125 (33.78) | |
| **Stroke Buckle (%)** | 181 (21.02) | 1,413 (23.36) | 1,921 (21.68) | 763 (20.72) | 68 (18.38) | |
| **Non-Belt (%)** | 3,353 (41.00) | 2,450 (40.50) | 3,927 (44.31) | 1,720 (46.71) | 177 (47.84) | |
| **Exercise** | | | | | | <0.01 |
| **4 or more times/week, (%)** | 206 (23.93) | 1,655 (27.36) | 2,780 (31.37) | 1,299 (35.28) | 131 (35.41) | |
| **1-3 times/week, (%)** | 301 (34.96) | 2,150 (35.54) | 3,390 (38.25) | 133 (37.07) | 133 (35.95) | |
| **None (%)** | 354 (41.11) | 2,245 (37.11) | 2,692 (31.37) | 106 (28.65) | 106 (28.65) | |
| **Total energy intake (kcal/day), Mean (SD)** | 1,444.36 (559.61) | 1,507.80 (623.55) | 1,750.87 (705.25) | 1,976.71 (767.97) | 1,914.73 (644.24) | <0.01 |
| **BMI (kg/m$^2$), Mean (SD)** | 28.11 (5.47) | 29.15 (5.99) | 29.16 (5.91) | 29.28 (5.97) | 29.42 (6.42) | <0.01 |

[a] Population for covariates: any participant with at least one biomarker (n=19,825)

[b] Analysis for biomarker limited to participants who were measured for that biomarker: CRP, n=19,531; Cholesterol, n= 19,790; HDL, n=19,685; LDL, n=19,416 ; Triglyceride, n= 19,782; Albumin, n= 14,475; WBC, n=13,716

Values are presented as mean (SD) or number (%)

Table 3a. Individual OBS components by OBS Equal Interval Categories

| OBS Components | Equal Interval OBS categories | | | | |
|---|---|---|---|---|---|
| | 3-7 (n=861) | 8-12 (n=6,050) | 13-17 (n=8,862) | 18-21 (n=3,682) | 22-26 (n=370) |
| **Total β–Carotene (mcg/day)** | | | | | |
| **Males (n=8,829)** | 1,498.0 (766.0) | 2,533.1 (2,325.4) | 5,269.1 (,5126.8) | 9,016.6 (7,621.3) | 10,396.4 (7,618.1) |
| **Females (n=10,996)** | 1,577.7 (741.1) | 2,660.7 (2393) | 5,503.3 (5023.8) | 9,144.8 (7,318.7) | 11,325.4 (8,306.1) |
| **β–Cryptoxanthin (mcg/day)** | | | | | |
| **Males** | 31.7 (50.7) | 77.1(92.5) | 142.3 (137.2) | 204.3 (160.3) | 262.3 (130.5) |
| **Females** | 25.6 (34.2) | 65.5 (82.5) | 128.7 (131.3) | 190.4 (160.4) | 217.6 (136.1) |
| **α-carotene (mcg/day)** | | | | | |
| **Males** | 221.5 (189.5) | 375.2 (319.1) | 730.4 (697.2) | 1,243.9 (1,129.6) | 1,415.7 (1,008.3) |
| **Females** | 211.3 (148.7) | 343.0 (288.1) | 720.5 (793.8) | 1,214.7 (1,098.0) | 1,588.1 (1,155.3) |
| **Lutein (µg/day)** | | | | | |
| **Males** | 584.4 (347.3) | 945.4 (677.2) | 1,738.7 (1466.2) | 2,810.9 (2491.0) | 3,135.2 (2,186.0) |
| **Females** | 690.7 (499.3) | 1087.5 (950.3) | 2,053.1 (1943.9) | 3,292.3 (2776.5) | 3,668.5 (2,607.7) |
| **Lycopene (µg/day)** | | | | | |
| **Males** | 2,147.8 (2,497.3) | 3,260.9 (3,390.7) | 5,020.9 (5,061.3) | 7,041.3 (7,083.0) | 8,346.4 (7,757.3) |
| **Females** | 1,776.7 (2,099.0) | 2,598.6 (2,891.8) | 3,940.4 (4,276.2) | 5,621.3 (2,684.1) | 7,575.8 (6,387.0) |
| **Total vitamin E, (mg – TE/day)** | | | | | |
| **Males** | 19.5 (57.8) | 46.2 (103.9) | 115.4 (177.1) | 185.5 (191.1) | 287.8 (196.8) |
| **Females** | 18.9 (45.7) | 50.14 (108.9) | 114.7 (170.5) | 181.1 (191.6) | 267.0 (198.3) |
| **Total vitamin C, (mg/day)** | | | | | |
| **Males** | 64.6 (73.7) | 156.6 (224.1) | 375.8 (440.0) | 629.3 (566.3) | 795.3(487.8) |
| **Females** | 76.4 (117.9) | 179.3 (255.5) | 383.7 (421.6) | 597.5 (503.6) | 838.6 (626.0) |
| **Total Selenium (µg/day)** | | | | | |
| **Males** | 72.9 (30.6) | 83.9 (38.2) | 110.8 (57.7) | 141.0 (74.8) | 151.8 (79.7) |
| **Females** | 59.3 (25.7) | 69.2 (33.8) | 92.2 (48.3) | 116.6 (61.4) | 135.2 (76.4) |
| **PUFA (g/day)** | | | | | |
| **Males** | 17.9 (9.1) | 18.5 (10.0) | 20.65 (10.9) | 22.0 (11.2) | 18.6 (8.7) |
| **Females** | 16.3 (9.0) | 15.7 (9.1) | 17.8 (10.1) | 19.3 (10.4) | 17.9 (9.6) |
| **Total iron (mg/day)** | | | | | |
| **Males** | 16.5 (14.7) | 19.1 (13.7) | 26.2 (17.1) | 31.1 (19.3) | 30.2 (20.3) |
| **Females** | 17.2 (18.2) | 19.0 (16.8) | 25.4 (18.9) | 30.1 (21.0) | 32.2 (23.6) |

Table 3b. Individual Lifestyle OBS components by OBS Equal Interval Categories

| OBS Components | Equal Interval OBS categories | | | | |
|---|---|---|---|---|---|
| | 3-7 (n=861) | 8-12 (n=6,050) | 13-17 (n=8,862) | 18-21 (n=3,682) | 22-26 (n=370) |
| **Smoking** | | | | | |
| **Never smoker, (%)** | 125 (14.8) | 2,221 (36.7) | 4,151 (46.8) | 2,211 (60.0) | 281 (76.0) |
| **Former smoker (%)** | 348 (40.4) | 2,649 (43.8) | 3,820 (43.1) | 1,298 (35.3) | 87 (23.5) |
| **Current smoker, (%)** | 386 (44.8) | 1,180 (19.5) | 891 (10.1) | 173 (4.7) | 2 (0.5) |
| **Alcohol** | | | | | |
| **Non-Drinker, (%)** | 343 (39.8) | 3,422 (56.6) | 5,318 (60.0) | 2,441 (66.3) | 277 (74.9) |
| **Moderate Drinker, (%)** | 386 (44.8) | 2,286 (37.8) | 3,239 (36.6) | 1,171 (31.8) | 86 (23.2) |
| **Heavy Drinker (%)** | 132 (15.4) | 342 (5.6) | 305 (3.4) | 70 (1.9) | 7(1.9) |
| **Regular NSAID use (miss 65)** | 17 (2.0) | 581 (9.6) | 1,327 (15.0) | 873 (23.8) | 198 (53.5) |
| **Regular Aspirin use (miss 9)** | 78 (9.1) | 2,040 (33.7) | 4,029 (45.5) | 2,311 (62.8) | 315 (25.1) |
| **Total OBS** | 6.3 (0.8) | 10.4 (1.3) | 15.0 (1.4) | 19.1 (1.0) | 22.5 (0.8) |

Values are presented as mean (SD) or number (%).

Abbreviations: PUFA= Polyunsaturated fatty acid; NSAID=Non-steroidal anti-inflammatory drugs; OBS=Oxidative balance score; SD=Standard deviation; Total intake=dietary intake plus supplements

Table 4. Association between OBS and biomarkers of inflammation in the REGARDS cohort

| Biomarker | LS Mean (SD) | Normal Biomarker Levels (n) | Abnormal Biomarker Levels (n) | OR (95% CI)[d] | p-trend[e] |
|---|---|---|---|---|---|
| **Elevated CRP[a]** | | | | | |
| **OBS Interval** | | | | | |
| **3-7** | 5.73 (7.75) | 454 | 383 | 1.0 | <0.01 |
| **8-12** | 5.05 (8.16) | 3,414 | 2,504 | 0.77 (0.66 - 0.90) | |
| **13-17** | 4.44 (8.57) | 5,370 | 3,274 | 0.62 (0.53 - 0.72) | |
| **18-21** | 4.15 (8.31) | 2,338 | 1,255 | 0.53 (0.45 - 0.62) | |
| **22-26** | 3.88 (7.71) | 236 | 123 | 0.50 (0.38 - 0.66) | |
| **Elevated WBC count [b]** | | | | | |
| **OBS Interval** | | | | | |
| **3-7** | 6.38 (3.01) | 377 | 214 | 1.0 | <0.01 |
| **8-12** | 6.05 (3.19) | 3,051 | 1,148 | 0.63 (0.53 – 0.76) | |
| **13-17** | 5.83 (3.39) | 4,688 | 1,431 | 0.51 (0.43 – 0.61) | |
| **18-21** | 5.80 (3.26) | 1,992 | 558 | 0.46 (0.38 – 0.56) | |
| **22-26** | 5.69 (3.00) | 196 | 61 | 0.50 (0.36 – 0.71) | |
| **Low Albumin[c]** | | | | | |
| **OBS Interval** | | | | | |
| **3-7** | 4.15 (0.32) | 612 | 7 | 1.0 | 0.65 |
| **8-12** | 4.16 (0.33) | 4,379 | 69 | 1.23 (0.56 – 2.71) | |
| **13-17** | 4.19 (0.35) | 6,377 | 70 | 0.87 (0.39 - 1.92) | |
| **18-21** | 4.19 (0.34) | 2,653 | 37 | 1.08 (0.47 - 2.50) | |
| **22-26** | 4.16 (0.31) | 266 | 5 | 1.44 (0.44 – 4.67) | |

[a] CRP cutoffs: Normal CRP, ≤3.0 mg/L (n=11,812); High CRP, >3.0 mg/L (n=7,539)

[b] WBC cutoffs: Normal WBC, $\leq$ 6.86 x $10^9$ cells/L (n=10,304); High WBC, >6.86 x $10^9$ cells/L (n=3,412)

[c] Albumin cutoffs: Normal albumin, >=3.5 g/dL (n=14,287); Hypoalbuminemia, <3.5 g/dL (n=188)

[d] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[e] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval; CRP= C-Reactive Protein; WBC= White Blood Cell Count

Table 5. Association between OBS and biomarkers of cardiovascular health in the REGARDS cohort

| Biomarker | LS Mean (SD) | Normal Biomarker Levels (n) | Abnormal Biomarker Levels (n) | OR (95% CI)[d] | p-trend[e] |
|---|---|---|---|---|---|
| **Elevated Total Cholesterol [a]** | | | | | |
| **OBS Interval** | | | | | |
| **3-7** | 193.97 (38.95) | 482 | 376 | 1.0 | 0.05 |
| **8-12** | 191.79 (41.01) | 3,622 | 2,422 | 0.89 (0.77 – 1.03) | |
| **13-17** | 190.25 (43.02) | 5,359 | 3,485 | 0.85 (0.74 – 0.99) | |
| **18-21** | 190.24 (41.79) | 2,232 | 1,442 | 0.84 (0.72 – 0.99) | |
| **22-26** | 189.73 (38.79) | 223 | 147 | 0.85 (0.66 – 1.10) | |
| **Elevated LDL [b]** | | | | | |
| **OBS Interval** | | | | | |
| **3-7** | 116.85 (34.47) | 263 | 585 | 1.0 | <0.01 |
| **8-12** | 114.54 (36.31) | 2,113 | 3,817 | 0.85 (0.73 – 0.99) | |
| **13-17** | 113.30 (38.07) | 3,204 | 5,465 | 0.80 (0.69 – 0.93) | |
| **18-21** | 113.02 (36.99) | 1,387 | 2,218 | 0.75 (0.64 – 0.89) | |
| **22-26** | 112.30 (34.33) | 141 | 223 | 0.75 (0.58 – 0.98) | |
| **Elevated Triglycerides [c]** | | | | | |
| **OBS Interval** | | | | | |
| **3-7** | 130.90 (88.24) | 613 | 345 | 1.0 | 0.17 |
| **8-12** | 129.45 (87.91) | 4,229 | 1,811 | 1.05 (0.89 – 1.23) | |
| **13-17** | 128.47 (92.22) | 6,344 | 2,496 | 0.98 (0.83 – 1.15) | |
| **18-21** | 128.31 (89.60) | 2,630 | 1,044 | 0.99 (0.83 – 1.18) | |
| **22-26** | 131.99 (83.15) | 255 | 115 | 1.11 (0.84 – 1.47) | |

[a] Cholesterol cutoffs: Normal cholesterol, < 200 mg/dL (n=11,918); High cholesterol, ≥200 mg/dL (n=7,872)

[b] LDL cutoffs: Normal LDL,< 100mg/dL (n=7,108); High LDL, ≥100 mg/dL (n=12,308)

[c] Triglyceride cutoffs: Normal triglyceride, < 150 mg/dL (n=14,071); High triglyceride, ≥150 mg/dL (n=5,711)

[d] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[e] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval

Table 6. Association between OBS and low HDL level[a] by sex in the REGARDS cohort

| Sex | LS Mean (SD) | Normal HDL | Low HDL | OR (95% CI)[d] | p-trend[e] |
|---|---|---|---|---|---|
| **Female[b]** | | | | | |
| **OBS Interval** | | | | | |
| **3-7** | 55.49 (0.75) | 380 | 62 | 1.0 | 0.05 |
| **8-12** | 57.12 (0.29) | 2,872 | 364 | 0.71 (0.53 – 0.95) | |
| **13-17** | 57.57 (0.24) | 4,376 | 538 | 0.72 (0.54 – 0.96) | |
| **18-21** | 57.59 (0.36) | 1,862 | 214 | 0.68 (0.50 – 0.93) | |
| **22-26** | 57.54 (1.04) | 209 | 19 | 0.48 (0.28 – 0.83) | |
| **Male[c]** | | | | | |
| **OBS Interval** | | | | | |
| **3-7** | 47.06 (0.64) | 277 | 136 | 1.0 | 0.13 |
| **8-12** | 45.99 (0.27) | 1,725 | 1,050 | 1.22 (0.97 – 1.53) | |
| **13-17** | 45.65 (0.24) | 2,420 | 1,464 | 1.25 (1.00 – 1.56) | |
| **18-21** | 45.56 (0.36) | 997 | 579 | 1.22 (0.96 – 1.55) | |
| **22-26** | 45.10 (1.09) | 83 | 58 | 1.63 (1.09 – 2.45) | |

[a] Normal HDL (≥40 mg/dL); Low HDL (<40 mg/dL)

[b] Normal HDL (n=9,699); Low HDL (n=1,197)

[c] Normal HDL (n=5,502); Low HDL (n=3,287)

[d] Adjusted for age, race, BMI, total daily energy, education, region, and exercise.

[e] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval

Table 7. Correlations among individual nutrients included in the OBS

| **Nutrients** | **Correlation Coefficients** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total iron | Total vitamin C | Lycopene | α-carotene | Total β-Carotene | Lutein | β-Cryptoxanthin | Total vitamin E | Total Selenium |
| **PUFA** | 0.18[b] | 0.04[b] | 0.24[b] | 0.17[b] | 0.14[b] | 0.17[b] | 0.04[b] | 0.03[b] | 0.48[b] |
| **Iron[a]** | - | 0.29[b] | 0.13[b] | 0.16[b] | 0.31[b] | 0.15[b] | 0.10[b] | 0.23[b] | 0.42[b] |
| **vitamin C[a]** | | - | 0.10[b] | 0.13[b] | 0.49[b] | 0.15[b] | 0.16[b] | 0.55[b] | 0.28[b] |
| **Lycopene** | | | - | 0.18[b] | 0.16[b] | 0.15[b] | 0.09[b] | 0.04[b] | 0.26[b] |
| **α-carotene** | | | | - | 0.48[b] | 0.43[b] | 0.14[b] | 0.07[b] | 0.24[b] |
| **β–carotene[a]** | | | | | - | 0.43[b] | 0.12[b] | 0.30[b] | 0.40[b] |
| **Lutein** | | | | | | - | 0.22[b] | 0.07[b] | 0.21[b] |
| **β – cryptoxanthin** | | | | | | | - | 0.03[b] | 0.10[b] |
| **vitamin E[a]** | | | | | | | | - | 0.24[b] |

[a]Total intake=dietary intake plus supplement
[b]$p<0.05$

Table 8. Sensitivity Analysis to evaluate the impact of individual OBS components on inflammatory markers

| **Model**[a] | **OR (95%CI)**[b] | | |
|---|---|---|---|
| | Elevated CRP | Elevated WBC | Low Albumin |
| **Original (reference)** | 0.956 (0.947-0.964) | 0.957 (0.947-0.968) | 0.971 (0.933-1.012) |
| **OBS excluding β-carotene** | 0.953 (0.942-0.965) | 0.952 (0.938-0.966) | 0.989 (0.936-1.044) |
| **OBS excluding β-cryptoxanthin** | 0.951 (0.942-0.960) | 0.955 (0.944-0.967) | 0.972 (0.929-1.016) |
| **OBS excluding α-carotene** | 0.953 (0.943-0.962) | 0.954 (0.942-0.966) | 0.955 (0.910-1.002) |
| **OBS excluding lutein** | 0.957 (0.947-0.967) | 0.966 (0.954-0.979) | 0.994 (0.947-1.044) |
| **OBS excluding lycopene** | 0.950 (0.941-0.959) | 0.953 (0.941-0.964) | 0.984 (0.942-1.028) |
| **OBS excluding vitamin C** | 0.952 (0.942-0.962) | 0.951 (0.938-0.964) | 0.980 (0.933-1.029) |
| **OBS excluding vitamin E** | 0.963 (0.954-0.973) | 0.956 (0.945-0.968) | 0.977 (0.933-1.022) |
| **OBS excluding selenium** | 0.953 (0.944-0.962) | 0.953 (0.942-0.965) | 0.971 (0.930-1.013) |
| **OBS excluding PUFA** | 0.954 (0.946-0.963) | 0.953 (0.943-0.964) | 0.967 (0.928-1.007) |
| **OBS excluding iron** | 0.957 (0.949-0.966) | 0.958 (0.947-0.969) | 0.967 (0.928-1.008) |
| **OBS excluding smoking** | 0.973 (0.964-0.982) | 0.991 (0.980-1.003) | 0.989 (0.948-1.032) |
| **OBS excluding alcohol** | 0.955 (0.946-0.963) | 0.951 (0.940-0.962) | 0.963 (0.923-1.004) |
| **OBS excluding NSAID use** | 0.953 (0.944-0.962) | 0.953 (0.943-0.964) | 0.969 (0.930-1.010) |
| **OBS excluding Aspirin use** | 0.956 (0.947-0.964) | 0.950 (0.997-1.082) | 0.949 (0.910-0.991) |

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

[a]Model controlled for excluded component

[b]OR represents change in odds for each additional OBS point.

All results are adjusted for age, sex, race, BMI, total daily energy intake, education, exercise, and region

Table 9. Sensitivity Analysis to evaluate the impact of individual OBS components on biomarkers of cardiovascular health

| **Model**[a] | **OR (95%CI)** [b] | | |
|---|---|---|---|
| | High Cholesterol | High LDL | HighTriglyceride |
| **Original (reference)** | 0.988 (0.980-0.996) | 0.981 (0.973-0.989) | 0.995 (0.986-1.004) |
| **OBS excluding β-carotene** | 0.976 (0.965-0.987) | 0.972 (0.961-0.983) | 1.000 (0.988-1.012) |
| **OBS excluding β-cryptoxanthin** | 0.986 (0.977-0.995) | 0.976 (0.967-0.985) | 0.995 (0.986-1.005) |
| **OBS excluding α-carotene** | 0.984 (0.975-0.994) | 0.972 (0.963-0.982) | 1.000 (0.989-1.010) |
| **OBS excluding lutein** | 0.985 (0.975-0.994) | 0.978 (0.968-0.987) | 1.011 (1.001-1.022) |
| **OBS excluding lycopene** | 0.985 (0.976-0.994) | 0.982 (0.973-0.990) | 0.993 (0.983-1.002) |
| **OBS excluding vitamin C** | 0.978 (0.968-0.988) | 0.974 (0.965-0.984) | 0.990 (0.979-1.001) |
| **OBS excluding vitamin E** | 0.982 (0.974-0.992) | 0.979 (0.970-0.988) | 0.989 (0.979-0.999) |
| **OBS excluding selenium** | 0.987 (0.978-0.995) | 0.980 (0.971-0.988) | 0.994 (0.985-1.004) |
| **OBS excluding PUFA** | 0.987 (0.979-0.995) | 0.980 (0.972-0.988) | 0.995 (0.986-1.004) |
| **OBS excluding iron** | 0.991 (0.983-1.000) | 0.985 (0.977-0.993) | 0.994 (0.985-1.003) |
| **OBS excluding smoking** | 0.988 (0.979-0.996) | 0.977 (0.968-0.985) | 1.007 (0.998-1.017) |
| **OBS excluding alcohol** | 0.993 (0.984-1.001) | 0.981 (0.973-0.989) | 0.989 (0.980-0.998) |
| **OBS excluding NSAID use** | 0.988 (0.980-0.996) | 0.981 (0.973-0.989) | 0.994 (0.985-1.003) |
| **OBS excluding Aspirin use** | 1.004 (0.995-1.012) | 1.000 (0.992-1.009) | 0.988 (0.979-0.997) |

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

[a]Model controlled for excluded component

[b]OR represents change in odds for each additional OBS point.

All results are adjusted for age, sex, race, BMI, total daily energy intake, education, exercise, and region

Table 10. Sensitivity Analysis to evaluate the impact of individual OBS components on HDL by sex

| **Model** | **OR (95% CI)** | |
|---|---|---|
| | Low HDL -females | Low HDL-males |
| **Original (reference)** | 0.988 (0.980-0.996) | 1.006 (0.994-1.019) |
| **OBS excluding β-carotene** | 0.976 (0.965-0.987) | 1.026 (1.009-1.044) |
| **OBS excluding β-cryptoxanthin** | 0.986 (0.977-0.995) | 1.003 (0.990-1.017) |
| **OBS excluding α-carotene** | 0.984 (0.975-0.994) | 0.998 (0.984-1.013) |
| **OBS excluding lutein** | 0.985 (0.975-0.994) | 1.019 (1.004-1.034) |
| **OBS excluding lycopene** | 0.985 (0.976-0.994) | 1.008 (0.995-1.021) |
| **OBS excluding vitamin C** | 0.978 (0.968-0.988) | 1.017 (1.002-1.033) |
| **OBS excluding vitamin E** | 0.982 (0.974-0.992) | 1.016 (1.002-1.031) |
| **OBS excluding selenium** | 0.987 (0.978-0.995) | 1.009 (0.996-1.023) |
| **OBS excluding PUFA** | 0.987 (0.979-0.995) | 1.006 (0.994-1.019) |
| **OBS excluding iron** | 0.991 (0.983-1.000) | 1.009 (0.996-1.022) |
| **OBS excluding smoking** | 0.988 (0.979-0.996) | 1.012 (0.999-1.025) |
| **OBS excluding alcohol** | 0.993 (0.984-1.001) | 0.989 (0.976-1.002) |
| **OBS excluding NSAID use** | 0.988 (0.980-0.996) | 1.007 (0.995-1.020) |
| **OBS excluding Aspirin use** | 1.004 (0.995-1.012) | 1.013 (0.968-1.060) |

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

[a]Model controlled for excluded component

[b]OR represents change in odds for each additional OBS point.

All results are adjusted for age, sex, race, BMI, total daily energy intake, education, exercise, and region

Table 11. Associations between individual OBS components and low HDL

| Variables | | Males OR (95% CI) [a] | Females OR (95% CI) [a] |
|---|---|---|---|
| **PUFA (g/day)** | 1 point relative to 0 points | 0.98 (0.87-1.11) | 1.02 (0.87-1.19) |
| | 2 points relative to 0 points | 1.01 (0.88-1.16) | 1.02 (0.85-1.21) |
| **Total iron (mg/day)** | 1 point relative to 0 points | 0.96 (0.86-1.08) | 1.05 (0.90-1.22) |
| | 2 points relative to 0 points | 1.09 (0.97-1.22) | 1.12 (0.96-1.30) |
| **Total vitamin C, (mg/day)** | 1 point relative to 0 points | 1.02 (0.91-1.14) | 0.96 (0.83-1.12) |
| | 2 points relative to 0 points | 0.93 (0.83-1.04) | 0.93 (0.79-1.08) |
| **Lycopene (µg/day)** | 1 point relative to 0 points | 1.01 (0.91-1.13) | 0.96 (0.82-1.11) |
| | 2 points relative to 0 points | 0.99 (0.88-1.11) | 0.89 (0.76-1.03) |
| **α-carotene (mcg/day)** | 1 point relative to 0 points | 1.01 (0.91-1.13) | 1.03 (0.89-1.20) |
| | 2 points relative to 0 points | 1.14 (1.02-1.28) | 0.96 (0.83-1.13) |
| **Total β-Carotene (mcg/day)** | 1 point relative to 0 points | 0.95 (0.85-1.06) | 0.99 (0.86-1.15) |
| | 2 points relative to 0 points | 0.90 (0.80-1.00) | 0.93 (0.80-1.08) |
| **Lutein (µg/day)** | 1 point relative to 0 points | 0.90 (0.80-1.00) | 0.87 (0.75-1.01) |
| | 2 points relative to 0 points | 0.87 (0.78-0.98) | 0.85 (0.73-0.99) |
| **β-Cryptoxanthin (mcg/day)** | 1 point relative to 0 points | 1.09 (0.98-1.22) | 0.98 (0.84-1.14) |
| | 2 points relative to 0 points | 1.07 (0.95-1.19) | 1.09 (0.94-1.27) |
| **Total vitamin E, (mg – TE/day)** | 1 point relative to 0 points | 0.95 (0.85-1.06) | 0.98 (0.84-1.13) |
| | 2 points relative to 0 points | 0.89 (0.80-0.99) | 0.79 (0.68-0.92) |
| **Total Selenium (µg/day)** | 1 point relative to 0 points | 0.95 (0.85-1.07) | 0.94 (0.81-1.10) |
| | 2 points relative to 0 points | 0.93 (0.81-1.07) | 0.92 (0.78-1.09) |
| **Smoking** | 1 point relative to 0 points | 0.73 (0.64-0.84) | 0.43 (0.36-0.51) |
| | 2 points relative to 0 points | 0.76 (0.66-0.88) | 0.41 (0.34-0.48) |
| **Regular Aspirin use** | 1 point relative to 0 points | 3.11 (0.28-34.44) | 0.0001 (0-$1.93e^{111}$) |
| | 2 points relative to 0 points | 1.05 (0.96-1.15) | 1.14 (1.01-1.30) |
| **Regular NSAID use** | 1 point relative to 0 points | 1.50 (0.63-3.58) | 0.71 (0.25-2.04) |
| | 2 points relative to 0 points | 0.96 (0.25-2.04) | 0.86 (0.74-1.01) |
| **Alcohol** | 1 point relative to 0 points | 2.48 (1.91-3.23) | 4.78 (2.11-10.84) |
| | 2 points relative to 0 points | 4.11 (3.17-5.34) | 7.52 (3.34-16.93) |

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

[a]All results are adjusted for age, sex, race, BMI, total daily energy intake, education, exercise, and region

Table 12. Association between OBS and Biomarkers (continuous) in the REGARDS cohort using linear regression

| Biomarker | Regression Coefficient[a] | Confidence Interval | P-value |
|---|---|---|---|
| **Ln (hsCRP)** | -0.02549 | **(-0.02968, -0.02130)** | **<0.01** |
| **Ln (Cholesterol)** | -0.00161 | **(-0.00239, -0.00083)** | **<.0.01** |
| **Ln (HDL) -All** | 0.00052 | (-0.00052, 0.00157) | 0.33 |
| **Ln (HDL) -Males** | -0.00124 | (-0.00282, 0.00034) | 0.12 |
| **Ln (HDL) -Females** | 0.00193 | **(0.00053, 0.00333)** | **<0.01** |
| **Ln (LDL)** | -0.00269 | **(-0.00393, -0.00146)** | **<0.01** |
| **Ln (Triglyceride)** | -0.00093 | (-0.00278, 0.00091) | 0.32 |
| **Serum Albumin** | 0.00341 | **(0.00199, 0.00483)** | **<0.01** |
| **Ln (WBC)** | -0.00597 | **(-0.00737, -0.00457)** | **<0.01** |

[a]Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise

**Appendix**

Associations between OBS and CRP using different categorizations of OBS

| OBS (Range: 3-26) | Normal CRP (≤ 3.0mg/L) | High CRP (> 3.0mg/L) | OR (95% CI)[a] | p-trend[b] |
|---|---|---|---|---|
| **5 Equal intervals** | | | | |
| **3-7** | 454 | 383 | 1.0 | <0.01 |
| **8-12** | 3,414 | 2,504 | 0.77 (0.66 - 0.90) | |
| **13-17** | 5,370 | 3,274 | 0.62 (0.53 - 0.72) | |
| **18-21** | 2,338 | 1,255 | 0.53 (0.45 - 0.62) | |
| **22-26** | 236 | 123 | 0.50 (0.38 - 0.66) | |
| **4 Equal intervals** | | | | |
| **3-8** | 829 | 674 | 1.0 | <0.01 |
| **8-14** | 5,175 | 3,597 | 0.78 (0.69-0.87) | |
| **15-20** | 5,281 | 3,005 | 0.62 (0.55-0.70) | |
| **21-26** | 527 | 263 | 0.51 (0.42-0.62) | |
| **Quartiles** | | | | |
| **Q1: 3-11** | 2,926 | 2,219 | 1.0 | <0.01 |
| **Q2: 12-14** | 3,078 | 2,052 | 0.83 (0.77-0.91) | |
| **Q3: 15-17** | 3,234 | 1,890 | 0.73 (0.67-0.80) | |
| **Q4: 18-26** | 2,574 | 1,378 | 0.64 (0.58-0.71) | |

[a] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[b] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval; CRP= C-Reactive Protein

Associations between OBS and WBC using different categorizations of OBS

| OBS (Range: 3-26) | Normal WBC ( $\leq$ 6.86 x $10^9$ cells/L) | High WBC ( > 6.86 x $10^9$ cells/L) | OR (95% CI)[a] | p-trend[b] |
|---|---|---|---|---|
| **5 Equal intervals** | | | | |
| **3-7** | 377 | 214 | 1.0 | <0.01 |
| **8-12** | 3,051 | 1,148 | 0.63 (0.53 – 0.76) | |
| **13-17** | 4,688 | 1,431 | 0.51 (0.43 – 0.61) | |
| **18-21** | 1,992 | 558 | 0.46 (0.38 – 0.56) | |
| **22-26** | 196 | 61 | 0.50 (0.36 – 0.71) | |
| **4 Equal intervals** | | | | |
| **3-8** | 718 | 367 | 1.0 | <0.01 |
| **8-14** | 4,634 | 1,595 | 0.65 (0.57-0.75) | |
| **15-20** | 4,520 | 1,313 | 0.55 (0.47-0.64) | |
| **21-26** | 432 | 137 | 0.57 (0.45-0.73) | |
| **Quartiles** | | | | |
| **Q1: 3-11** | 2,600 | 1,079 | 1.0 | <0.01 |
| **Q2: 12-14** | 2,752 | 883 | 0.77 (0.69-0.86) | |
| **Q3: 15-17** | 2,764 | 831 | 0.72 (0.64-0.80) | |
| **Q4: 18-26** | 2,188 | 619 | 0.66 (0.59-0.75) | |

[a] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[b] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval; WBC= White Blood Cell Count

Associations between OBS and albumin using different categorizations of OBS

| OBS (Range: 3-26) | Normal albumin (≥ 3.5 g/dL) | Low albumin (< 3.0mg/L) | OR (95% CI)[a] | p-trend[b] |
|---|---|---|---|---|
| **5 Equal intervals** | | | | |
| **3-7** | 612 | 7 | 1.0 | 0.65 |
| **8-12** | 4,379 | 69 | 1.23 (0.56 – 2.71) | |
| **13-17** | 6,377 | 70 | 0.87 (0.39 - 1.92) | |
| **18-21** | 2,653 | 37 | 1.08 (0.47 - 2.50) | |
| **22-26** | 266 | 5 | 1.44 (0.44 – 4.67) | |
| **4 Equal intervals** | | | | |
| **3-8** | 1,119 | 19 | 1.0 | 0.05 |
| **8-14** | 6,505 | 96 | 0.79 (0.48 - 1.31) | |
| **15-20** | 6,073 | 63 | 0.54 (0.32 – 0.93) | |
| **21-26** | 590 | 10 | 0.84 (0.38 – 1.86) | |
| **Quartiles** | | | | |
| **Q1: 3-11** | 3,834 | 56 | 1.0 | 0.21 |
| **Q2: 12-14** | 3,790 | 59 | 1.04 (0.71-1.51) | |
| **Q3: 15-17** | 3,744 | 31 | 0.55 (0.35-0.87) | |
| **Q4: 18-26** | 2,919 | 42 | 0.94 (0.61-1.44) | |

[a] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[b] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval

Associations between OBS and total cholesterol using different categorizations of OBS

| OBS (Range: 3-26) | Normal total cholesterol (< 200 g/dL) | High total cholesterol (≥ 200 mg/dL) | OR (95% CI) [a] | p-trend [b] |
|---|---|---|---|---|
| **5 Equal intervals** | | | | |
| **3-7** | 482 | 376 | 1.0 | 0.05 |
| **8-12** | 3,622 | 2,422 | 0.89 (0.77 – 1.03) | |
| **13-17** | 5,359 | 3,485 | 0.85 (0.74 – 0.99) | |
| **18-21** | 2,232 | 1,442 | 0.84 (0.72 – 0.99) | |
| **22-26** | 223 | 147 | 0.85 (0.66 – 1.10) | |
| **4 Equal intervals** | | | | |
| **3-8** | 876 | 655 | 1.0 | 0.02 |
| **8-14** | 5,405 | 3,576 | 0.91 (0.81 - 1.02) | |
| **15-20** | 5,136 | 3,336 | 0.88 (0.78 – 0.98) | |
| **21-26** | 501 | 305 | 0.83 (0.69 – 0.99) | |
| **Quartiles** | | | | |
| **Q1: 3-11** | 3,067 | 2,192 | 1.0 | 0.01 |
| **Q2: 12-14** | 3,214 | 2,039 | 0.89 (0.82-0.96) | |
| **Q3: 15-17** | 3,182 | 2,052 | 0.89 (0.82-0.97) | |
| **Q4: 18-26** | 2,455 | 1,589 | 0.89 (0.81-0.97) | |

[a] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[b] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval

Associations between OBS and LDL using different categorizations of OBS

| OBS (Range: 3-26) | Normal LDL (< 100 g/dL) | High LDL (≥ 100 mg/L) | OR (95% CI)[a] | p-trend[b] |
|---|---|---|---|---|
| **5 Equal intervals** | | | | |
| **3-7** | 263 | 585 | 1.0 | <0.01 |
| **8-12** | 2,113 | 3,817 | 0.85 (0.73 – 0.99) | |
| **13-17** | 3,204 | 5,465 | 0.80 (0.69 – 0.93) | |
| **18-21** | 1,387 | 2,218 | 0.75 (0.64 – 0.89) | |
| **22-26** | 141 | 223 | 0.75 (0.58 – 0.98) | |
| **4 Equal intervals** | | | | |
| **3-8** | 488 | 1,024 | 1.0 | <0.01 |
| **8-14** | 3,156 | 5,645 | 0.88 (0.78 – 0.99) | |
| **15-20** | 3,147 | 5,166 | 0.80 (0.71 – 0.91) | |
| **21-26** | 317 | 473 | 0.75 (0.62 – 0.90) | |
| **Quartiles** | | | | |
| **Q1: 3-11** | 1,778 | 3,393 | 1.0 | <0.01 |
| **Q2: 12-14** | 1,866 | 3,276 | 0.93 (0.85-1.01) | |
| **Q3: 15-17** | 1,936 | 3,198 | 0.87 (0.80-0.95) | |
| **Q4: 18-26** | 1,528 | 2,441 | 0.84 (0.77-0.92) | |

[a] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[b] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval

Associations between OBS and triglyceride using different categorizations of OBS

| OBS (Range: 3-26) | Normal triglyceride (< 150 g/dL) | High triglyceride (≥ 150 mg/L) | OR (95% CI)[a] | p-trend[b] |
|---|---|---|---|---|
| **5 Equal intervals** | | | | |
| **3-7** | 613 | 345 | 1.0 | 0.17 |
| **8-12** | 4,229 | 1,811 | 1.05 (0.89 – 1.23) | |
| **13-17** | 6,344 | 2,496 | 0.98 (0.83 – 1.15) | |
| **18-21** | 2,630 | 1,044 | 0.99 (0.83 – 1.18) | |
| **22-26** | 255 | 115 | 1.11 (0.84 – 1.47) | |
| **4 Equal intervals** | | | | |
| **3-8** | 1,091 | 438 | 1.0 | 0.29 |
| **8-14** | 6,327 | 2,651 | 1.04 (0.92-1.18) | |
| **15-20** | 6,073 | 2,396 | 1.00 (0.88-1.14) | |
| **21-26** | 580 | 226 | 0.93 (0.76-1.13) | |
| **Quartiles** | | | | |
| **Q1: 3-11** | 3,681 | 1,574 | 1.0 | 0.23 |
| **Q2: 12-14** | 3,737 | 1,515 | 0.95 (0.87-1.04) | |
| **Q3: 15-17** | 3,768 | 1,463 | 0.92 (0.84-1.01) | |
| **Q4: 18-26** | 2,885 | 1,159 | 0.95 (0.86-1.05) | |

[a] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[b] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval

Associations between OBS and HDL using different categorizations of OBS

| OBS (Range: 3-26) | Normal HDL (≥ 40 mg/dL) | High triglyceride (< 40 mg/dL) | OR (95% CI)[a] | p-trend[b] |
|---|---|---|---|---|
| **5 Equal intervals ***significant interaction (p=0.0048 ) with sex** | | | | |
| **3-7** | 657 | 198 | 1.0 | 0.91 |
| **8-12** | 4,597 | 1,414 | 1.02 (0.85 – 1.22) | |
| **13-17** | 6,796 | 2,002 | 1.04 (0.87 – 1.25) | |
| **18-21** | 2,859 | 793 | 1.00 (0.83 – 1.22) | |
| **22-26** | 292 | 77 | 1.05 (0.76 – 1.45) | |
| **4 Equal intervals***significant interaction (p=0.0177 ) with sex** | | | | |
| **3-8** | 1,161 | 366 | 1.0 | 0.44 |
| **9-14** | 6,840 | 2,088 | 0.98 (0.86-1.13) | |
| **15-20** | 6,565 | 1,865 | 0.97 (0.84-1.12) | |
| **21-26** | 635 | 165 | 0.90 (0.72-1.13) | |
| **Quartiles*** NO significant interaction (p=0.248) with sex** | | | | |
| **Q1: 3-11** | 4,018 | 1,212 | 1.0 | 0.98 |
| **Q2: 12-14** | 3,983 | 1,242 | 1.08 (0.98-1.19) | |
| **Q3: 15-17** | 4,049 | 1,160 | 1.03 (0.93-1.14) | |
| **Q4: 18-26** | 3,151 | 870 | 1.02 (0.91-1.14) | |

[a] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[b] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval

Associations between OBS and HDL using different categorizations of OBS among females

| **OBS (Range: 3-26)** | **Normal HDL (≥ 40 mg/dL)** | **High triglyceride (< 40 mg/dL)** | **OR (95% CI)[a]** | **p-trend[b]** |
|---|---|---|---|---|
| **5 Equal intervals ***significant interaction (p=0.0048 ) with sex** | | | | |
| **3-7** | 380 | 62 | 1.0 | 0.05 |
| **8-12** | 2,872 | 364 | 0.71 (0.53 – 0.95**)** | |
| **13-17** | 4,376 | 538 | 0.72 **(**0.54 – 0.96**)** | |
| **18-21** | 1,862 | 214 | 0.68 **(**0.50 – 0.93**)** | |
| **22-26** | 209 | 19 | 0.48 **(**0.28 – 0.83**)** | |
| **4 Equal intervals***significant interaction (p=0.0177 ) with sex** | | | | |
| **3-8** | 690 | 109 | 1.0 | .09 |
| **9-14** | 4,339 | 532 | 0.76 (0.61 – 0.95**)** | |
| **15-20** | 4,247 | 512 | 0.77 **(**0.61 – 0.97**)** | |
| **21-26** | 423 | 44 | 0.59 **(**0.40 – 0.87**)** | |
| **Quartiles *** NO significant interaction (p=0.248) with sex** | | | | |
| **Q1: 3-11** | 2,466 | 320 | 1.0 | 0.50 |
| **Q2: 12-14** | 2,563 | 321 | 0.97 (0.82-1.15) | |
| **Q3: 15-17** | 2,599 | 323 | 1.00 (0.85-1.19) | |
| **Q4: 18-26** | 2,027 | 233 | 0.90 (0.74-1.08) | |

[a] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[b] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval

Associations between OBS and HDL using different categorizations of OBS among males

| OBS (Range: 3-26) | Normal HDL (≥ 40 mg/dL) | High triglyceride (< 40 mg/dL) | OR (95% CI)[a] | p-trend[b] |
|---|---|---|---|---|
| **5 Equal intervals ***significant interaction (p=0.0048 ) with sex** | | | | |
| **3-7** | 277 | 136 | 1.0 | 0.13 |
| **8-12** | 1,725 | 1,050 | 1.22 (0.97 – 1.53) | |
| **13-17** | 2,420 | 1,464 | 1.25 (1.00 – 1.56) | |
| **18-21** | 997 | 579 | 1.22 (0.96 – 1.55) | |
| **22-26** | 83 | 58 | 1.63 (1.09 – 2.45) | |
| **4 Equal intervals***significant interaction (p=0.0177 ) with sex** | | | | |
| **3-8** | 671 | 257 | 1.0 | .85 |
| **9-14** | 2,501 | 1,556 | 1.11 (0.94 – 1.31) | |
| **15-20** | 2,318 | 1,353 | 1.08 (0.91 – 1.29) | |
| **21-26** | 212 | 121 | 1.10 (0.83 – 1.46) | |
| **Quartiles*** NO significant interaction (p=0.248) with sex** | | | | |
| **Q1: 3-11** | 1,552 | 892 | 1.0 | 0.63 |
| **Q2: 12-14** | 1,420 | 921 | 1.14 (1.01-1.28) | |
| **Q3: 15-17** | 1,450 | 837 | 1.04 (0.92-1.17) | |
| **Q4: 18-26** | 1,080 | 637 | 1.08 (0.95-1.24) | |

[a] Adjusted for age, sex, race, BMI, total daily energy, education, region, and exercise.

[b] $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval

Association between OBS and low HDL level[a] (according to AHA guidelines) among females

| OBS Interval | LS Mean (SD) | Normal HDL (n=7,106) | Low HDL (n=3,790) | OR (95% CI)[b] | p-trend[c] |
|---|---|---|---|---|---|
| **3-7** | 55.49 (0.75) | 262 | 180 | 1.0 | <0.01 |
| **8-12** | 57.12 (0.29) | 2,045 | 1,191 | 0.78 (0.63 – 0.96) | |
| **13-17** | 57.57 (0.24) | 3,258 | 1,656 | 0.70 (0.57 – 0.86) | |
| **18-21** | 57.59 (0.36) | 1,390 | 686 | 0.69 (0.55 – 0.86) | |
| **22-26** | 57.54 (1.04) | 151 | 77 | 0.65 (0.46 – 0.92) | |

[a] Normal HDL (≥50 mg/dL); Low HDL (<50 mg/dL)

[b]Adjusted for age, race, BMI, total daily energy, education, region, and exercise

c $X^2$ test

Abbreviations: OBS=Oxidative balance score; OR=Odds ratio; CI= Confidence interval

# CHAPTER 3

## Conclusions

This dissertation consisted of three closely related but distinct studies, which examined the associations of OBS with several health-related and biochemical endpoints. The following is a summary of our findings.

The first dissertation aim examined the association between OBS and prostate cancer incidence in the Cancer Prevention Study-II Nutrition Cohort. This project was motivated by the disagreement between a previously published case-control study conducted in North Carolina, which found an inverse relation of OBS to prostate cancer risk [122] and a Canadian cohort study, which reported null results [123]. We expected to resolve this disagreement by conducting an analysis of the data from a large national US-based cohort, and by using a more comprehensive version of OBS.

Using both weighted (literature based) and equally weighted OBS components, we found no statistically significant association between OBS and prostate cancer risk. Contrary to expectation, we observed a positive association between OBS and risk of any prostate cancer, although the corresponding result for aggressive cancer was not statistically significantly different from the null. The most plausible explanation for the unexpected findings is that men with higher OBS (and presumably healthier lifestyle) are more likely to be diagnosed with prostate cancer that those with low OBS, even if the true incidence of disease in the two groups is the same. It is important to keep in mind that prostate cancer diagnosis depends on a number of factors in addition to the presence of the disease. The probability of prostate cancer diagnosis is clearly a function of the likelihood of having a prostate biopsy, which in turn depends on PSA screening [263],

and on the clinical cutoffs used to trigger a referral for the procedure [264]. The probability of finding prostate cancer on biopsy further depends on the number of tissue cores obtained during the procedure [265]. All of these factors may reflect not only access to care, but also care utilization, as well as clinical practices, all of which may depend on a person's social and behavioral characteristics and could be associated with lifestyle factors including OBS.

Regardless of the interpretation of the results observed in study 1, it is clear that the hypothesized protective effect of OBS on prostate cancer risk does not find support in the current literature. The discrepancy between the results observed in the MPC study and in the two cohort studies is most likely explained by the limitations of the care-control design.

The second aim of this dissertation examined the association between OBS and specific biomarkers of oxidative stress (FIP, FOP, and mtDNA copy number) in the cross-sectional SRSH study. We hypothesized that OBS would be inversely associated with the three biomarkers. This was confirmed for FIP, but not for mtDNA copy number and the association was opposite of the hypothesized direction for FOP. The three biomarkers were also not correlated with each other, indicating that they likely measure different biological processes.

The role of oxidative stress in human pathophysiology may be organ-, or disease-specific, since OBS was previously found to be associated with colorectal adenoma [95, 96, 122] and morality [98, 121], but not with prostate cancer [123] and not with stroke [121]. Moreover, the mechanisms by which OBS may affect health outcomes were unknown. To address this, the third aim of the dissertation examined the association

between OBS and markers of cardiovascular disease (cholesterol, LDL, HDL, and triglycerides) and inflammation (CRP, white blood cell count, and albumin) in the Reasons for Geographic and Racial Differences in Stroke cohort. We found statistically significant inverse associations between OBS and abnormal biomarker levels of CRP, WBC, and LDL, but not with cholesterol, triglycerides, or albumin. Gender modified the association between OBS and low HDL; among women there was an inverse association, but among men the association was in the opposite direction. Our findings indicate that OBS may affect some biological mechanisms more strongly than others.

**Implications for Future Research**

This dissertation adds to the growing literature on OBS. One of the most puzzling findings was the positive association between OBS and FOP, and the lack of correlation between FOP and any biomarkers of oxidative stress. Although the relation between OBS and FOP is in the opposite of the hypothesized direction, ours is the second study to report this observation [121]. As reviewed previously the discrepancy between the results for FIP and FOP may be explained by the fact that FIP only reflects lipid peroxidation whereas FOP could be a measure of other oxidation processes [121]. It is also possible, that FOP is not a valid biomarker for oxidative damage and reflects other biochemical processes that still need to be understood [69].

Other studies found that FOP is associated adverse health outcomes [68, 69, 121], yet there is not a clear understanding of what is being measured by this marker [69]. Based on these findings, the value of using FOP in population based studies as a marker of oxidative stress should be questioned. Since FOP is non-specific biomarker of

oxidative stress, future studies should investigate the amount of oxidative product actually measured by FOP.

Overall, the value of using FOP in population based studies as a marker of oxidative stress should be questioned, particularly considering that it is not correlated with FIP, the most accepted and validated biomarker to date. It is important that several studies found FOP to be associated adverse health outcomes [68, 69, 121], But it is possible that this association, if confirmed is not related to oxidative stress since there is no clear understanding of what is being measured by FOP [69].

While it is unlikely that OBS will change dramatically over time in adults, biomarkers of oxidative stress markers may vary due to a number of factors. Ways of measuring long term profile of oxidative stress should be investigated. One approach would be to conduct longitudinal analyses based on multiple samples. Changes in the oxidative stress profile may more accurately reflect the association with adverse health outcomes, compared to a one-time measurement. Another approach would be to measure several markers of oxidative stress as is done using the recently proposed OXYSCORE, which incorporates plasma malondialdehyde (MDA), oxidized and reduced glutathione, individual antioxidant capacity, α- and γ-tocopherol and urinary isoprostanes [266]. Previous studies have demonstrated that the OXYSCORE can differentiate healthy subjects from patients with coronary artery disease [266].

Metabolomics research indicates that oxidative stress, as measured by redox circuitry, is not uniform and may be tissue or even cell specific [2]. One approach would be to use metabolomics, a relatively new methodology that provides the most accurate profile of redox status. Although this method is expensive and difficult to apply to large

population based studies, further research should aim to integrate metabolomics and epidemiology.

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