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Dietary Factors, Biomarkers and Type 2 Diabetes Risk in Postmenopausal Women: An Investigation of the Biologic Pathway for Reduced Diabetes Risk by Diet

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Dietary Factors, Biomarkers and Type 2 Diabetes Risk in Postmenopausal Women: An Investigation of the Biologic Pathway for Reduced Diabetes Risk by Diet

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Epidemiology

by

Rachelle Dawn Rodriguez

2012

ABSTRACT OF THE DISSERTATION

Dietary Factors, Biomarkers and Type 2 Diabetes Risk
in Postmenopausal Women: An Investigation of the Biologic
Pathway for Reduced Diabetes Risk by Diet

by

Rachelle Dawn Rodriguez
Doctor of Philosophy in Epidemiology
University of California, Los Angeles, 2012
Professor Frank Sorvillo, Chair

Oxidative stress and chronic subclinical inflammation are implicated in the pathology of type 2 diabetes (T2D). Consumption of vitamins and nutrients may dampen the harmful oxidative stress, which normally perpetuates inflammation. Reduction of inflammation may delay or reduce the risk of type 2 diabetes. However current research has not been able to confirm the nutrient-inflammation inverse association. Further, postmenopausal women have a different T2D risk and average C-reactive protein (CRP) concentrations than premenopausal women or men of a similar age.

Moderate alcohol consumption is another dietary factor which has been associated with a reduced type 2 diabetes risk. Limited evidence also suggests alcohol consumption maybe associated with some sex hormones. Additionally, sex hormones have been associated with type

2 diabetes risk. However, sex hormones as mediators of the alcohol-type 2 diabetes association have not been established.

The overall objectives of this dissertation were to examine the role of certain dietary factors (vitamins, nutrients, and alcohol) and inflammation or sex hormones in the pathology of type 2 diabetes in postmenopausal women.

Three separate study designs, using 3 distinct datasets, were selected to evaluate the objectives of this dissertation. A cross-sectional study was employed to assess vitamins, nutrients, inflammation and T2D risk with data from the NHANES survey, a stratified, multistage probability sample of the civilian noninstitutionalized U.S. population in 2003-2006. Cross-sectional data from a nested, matched case-control study within the Women's Health Initiative-Observational Study (WHI-OS) were used to examine the separate relations of dietary or supplemental nutrients to biomarkers of inflammation. Prospective data from a matched, nested case-control study within the Women's Health Study (WHS) were used to examine whether circulating concentrations of sex hormones were associated with alcohol intake or mediated the alcohol-T2D association.

Nutrient concentrations measured in NHANES were different in postmenopausal women than in premenopausal women, but were similar to men. The nutrients were not associated with reduced inflammation and T2D risk in postmenopausal women. WHI-OS data results indicate that dietary vitamin C, beta-carotene, and alpha-carotene as well as supplemental vitamin E and beta-carotene are modestly inversely associated with concentrations of systemic inflammatory biomarkers among postmenopausal women. The WHS cross-sectional analysis showed positive associations between alcohol intake and endogenous estradiol concentrations. Prospective WHS

data suggested that baseline concentrations of estradiol and SHBG might influence the alcohol-T2D association in postmenopausal women.

The dissertation of Rachelle Dawn Rodriguez is approved.

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2012

DEDICATION

This dissertation is dedicated to my family who both supported me and sacrificed for me so that I could achieve this accomplishment. I sincerely thank my academic advisor, Frank Sorvillo, and doctoral committee for their guidance and support. This support greatly improved the scope and quality of this dissertation. I would also like to recognize the UCLA Program for Genomics and Nutrition, particularly Simin Liu, Yiqing Song, Nai-Chieh You, and Sara Chako for providing me with the opportunity to use their data and for their guidance. Thanks to Amgen and my management at Amgen for supporting this endeavor with both flexibility of my work schedule and tuition reimbursement.

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Chapter 1 is a version of “Associations of vitamins, nutrients and CRP with type 2 diabetes risk in US postmenopausal women differ from premenopausal women and men >50”, a co-authored paper in progress. Co-authors include Nai-Chieh You and Sara Chacko. Earl Ford was principal investigator for NHANES and biomarker measurements used for this study.

Chapters 2 is a version of “Dietary and supplemental nutrient consumption and inflammatory biomarkers in Type 2 diabetic and non-diabetic postmenopausal women”, a co-authored paper in progress. Co-authors include Nai-Chieh You, Sara Chacko, Lawrence S. Phillips, James M. Shikany, Lesley Tinker, and JoAnn E. Manson. Simin Liu was the principal investigator for original matched case control study from which data was used for this study. This work is supported by grants DK066401, HL-43851, and CA-47988 from the National Institutes of Health.

Chapter 3 is a version of “Interrelationship between alcohol intake and endogenous sex-steroid hormones on diabetes risk in postmenopausal women” a co-authored paper in progress. Co-authors include Simin Liu, Yiqing Song, Julie Buring, JoAnn E. Manson, and Nai-Chieh You. Simin Liu was the principal investigator for original matched case control study from which data was used for this study. This work is supported by grants DK066401, HL-43851, and CA-47988 from the National Institutes of Health. Dr. Song is supported by a grant (K01-DK078846) from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.

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Rachelle Rodriguez, Khaled Sarsour, Beth Mitchell, Lee Bowman **(**November 5-8, 2011) Natural History of Beta Cell Rate of Decline and its Effect on Development of Secondary Complications in Type 1 Diabetes. Poster presentation at the International Society for Pharmacoeconomics and Outcomes Research, European Congress, Madrid, Spain

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Rachelle Rohwer (October 6, 2000) Surviving Tenant Appreciation Day: A *Salmonella* Thompson Outbreak. Presentation at the meeting of the Southern California Epidemiology Exchange

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CHAPTER 1
Background

1.1 *Epidemiology of Diabetes*

Diabetes is a growing concern for the United States population as the prevalence and incidence continues to increase. In 2007, an estimated 23.6 million (7.8%) people in the United States had diabetes and an estimated 57 million had prediabetes.(1) In the United States, 11.2% of all men and 10.2% of all women (aged >20 years) have diabetes.(1) Accounting for approximately 90% to 95% of all diagnosed cases of diabetes, type 2 diabetes (non-insulin-dependent diabetes mellitus) is associated with obesity, older age, family history, impaired glucose metabolism, physical inactivity and race/ethnicity.(1) Figure 1-1 highlights the increased prevalence of diabetes in those 65 years and older and in men 45 years and older. Since the mid 1990s the US has experienced a profound trend for increasing diabetes prevalence.

The populations at highest risk are American Indians, African Americans, and Hispanic/Latino Americans.(1) The total prevalence of diabetes by race/ethnicity is 14.2% for American Indians and Alaska Natives, 11.8% for non-Hispanic blacks, 10.4% for Hispanic/Latino Americans, and 6.6% for non-Hispanic whites. Diabetes is listed as the seventh leading cause of death among Americans, but is listed 3rd-4th among older (65+) ethnic minorities, not accounting for underlying or associated causes.(2) Diabetic individuals have almost twice the risk of death compared to non-diabetic individuals according to the United States 2006 death certificates.(1)

Among 65 to 74 year old Americans the prevalence of diabetes among women is greater than that for men (Table 1-1), which is not evident in Figure 1-1. Diabetes is a leading cause of death among middle-aged American women.(2) Diabetes is one of the leading underlying causes

of death among women aged 65 years and older.(2) Further, the secondary illnesses associated with type 2 diabetes are of major concern due to the severity (and cost) of those diseases including: heart disease, stroke, high blood pressure, blindness, kidney disease, nervous system disease, dental disease, pregnancy complications, and amputations.(1)

1.2 *Biology of Diabetes*

Type 2 diabetes is diagnosed through an impaired glucose tolerance (IGT: a glucose level after 2-hour oral glucose tolerance test of 140-199 mg/dL) or impaired fasting glucose (fasting glucose level of 100-125 mg/dL), also described as “insulin resistance” or “prediabetes.” “Insulin resistance is defined as a defect in the ability of insulin to drive glucose into its major target tissue, skeletal muscle. In the early stages, insulin resistance is compensated by an increase in pancreatic insulin secretion”.(3) Prediabetes can progress into type 2 diabetes which is characterized by increased levels of glucose (fasting blood sugar>125 mg/dL or 2-hour oral glucose tolerance test level>200 mg/dL).(4)

β -cell function and insulin sensitivity are involved in maintaining glucose levels.(5) Reduced action by decreased numbers of β-cells of the pancreas, which secrete insulin, eventually results in type 2 diabetes.

An increase in inflammatory biomarker concentrations is present in obese individuals (Table 1-2). Biomarkers of inflammation, including tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and C-reactive protein (CRP) have been associated with measures of insulin resistance, insulin secretion and the development of type 2 diabetes. (6-11) Reactive oxygen species(12;13) and free fatty acids(12) may play a role in the insulin signaling pathways.

Small increases in TNF-α inhibit the insulin signaling pathway and interfere with glucose transport(14). TNF-α is suggested to only act locally to inhibit insulin signaling.(15) IL-6

suppresses insulin-dependent insulin receptor autophosphorylation (interfering with insulin sensitivity). IL-6 is systemic and enhances hepatic glucose production(15), stimulates LDL production(12;15) and triglycerides in liver(12), and insulin resistance in muscle(12;15). IL-6 induces IL-1β production which impairs insulin signaling and action in vitro.(16)

The onset of menopause influences a change in the distribution of fat stores from the gluteal-femoral area (subcutaneous adipose tissue), to the abdomen (visceral adipose tissue), thereby increasing diabetes risk in menopausal women compared to premenopausal women. The visceral adipose tissue is responsible for the majority of the proinflammatory cytokine (IL-6 and TNF-α) production in the body.(17) These cytokines induce CRP production by the liver. CRP concentrations have been inversely associated with antioxidant blood concentrations in a representative sample of the US general population (NHANES).(18) Additionally, men of a similar age to postmenopausal women may not share the same risk due to the hormonal differences earlier in life. Therefore postmenopausal women comprise a unique diabetes risk group.

1.3 *Diet, Inflammation and Diabetes*

1.3.1 Overview

Many foods contain nutritional factors which may have potent antioxidant and/or anti-inflammatory effects. Specifically, food groups and nutrients high in antioxidants: [decaffeinated filtered coffee(19), regular filtered coffee (20), antioxidant vitamins and antioxidant nutrients(21;22)], dietary sources of certain unsaturated fatty acids [nuts and seeds(23), monounsaturated fatty acids(22)], minerals [magnesium(24)], fiber(25), and moderate to light alcohol consumption(26-33) [total ethanol intake(34) and red wine(26)] have been identified as potential dietary components which may inhibit chronic inflammation. The benefit for reducing

inflammation is more pronounced for those in a state of chronic inflammation(28), such as overweight individuals or smokers.(24;35) CRP concentrations were inversely associated with antioxidant blood concentrations in the US general population.(36)

Current research has also identified dietary factors which overlap with foods that reduce inflammation. Many plant-based foods and nutrients provide a reduced risk for type 2 diabetes including: vegetables (green leafy; dark yellow(37)), carotenoids(38;39), magnesium(35;40-43), coffee(44-48), vegetable fat(49;50), nuts(51), fiber(40;52), whole-grain(40;41;49;53), and glycemic index(52). In other studies, the following were unrelated to diabetes risk: refined grains(40), fruit and vegetables(40), soluble fiber(40), glycemic load(52), glycemic index(40), monounsaturated fatty acids (Mediterranean diet, olive oil(54)), carotenoids(55-57), and vitamin E(58). In other studies glycemic index, glycemic load(59;60) and refined grains(49;53) were associated with an increased risk for type 2 diabetes.

1.3.2 Antioxidants, Vitamins and Nutrients

Individuals consuming a healthier diet rich in antioxidants are at lower risk for type 2 diabetes. Antioxidants quench damaging reactive oxygen species and radical chain reactions. Current research, however, has not yet fully determined if dietary antioxidants or nutrients play a role in the prevention of type 2 diabetes. CRP concentrations have been inversely associated with antioxidant blood concentrations in a representative sample of the US general population.(61) However, some studies did not find an association between several dietary antioxidants and nutrients and risk of type 2 diabetes.(62;63)

Dietary vitamins and nutrients(21;22;38;39;64) may reduce the risk of developing type 2 diabetes, increase insulin sensitivity and improve glucose tolerance through reduced inflammation. Vitamin E, vitamin C, beta-carotene, alpha-carotene, and lycopene have

antioxidant properties. Selenium is an antioxidant nutrient which alone has no antioxidant function, but when it interacts with vitamin E, it enhances vitamin E's antioxidant effects.(65) The bioavailability, clearance, regulation, antagonism and potency (bioactivity) of the dietary nutrients will determine the effective exposure of normal dietary intakes of the nutrients on oxidative stress.

Vitamins in the body are either water soluble and react inside the cell, in the plasma or within cells or are lipid soluble and react in the cell membrane. Common water soluble nutrients include vitamin C, glutathione and lipoic acid. Lipid soluble nutrients include vitamin E, coenzyme Q, vitamin A, retinyl palmitate, carotenes, and lycopene.

Vitamin E supplementation increased plasma levels by 84% when taken with a meal as compared to 29% when taken alone because it is lipophilic.(66) Another source suggests vitamin E supplementation bioavailability is around 50%.(65;67) Alpha-tocopherol is the most bioavailable among the eight vitamin E vitamers. Additionally the clearance is the slowest. Alpha-tocopherol levels are regulated however; large quantities of alpha-tocopherol consumption can only double the serum levels. Gamma-tocopherol is also a highly active vitamer of vitamin E and is one of the highest dietary vitamers available in the Western diet, however, the absorbance is slower and the half-life is much shorter than alpha-tocopherol. Further levels of alpha-tocopherol inhibit the levels of gamma-tocopherol, but the reverse is not true. Other vitamers also have antioxidant activity, but because of the limited information available and the low quantities consumed, they were not evaluated further in this research.

Beta-carotene is a dietary nutrient which has a substantial bioactivity compared to other antioxidant vitamins. The bioavailability of beta-carotene is reduced substantially because of degradation in the gut to constituents which are later converted into vitamin A. (67)

Approximately 35% of beta-carotene is absorbed intact. Beta-carotene levels are directly related to levels of consumption. Alpha-carotene has similar bioactivity as beta-carotene(68) and is also a precursor to vitamin A at about half the rate of beta-carotene.(69)

1.3.3 Antioxidant Index

Perhaps the difficulty in determining an association between vitamins and nutrients and diabetes risk is due to the small contribution each provides in reducing inflammation. Adding the effects of all potential contributors together may demonstrate a collective measurement that could be potent enough to clearly detect a difference in diabetes risk. Because the bioavailability and potency (bioactivity) of dietary nutrients interact to influence the effectiveness of other antioxidants and nutrients on oxidative stress, antioxidant scores have been created to capture the cumulative effects of dietary nutrients. However, existing scores do not adequately account for exposure levels, such as the bioavailability of the nutrient.

For example, Wright and associates(70) developed a total dietary antioxidant index (AI) including vitamin E, carotenoids, flavonoids, vitamin C and selenium that was shown to be inversely associated with lung cancer risk. Other dietary antioxidant indices follow a similar approach with equal weighting of the individual antioxidants.(71;72) This method is not considered optimal, however, because it does not take into account the biology of the nutrients, such as the potency and bioavailability.(73) Others have improved the antioxidant index by including the antioxidant potency of the nutrient in calculating the dietary total antioxidant capacity of foods (TAC).(74;75) However, bioavailability has not been accounted for in these antioxidant indices.

1.3.4 Alcohol

Moderate to light alcohol consumption has been found to have an inverse association with glucose intolerance, insulin resistance, and incidence of type 2 diabetes; (30;76) however, the mechanisms underlying the benefits from alcohol intake are not completely understood. Conversely, light to moderate alcohol consumption in a multi ethnic cross-sectional study demonstrated that previously reported beneficial associations with insulin sensitivity may be due to body mass index (BMI) and central adiposity profiles which are more favorable to higher insulin sensitivity.(76)

There are likely many factors within each type of alcohol that interplay to produce the u-shaped response curve previously reported(26-33). Ethanol has been suggested as one possible constituent in alcohol beverages which could be responsible for the observed protective effect.(34) The types of alcohol consumed require further evaluation to better ascertain if any specific constituents in an alcoholic beverage contributes to its apparent protective effect against diabetes.

1.4 *Postmenopausal Women, Hormones and Diabetes Risk*

An increased incidence of diabetes occurs in middle-aged and elderly women after menopause occurs(77), suggesting that hormones have a role in the natural history of diabetes. Sex hormones have been linked with the occurrence of diabetes, including estrogen (+), testosterone (+), sex hormone-binding globulin (SHBG) (-), and dehydroepiandrosterone sulfate (DHEAS) (+).(78)

Estrogens, in conjunction with low levels of progesterone, have been shown to improve insulin sensitivity.(77) However, high estradiol levels alone increased diabetes risk in both men and women.(79) Studies have determined that 17 β -estradiol increases insulin secretion through

its effects on pancreatic beta-cells. (77;80) A cohort study found that high levels of testosterone in women was associated with the increased incidence of insulin resistance.(81) Low testosterone levels in men is associated with increased insulin resistance and incidence of type 2 diabetes.

Older studies suggest synthetic estrogens and progestins (oral contraceptives) cause IGT, reduced insulin sensitivity and low beta-cell function while other more recent studies disagree.(5;77) The effect of synthetic estrogens and progestins may depend on the steroid used and route of administration.(77;80) Transdermal hormone therapy (estradiol and norethisterone) resulted in a lower prevalence of IGT than in the combined oral regimen in Swedish women.(82)

A study of postmenopausal women found that low levels of SHBG were associated with increased risk of IGT and type 2 diabetes.(83) High levels of SHBG were more protective in women than in men.

Studies have reported mixed results of no effect or improved effect of DHEAS on insulin sensitivity.(84) DHEAS has been shown to enhance insulin secretion in β-cells in vitro and in vivo.(84) DHEAS has been found to improve insulin sensitivity by inducing an increase glucose transport activity.(84) Conversely, DHEAS may be a non-specific biomarker of poor health.(85;86)

Estrogen decreases testosterone levels.(85) DHEAS is a precursor for all sex hormones, converting to testosterone then subsequently estradiol (Figure 1-2).(84;87) A study was conducted which found that testosterone was positively associated with SHBG.(88)

Recently, sex-steroid hormones , including estrogen(5;77-79;81;89), testosterone(78;81), SHBG(78;83;84), and DHEAS(78;84;90), have been linked with the occurrence of diabetes, insulin resistance or IGT. Diabetes risk in women increases with the onset of menopause and the

concurrent decrease in estrogen,(77) highlighting the importance of this pathway for postmenopausal women. There is evidence that estradiol increases insulin secretion through its effects on pancreatic beta-cells.(77;80) DHEAS has been shown to enhance insulin secretion in beta-cells in vitro and in vivo(84) and improve insulin sensitivity by inducing an increase glucose transport activity.(84)

Such diabetes-related hormonal markers may be important mediators in the association of alcohol consumption and diabetes risk. There is some evidence suggesting that alcohol consumption might be associated with increased concentrations of the sex hormones estrogen(89) and DHEAS; (86) however, due to limited data, the evidence is inconclusive. Alcohol consumption, did not have an association with levels of testosterone in aging men.(91) SHBG levels were not associated with alcohol intake in a cross-sectional study of postmenopausal women who were not taking hormone replacement therapy (HRT).(83) Furthermore, it also remains uncertain whether the association between alcohol and sex-steroid hormones differs by type of alcoholic beverage consumed.

1.5 *Dissertation Objectives*

The overall objective of this dissertation is to examine the role of certain dietary factors with anti-inflammatory properties and inflammation or sex hormones in the pathology of type 2 diabetes and to investigate whether these dietary factors are potent enough to inhibit inflammation or influence sex hormone concentrations thereby reducing the risk of developing type 2 diabetes in postmenopausal women

1.6 *Tables*

Table 1- 1. Age-Specific Percentage of Civilian, Noninstitutionalized Population with Diagnosed Diabetes, by Race and Sex, United States, 2006

Race/Ethnicity	Sex	Age (years) 0–44	45–64	65–74	75+
White	Male	1.4	10.2	18.7	17.4
	Female	1.6	9.2	15.6	14.3
Black	Male	2.2	16.7	24.2	23.7
	Female	2.5	15.2	34	25.7
Asian/ Pacific Islander	Male	1.2	11.1	15.2	30.6
	Female	0.9	8.3	17.1	25.4
Hispanic	Male	1.4	14.1	23.5	24.8
	Female	1.2	16.5	27.9	23.8

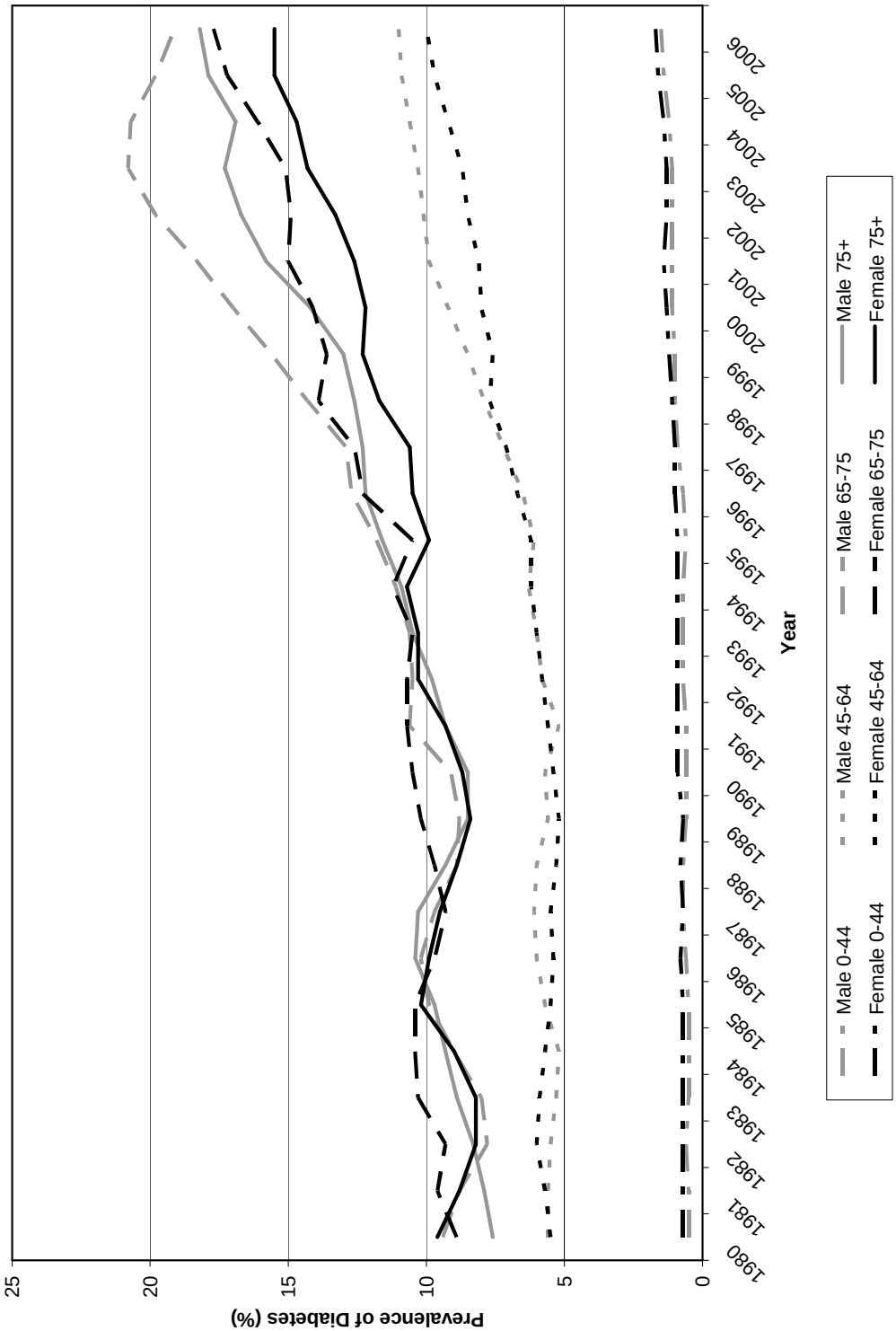
Data Source: http://www.cdc.gov/diabetes/statistics/prev/national/tprevhmemage.htm; http://www.cdc.gov/diabetes/statistics/prev/national/tprevhfemage.htm; http://www.cdc.gov/diabetes/statistics/prev/national/fig2004.htm

Table 1- 2. Circulating Levels of Inflammatory Biomarkers in Normal Weight and Obese Individuals.

Inflammatory Biomarker	Normal Weight/ Non-Obese	Obese
IL-6 (pg/mL)		
Khaodhiar 2004(14)	0.1 ± 0.1	3.2 ± 2.5
Ziccardi 2002(15)	1.4 ± 0.5	3.2 ± 0.9
Van Guilder 2006(16)	~1.7	1.4 ± 0.2
TNF-α (pg/mL)		
Khaodhiar 2004	0.3 ± 0.3	1.0 ± 0.8
Ziccardi 2002	3.6 ± 0.9	5.8 ± 1.5
Van Gulider 2006	1.5 ± 0.1	1.6 ± 0.1
sTNF RII (ng/mL)		
Khaodhiar 2004	1.1 ± 0.9	12.9 ± 6.9
CRP (mg/L)		
Khaodhiar 2004	0.8 ± 1.0	4.3 ± 2.3
Van Guilder 2006	0.9 ± 0.2	1.5 ± 0.3

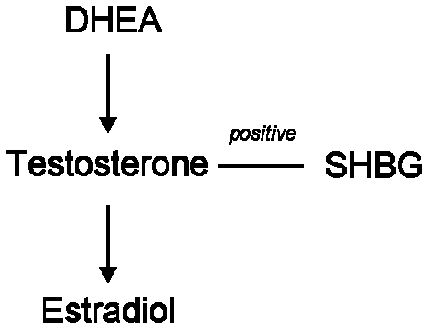
1.7 *Figures*

Figure 1- 1. Percentage of Civilian, Noninstitutionalized Population with Diagnosed Diabetes, by Sex and Age, United States, 1980–2006



Data Source: http://www.cdc.gov/diabetes/statistics/prev/national/tprevfemage.htm; http://www.cdc.gov/diabetes/statistics/prev/national/tprevmage.htm

Figure 1- 2. Diagram of Sex Hormone Relationships



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CHAPTER 2

Associations of vitamins, nutrients and CRP with type 2 diabetes risk in US postmenopausal women differ from premenopausal women and men >50

2.1 *Abstract*

Subclinical chronic inflammation as measured by C-reactive protein (CRP) has been shown to increase type 2 diabetes (T2D) risk. Postmenopausal women have a different T2D risk and average CRP concentrations than premenopausal women or men of a similar age. Nutrients are thought to be able to reduce inflammation through inactivation of reactive oxygen species. However, the possible role of nutrients on inflammation and T2D remains uncertain. We evaluated vitamin (vitamin C, alpha tocopherol), nutrient (lycopene, trans beta carotene, cis beta carotene, alpha carotene), and CRP serum levels with T2D risk in postmenopausal women and compared these results with premenopausal women and men 50 years or older. The data used in this cross-sectional study were from the NHANES survey, a stratified, multistage probability sample of the civilian noninstitutionalized U.S. population in 2003-2006, including 1471 postmenopausal women, 2494 premenopausal women and 1442 men.

Nutrient concentrations were different in postmenopausal women than in premenopausal women, but were similar to men. Both T2D and non-diabetic (ND) postmenopausal women had higher nutrient concentrations (vitamin C: T2D=1.26±0.05, ND=1.30±0.03 mg/dL; alpha tocopherol: T2D=1.65±0.04, ND=1.55±0.02 mg/dL; trans beta carotene: T2D=20.47±0.87, ND=23.51±0.46 μg/dL; cis beta carotene: T2D=1.62±0.05, ND=1.75±0.03 μg/dL; alpha carotene: T2D=4.62±0.16, ND=5.37±0.08 μg/dL) than premenopausal women (vitamin C: T2D=0.91±0.07, ND=0.87±0.01 mg/dL; alpha tocopherol: T2D=1.28±0.05, ND=1.06±0.01

mg/dL; trans beta carotene: T2D=11.08±1.33, ND=11.73±0.25 μg/dL; cis beta carotene: T2D=0.95±0.08, ND=0.85±0.02 μg/dL; alpha carotene: T2D=3.22±0.29, ND=4.23±0.05 μg/dL), except for lycopene which was lower (postmenopausal: T2D=38.26±1.26, ND=43.20±0.68 μg/dL; premenopausal: T2D=43.81±1.94, ND=51.70±0.39 μg/dL). Nutrient concentrations were similar between postmenopausal women and men ≥50 years. Adjusted cross-sectional associations (odds ratios) between nutrients and inflammation in postmenopausal women ranged from 0.44 to 0.57 however, the 95% confidence intervals were very wide. Premenopausal women and men had similar results, except positive non-significant associations with T2D were observed with lycopene in both and with alpha tocopherol in men. The nutrients examined were not associated with reduced inflammation and T2D risk in postmenopausal women.

2.2 *Introduction*

Type 2 diabetes (T2D) is characterized by insulin resistance, subclinical chronic inflammation and oxidative stress.(1-3) Damaging reactive oxygen species, the hallmark of oxidative stress, are present with chronic subclinical inflammation and perpetuate inflammation by damaging tissue, including beta cells, and increasing insulin resistance. (3;4) Antioxidants, by their nature, can inactivate reactive oxygen species, rendering them harmless and thus can reduce the level of inflammation.(4) A diet rich in antioxidants can increase the antioxidant capacity of the blood.(5-9) However, conflicting evidence exists whether dietary supplementation or blood concentrations of vitamins and nutrients can reduce concentrations of the inflammatory biomarker, C-reactive protein (CRP). (10-12) Further, many studies have examined the relationship between individual nutrients and T2D risk, definitive results have not yet been established, particularly in postmenopausal women.(13-17)

Women of postmenopausal age have a dramatically increased T2D risk compared to premenopausal women but variable T2D risk by race compared to men(18), rendering them a unique T2D risk group. Subclinical chronic inflammation, as measured by CRP, is thought to be partially responsible for the increased T2D risk.(19;20) Postmenopausal women are of particular interest because the transition into menopause causes an increase in CRP that stems from an increase in visceral adipose tissue storage.(21) and CRP concentrations have been observed to be higher in women than in men.(22;23) Determining whether antioxidants influence CRP levels and T2D risk in postmenopausal women may provide insights on prevention approaches.

To further evaluate the possible role of nutrients in reducing CRP levels and T2D risk in postmenopausal women, we examined serum vitamin, nutrient, and CRP concentrations and assessed possible associations with T2D. We also evaluated whether these associations of nutrients on CRP levels and T2D risk are modified in postmenopausal women by comparing the findings in this risk group with premenopausal women and men over 50 years of age.

2.3 *Subjects and Methods*

2.3.1 NHANES Subjects

The data for this study were obtained from the NHANES survey, a complex stratified, multistage probability sample of the civilian noninstitutionalized U.S. population in 2003-2004(24) (n=10,122 participants) and 2005-2006(25) (n=10,348 participants). Three stages of data collection were conducted. First the participants were screened for inclusion in the study sample. Then the participants were interviewed on a variety of health-related topics. Finally the participants underwent a medical examination in which blood and urine samples were collected in the mobile examination center (2003-2004: n=9,643, 2005-2006: n=9,950). Several subpopulations were oversampled during the 2003-2006 study years: elderly individuals,

adolescents, pregnant women, Mexican Americans, African Americans, and low-income non-Hispanic white persons. Details of the design and variables of the NHANES 2003-2004(26) and 2005-2006(27) datasets have previously been described. Postmenopausal women, nonpregnant premenopausal women and men 50 years or older who underwent the health examination and did not have childhood onset diabetes, pre-diabetes or type 1 diabetes are included in this analysis. Only individuals with fasting blood samples were included because fasting blood glucose levels were used to determine if the individual had T2D. The final sample included 1471 postmenopausal women (1347 free of diabetes and 124 with type 2 diabetes); 2494 premenopausal women (2453 free of diabetes and 41 with type 2 diabetes); and 1442 men aged 50 or greater (1294 free of diabetes and 148 with type 2 diabetes).

2.3.2 Laboratory Methods

All NHANES laboratory methods are detailed in laboratory manuals by each 2-year survey. In summary, CRP was measured in serum using latex-enhanced nephelometry on a Behring Nephelometer.(28) Particles consisting of a polystyrene core and a hydrophilic shell were used to enhance the assay. The particles are used to covalently bind mouse monoclonal anti-CRP antibodies. The test sample containing unknown CRP was diluted then mixed with the anti-CRP antibodies linked to the particles so CRP can bind to the anti-CRP antibody-linked particles. The light scattered was measured by nephelometry and was proportional to the concentration of CRP in the test sample. An automatic blank was subtracted from the measured value then calculated using a calibration curve. A logit-log function was used to determine the concentration of CRP. Based on the inflammatory biomarker concentrations reported by Khaodhiar et. al.(29), CRP levels greater than or equal to 1.3 mg/L are considered elevated (inflamed) for this study.

The following vitamin and nutrients were measured in serum: alpha tocopherol, lycopene, trans beta carotene, cis beta carotene, and alpha carotene.(30) Alpha tocopherol and carotenoids were measured using high performance liquid chromatography (HPLC) with photodiode array detection. Serum (100 μL) was mixed with a solution of ethanol and 2 internal standards. The aqueous phase containing the micronutrients was removed, re-dissolved in ethanol and acetonitrile, then filtered. The filtrate was then injected into a C-18 reversed phase column, then isocratically eluted with a solution containing equal parts of ethanol and acetonitrile (mobile phase). Spectrophotemetry was used to measure absorbance. The absorbance measurements of the micronutrients were linearly proportional to the concentration. Absorbance peaks of each micronutrient are then compared to known concentrations of the micronutrient to quantify the concentration.

Vitamin C was measured in serum by isocratic HPLC with electrochemical detection.(31) To stabilize the ascorbate, the serum was acidified by mixing 1 part serum with 4 parts 6% MPA. The test sample was then frozen at -70ºC. Upon analysis, the sample was thawed at room temperature then centrifuged. The supernatant was removed for analysis and mixed with a solution of trisodium phosphate and dithiothreitol then 1-methyl uric acid, as an internal standard. Again to stabilize the ascorbate, the test sample was mixed with 40% MPA. The test sample was then filtered and injected onto a C-18 reversed-phase column. The test sample was eluted with a mobile phase solution then adjusted to pH 3.0 ± 0.03 using a 10N sodium hydroxide solution. The peak area was measured, then the concentration of the test sample was determined using a standard curve developed with 3 external standards.

Triglycerides were measured in serum through a series of reactions.(32) The triglycerides were first converted into glycerol then, oxidized to generate H_2O_2. The H_2O_2

concentration is quantified by measuring the absorbance of a color indicator. The intensity of the color is proportional to the triglyceride concentration. Triglycerides were only measured in the half of the MEC population who were fasting at the time of the examination. LDL cholesterol was calculated using concentration measurements of total cholesterol, triglycerides, and HDL cholesterol. As a result LDL cholesterol concentration values were only available for the population who had triglycerides measurements.

2.3.3 Diabetes Definition

An individual was classified as having T2D if previously diagnosed with diabetes by a physician after age 18 and was not taking insulin at the time of the interview. Additionally, an individual was categorized as being diabetic if their fasting plasma glucose level was greater than or equal to 126 mg/dL, consistent with the American Diabetes Association definition.(33) An individual was classified as being pre-diabetic if fasting plasma glucose was greater than or equal to 100 but less than 126 mg/dL.(33) Pre-diabetic individuals were not included in the analysis because they did not meet the diagnosis criteria for T2D (the disease of interest) and were not an appropriate comparison group, as their risk of developing T2D was much larger than the general population. An individual was classified as being non-diabetic if fasting plasma glucose was less than 100 mg/dL, consistent with the American Diabetes Association definition(33), and the individual was not previously diagnosed with diabetes.

2.3.4 Variables

Additional important covariates that are recognized correlates of type 2 diabetes and represent potential confounders include age, ethnicity, BMI, physical activity, hormone replacement therapy (HRT) use, smoking, family history of diabetes, and gender.

- Age in years was measured at the time of the evaluation.

- Ethnicity was categorized into the following groups: Non-Hispanic white, Non-Hispanic black, Mexican American and other. These ethnicity groups were consistent with the original NHANES categorization except the group Other Hispanic was combined with other.
- BMI (kg/m2) was categorized according to the World Health Organization definitions for overweight and obese for descriptive purposes, but used as a continuous variable in the models.
- Physical activity was assessed combining various physical activity evaluations into the following 2 general categories: sedentary to light activity (activity less than 9 times per month) and moderate to greater activity (activity 9 or more times per month). The US Department of Health and Human Services(34) states that health benefits are observed for adults engaging in aerobic activity at least 3 times per week. Since 2 times per week would equal 8 times per month, individuals who engaged in physical activity less than 9 times per month were categorized as sedentary to light activity. If a person engaged in physical activity 9 or more times per month then that individual was categorized as moderate to greater activity.
- Recently, estrogen(35-40) has been linked with the occurrence of diabetes, insulin resistance or IGT. Diabetes risk in women increases with the onset of menopause and the concurrent decrease in estrogen,(35) highlighting the importance of this pathway for postmenopausal women. There is evidence that estradiol increases insulin secretion through its effects on pancreatic beta-cells.(35;41) Since type 2 diabetes is not an acute onset disease, the use of HRT was only considered a relevant exposure in this study if used for one year or longer. Therefore HRT use, including both patch

and pill, of greater than one year was divided into: any estrogen HRT or non-estrogen HRT. An individual with less than one year exposure to HRT was categorized as no HRT.

- Smoking was categorized into the following 2 groups: never or former smoker and current smoker.
- Family history of diabetes was a binary variable: yes or no.
- Gender was categorized as men and women then further subcategorized into: premenopausal women, postmenopausal women, and men greater than or equal to 50 years old. Men were subcategorized according to age groups that aligned with those age groups used for postmenopausal women. Men less than 50 years are not reported in this study because they were not considered a relevant comparison group for postmenopausal women. However, young men were not excluded from the analysis due to statistical requirements for the use of weighting in the complex stratified multistage probability sampling method used in NHANES.(42)

2.3.5 Statistical Methods

Frequencies and percents of select participant characteristics were computed using SAS proc surveyfreq and unadjusted means. The means and 95% confidence intervals were calculated.

Since this was a complex sampling design, the weights for the mobile examination center evaluations were used. SAS software version 9.2 was used for this analysis. The weights account for oversampling, non-response and non-coverage.(3)

Micronutrient means and 95% CIs were adjusted for age, BMI, race, family history of diabetes, physical activity, HRT with estrogen, triglycerides, LDL cholesterol, smoking, and

gender (for male versus female comparisons) using proc surveyreg. GraphPad Prism Software (version 5.03, GraphPad Software Inc., La Jolla, CA) was used to generate graphs. T-tests on the adjusted data were used to test differences between non-diabetic and T2D postmenopausal women and to test differences between postmenopausal women and both premenopausal women and men 50 years or older.

Logistic regression was used to evaluate the association between nutrients and CRP levels and T2D for postmenopausal women. BMI, age, race, physical activity, HRT with estrogen, LDL cholesterol, triglycerides, smoking status and family history of diabetes were included in the models as potential confounding variables. Adjusted odds ratios and 95% confidence intervals were calculated for each variable of interest. Given that raw nutrient levels were skewed, we compared the first and the fourth quartiles for the odds ratios. (Table 2- 1) The odds ratio indicates the proportional change in risk of the outcome (either inflammation or T2D) for the fourth (highest) quartile versus the first (lowest) quartile. For example, an odds ratio of 0.85 would indicate a 15% decrease in outcome occurrence in the fourth quartile versus the first quartile of a nutrient concentration. All statistical analyses were conducted using SAS (version 9.2; SAS Institute, Cary, NC).

2.4 *Results*

The majority (47.8% - 81.7%, range by group) of this population was non-Hispanic white. (Table 2- 2) Known T2D risk factors were evaluated (unadjusted) among all 5407 individuals who met the inclusion criteria for this study from NHANES years 2003-2006. Individuals with T2D were older (mean range: 48.8-66.9 years) than those without diabetes (35.4-62.6 years). A majority of the study population was non-Hispanic white (range: 47.8% - 81.8), followed by non-Hispanic black (8.8% - 13.3%). Individuals with T2D had a higher prevalence of obesity

(range: 44.3%-71.0%) compared to non-diabetic individuals (27.8%-31.3%). Individuals with T2D reported a family history of diabetes (range: 59.0% - 81.9%) more often than non-diabetic individuals (35.1% - 47.0%).

Since CRP is generally accepted as a predictor for T2D, we tested whether the data in our population of postmenopausal women was consistent with this claim. The adjusted mean [95% CI] serum CRP concentration was indeed higher in postmenopausal women with T2D (4.7 [4.0-5.4] mg/L) than in non-diabetic postmenopausal women (4.2 [3.9-4.6] mg/L). (Figure 2- 1) CRP concentrations in T2D premenopausal women were higher than in non-diabetic women, but each of the group means were substantially higher than the corresponding groups for postmenopausal women. Men 50 years or older did not have a discernable difference between non-diabetic and T2D men. Mean CRP concentrations were comparable to those in postmenopausal women.

Additionally, the unadjusted association of CRP (highest quartile versus lowest quartile) with T2D in postmenopausal women was consistent with this claim (OR 3.6 [95% CI 1.0-13.2]). (Table 2- 3) However, the adjusted (including BMI) association of CRP with T2D was not consistent with the claim (0.6 [0.2-2.3]). Similar results were observed in men 50 years or older, but due to the small number of premenopausal women with T2D the CRP-T2D association could not be determined.

We tested whether each serum nutrient concentration (highest quartile versus lowest quartile) was associated with inflammation (CRP). We found inverse associations between nutrients and inflammation in postmenopausal women with adjusted odds ratios ranging from 0.44-0.57, however none were statistically significant. (Table 2- 3) In premenopausal women all nutrients except lycopene were inversely associated with inflammation in the adjusted models. Lycopene was positively associated with inflammation (OR 5.2 95% CI [1.6, 17.0]), although

there were only five women in the lowest quartile of lycopene with CRP≥1.3 mg/mL and there were no women in the highest quartile of lycopene with CRP≥1.3 mg/mL. Men 50 years or older had inverse associations between nutrients and inflammation ranging from 0.03-0.64. Only lycopene and alpha carotene were significantly associated with reduced subclinical inflammation risk in men 50 years or older (OR 0.03, 95% CI [0.0-0.42]; OR 0.17, 95% CI [0.05-0.6], respectively).

To further evaluate the hypothesized pathway where nutrients reduce inflammation and thereby reduce T2D risk, we examined the relationships between nutrients and T2D. Among T2D postmenopausal women, the adjusted nutrient concentrations were statistically different than those in non-diabetic women ($p<0.05$). (Figure 2- 1) Lycopene, trans beta carotene, cis beta carotene, alpha carotene, and vitamin C mean adjusted concentrations were lower in T2D postmenopausal women than non-diabetic women, while alpha tocopherol was higher. Mean adjusted nutrient concentrations in men 50 years and older were similar to postmenopausal women, although statistically significantly different due to the large sample size in NHANES. Overall premenopausal women (non-diabetic and T2D) had higher mean adjusted concentrations of lycopene and lower trans beta carotene, cis beta carotene, alpha carotene, vitamin C, and alpha tocopherol than postmenopausal women and men 50 years or older. Contrary to postmenopausal women and men 50 years and older, T2D premenopausal women had higher mean adjusted cis beta carotene concentrations than non-diabetic women.

Correspondingly, all nutrients had inverse adjusted associations with T2D in postmenopausal women (range: 0.24-0.75), however none were statistically significant. (Table 2-3) Only trans beta carotene in premenopausal women (OR 0.3, 95% CI [0.0-0.3]) and men 50

years or older (OR 0.5, 95% CI [0.2-0.98] or vitamin C in men 50 years or older (OR 0.3, 95% CI [0.1-0.7]) had statistically significant inverse associations with T2D risk.

2.5 *Discussion*

Our study highlights important differences, as well as some similarities, in plasma nutrient profiles and CRP concentrations and cross-sectional associations between postmenopausal women and two comparison groups (premenopausal women and men of a similar age) by T2D status. Postmenopausal women had higher adjusted nutrient concentrations than premenopausal women, except for lycopene where the concentrations were lower. Postmenopausal women had similar adjusted nutrient concentrations as men 50 years or older. However, adjusted mean CRP concentrations were higher in T2D postmenopausal women compared to T2D men. The inverse associations observed in all three groups for nutrients with inflammation were only significant in men 50 years or older for lycopene and alpha carotene. Similarly, inverse associations (although most were not significant) were observed in all three groups for most nutrients with T2D.

Each vitamin or nutrient has unique properties which influence blood concentrations and antioxidant capabilities. We found that lycopene concentrations were lower in postmenopausal women than premenopausal women. This finding may be explained given that lycopene is not as common in supplements as the other vitamins and nutrients. The adjusted mean lycopene concentration in postmenopausal women from NHANES was contained within the range of 23.1 to 105.8 μg/dL reported in the literature.(43-45) Previous studies examining dietary and plasma lycopene and T2D did not detect an association. (15;16) Lycopene did not clearly demonstrate an association with reduced T2D risk in our study population.

Trans and cis beta carotene concentrations were higher in postmenopausal women than premenopausal women, but similar to men. The adjusted mean concentration of trans and cis beta carotene was lower than the total beta carotene range of 45.6 to 83.2 μg/dL reported in the literature. (43-45) Previous studies did not detect an association between supplemental and plasma beta carotene and type 2 diabetes risk.(14;16;17) In contrast, we detected an inverse association of serum trans beta carotene with T2D in premenopausal women and men 50 years or older.

Speculation about the slightly elevated alpha carotene concentration in postmenopausal women compared to premenopausal women is more difficult to explain than lycopene, as less in known about this antioxidant. Postmenopausal women may have slightly elevated alpha carotene because this constituent is often found in multivitamins that also contain beta carotene. The adjusted mean concentration of alpha carotene in postmenopausal women from NHANES was lower than the range of 9.1 to 19.3 μg/dL reported in the literature. (43-45) A previous study examining the association between plasma alpha carotene and T2D risk did not find an association.(16) However, alpha carotene had a marginal inverse association with T2D in postmenopausal women in this study.

Following oxidation, vitamin C may be regenerated intracellularly then released back into the blood, although hyperglycemia limits the cellular uptake. (46;47) Since vitamin C has less regeneration in individuals with hyperglycemia, we would expect it to have lower concentrations among those with highest inflammation and highest glucose concentrations. However, we did not observe notable differences in vitamin C between T2D and non-diabetic individuals. Estimation of adjusted vitamin C concentrations in NHANES III also showed no differences between T2D individuals and non-diabetic individuals (48), however such differences were

reported from another cross-sectional study.(49) Nonetheless, we found that vitamin C was inversely associated with T2D in the highest CRP concentration group, premenopausal women (OR 0.46 95% CI [0.24-0.88]). The adjusted mean serum vitamin C concentrations we estimated were within the range of 0.39 to 1.19 mg/dL reported in the literature.(45;48-50)

Alpha tocopherol can be regenerated by vitamin C.(51) Additionally, alpha tocopherol is thought to be regenerated through other mechanisms as well and it is known to be difficult to deplete concentrations.(51) Therefore we would expect the alpha tocopherol concentrations to be somewhat stable and similar among the groups investigated. Indeed we did observe similar concentrations among postmenopausal women and men 50 years or older. Slightly lower concentrations of alpha tocopherol were observed in premenopausal women. The adjusted mean alpha tocopherol concentrations were within the range of 0.64 to 1.82 mg/dL reported in the literature. (43;45;50;52;53) Data on the possible role of alpha tocopherol in reducing inflammatory biomarkers have been conflicting. (54;55) Additionally, two studies examining supplemental vitamin E failed to show an association with T2D risk. (13;14) Similarly, we were not able to detect a statistically significant association between alpha tocopherol and either inflammation or T2D risk.

The differences observed in postmenopausal women compared to premenopausal women may stem in part from differences in multivitamin use. Concentrations of nutrients were consistently higher in postmenopausal women than premenopausal women, which is seemingly contradictory to what we would expect in this population with an increased T2D risk. However, postmenopausal women are known to consume multivitamins more frequently than premenopausal women and older men.(56;57) A diagnosis of T2D may further increase this consumption.(58)

Among women adjusted CRP concentrations were highest among T2D than non-diabetic individuals, but were similar for men 50 years or older. However, among T2D individuals, the adjusted CRP concentrations were higher among premenopausal women, than postmenopausal women and men, with the lowest levels observed among males. The non-significant adjusted inverse association of CRP with T2D risk was unexpected. Perhaps upon controlling for BMI and other covariates the unadjusted positive CRP-T2D association was diminished. The adjusted mean CRP concentration in non-diabetic and T2D men and women was generally higher than the values [4.3 ± 2.3(29) and 1.5 ± 0.3 mg/L(59)] reported in the literature for non-diabetic obese individuals.

A possible explanation for the CRP differences observed in postmenopausal women relative to premenopausal women or men 50 years or older may be due to the changes that occur at menopause. Menopause initiates a transition from gluteal-femoral adipose tissue storage to abdominal adipose tissue storage.(21) Abdominal adipose tissue is responsible for the production of proinflammatory cytokines, such as IL-6 (interleukin-6). IL-6 stimulates the production of CRP, an inflammatory biomarker. The inflammatory response in the body also produces reactive oxygen species which can damage the body and further perpetuate inflammation. Extended exposure to this increased state of inflammation, subclinical chronic inflammation, can have adverse effects (3;4) and increased IL-6 and CRP concentrations have been associated with an increase in T2D risk.(19)

Nutrients have been studied extensively for their ability to quench ROS,(11;60;61) which has potential to reduce inflammation and T2D risk. However, definitive evidence supporting the ability of nutrients to effectively reduce oxidative stress (61) or T2D risk (13-17) has been contradictory. Most nutrients examined resulted in an inverse association (most were not

statistically significant) between inflamed individuals (≥1.3 mg/L) and non-inflamed individuals (<1.3 mg/L) among all three groups studied in the adjusted models. The exceptions included vitamin C in premenopausal women and alpha tocopherol in all groups. For alpha tocopherol, the 95% confidence intervals were very wide, indicating that the point estimates of the odds ratios may not be precise and therefore judgments about the direction of the association could not be made.

Similar to the results with inflammation, most nutrients had an inverse association (most were not statistically significant) with T2D risk using the adjusted models. The only exceptions were lycopene in premenopausal women and alpha tocopherol and lycopene in men 50 years and older. For these exceptions, the 95% confidence intervals were very wide, not allowing a judgment about the direction of the association to be made.

Furthermore, an approach of tailoring nutrient dietary intake based upon genetic factors could determine whether the particular nutrient will effectively improve the health of an individual.(62) Our findings are consistent with this tailored treatment regimen, suggesting that nutrients may play a role in reducing inflammation and T2D especially in women. Since postmenopausal women have increased inflammation (as measured by CRP) compared to men (22;23) and increased T2D risk compared to premenopausal women(18), they could have more potential for an observable effect in this pathway to reduce T2D risk.

Our study has many strengths including a large representative sample of the US population, health examinations and plasma concentrations rather than simply using dietary intake to estimate plasma exposure. However our findings must be interpreted with caution, given the cross-sectional study design and subsequent temporal ambiguity. Moreover the inflammation measured may be short term in nature and may not be representative of past

inflammation levels. Alpha tocopherol and carotenoids are lipid soluble, and therefore, plasma levels of biomarkers of those nutrients may not accurately reflect their physiologic status. Additionally there were only 41 premenopausal women with T2D representing the US population and therefore the results presented in this study may not be entirely reflective of the true population values.

Our findings indicate that postmenopausal women are a unique T2D risk group with respect to nutrient concentrations and CRP. Specifically, diabetic postmenopausal women have less inflammation and have higher nutrient biomarker levels than their premenopausal counterparts, while diabetic postmenopausal women have more inflammation and similar nutrient biomarker levels compared to their male counterparts. Our data underscore that stratification by menopausal status for women could be important for diabetes research. However, it is still unclear if the nutrients are potent enough at the levels consumed to actually prevent T2D. These findings may provide guidance for future research in nutrition and diabetes research. Prospective studies are needed to further elucidate possible dietary recommendations for vitamins and nutrients in postmenopausal women.

2.6 *Acknowledgements*

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2.7 *Tables*

Table 2- 1. Vitamin, nutrient, and CRP concentration quartiles, NHANES 2003-2006 (n=19,593)

	Unadjusted Estimate (95% CI)		
	25th percentile	Median	75th percentile
Lycopene (μg/dL)	29.0	40.9	54.7
Trans beta carotene (μg/dL)	6.7	11.2	19.6
Cis beta carotene (μg/dL)	0.5	0.8	1.3
Alpha carotene (μg/dL)	1.3	2.5	4.8
Vitamin C (mg/dL)	0.6	1.0	1.3
Alpha tocopherol (mg/dL)	0.8	1.1	1.4
CRP (mg/L)	0.4	1.4	3.8

The entire NHANES 2003-2006 population with nutrient concentrations were included.

Table 2- 2. Select characteristics of postmenopausal women, premenopausal women and men ≥50 years and occurrence of Type 2 Diabetes, NHANES 2003-2006

	Frequency (%, SE [a])					
	Postmenopausal Women		Premenopausal Women		Men ≥ 50 years	
	Non-diabetic	T2D	Non-diabetic	T2D	Non-diabetic	T2D
N	1347	124	2453	41	1294	148
Age (years)						
Mean (95% CI)	61.7 (60.6-62.8)	66.9 (64.2, 69.5)	35.4 (34.9, 35.9)	48.8 (43.1-54.5)	62.6 (61.8, 63.4)	64.4 (62.3, 66.5)
Ethnicity						
Non-Hispanic White	838 (81.8, 2.0)	55 (69.1, 5.0)	1053 (67.7, 2.4)	12 (47.8, 11.3)	809 (81.7, 2.3)	71 (70.6, 5.5)
Non-Hispanic Black	243 (9.2, 1.3)	25 (13.3, 3.0)	638 (13.0, 1.6)	6 (9.9, 4.4)	231 (8.8, 1.4)	33 (11.3, 2.3)
Mexican American	196 (3.2, 0.8)	37 (6.9, 2.4)	545 (8.7, 1.1)	18 (19.1, 6.5)	196 (3.7, 0.8)	35 (6.6, 2.1)
Other	70 (5.8, 0.8)	7 (10.7, 4.0)	217 (10.6, 1.1)	5 (23.2, 9.7)	58 (5.8, 1.0)	9 (11.5, 3.9)
BMI						
Normal	450 (36.4, 1.6)	22 (19.4, 3.7)	1075 (48.6, 1.7)	4 (10.9, 5.8)	399 (27.9, 1.7)	29 (19.4, 4.4)
Overweight	433 (32.5, 1.5)	33 (27.6, 5.1)	608 (23.6, 1.0)	5 (18.0, 8.4)	543 (43.7, 1.9)	56 (36.3, 4.7)
Obese	431 (31.3, 1.5)	67 (52.9, 6.0)	725 (27.8, 1.4)	30 (71.0, 8.9)	326 (28.3, 1.4)	58 (44.3, 4.4)
Physical Activity						
Sedentary or Light	1004 (72.9, 1.6)	97 (72.0, 3.9)	1578 (67.3, 1.6)	32 (80.6, 7.7)	908 (69.2, 1.8)	112 (71.9, 3.7)
Moderate or Greater	343 (27.1, 1.6)	27 (28.0, 3.9)	875 (32.7, 1.6)	9 (19.4, 7.7)	386 (30.8, 1.8)	36 (28.1, 3.7)
Smoking						
Never or Former	1133 (82.3, 1.3)	104 (85.6, 4.9)	1509 (78.1, 1.3)	27 (64.9, 11.5)	1005 (78.5, 1.5)	127 (84.2, 3.8)
Current Smoker	188 (17.7, 1.3)	16 (14.4, 4.9)	377 (21.9, 1.3)	9 (35.1, 11.5)	257 (21.5, 1.5)	17 (15.8 , 3.8)
HRT with Estrogen	468 (39.8, 1.4)	34 (33.5, 4.1)	15 (0.9, 0.3)	0 (---, ---)	---	---
Family History of Diabetes	581 (43.2, 1.5)	80 (63.1, 5.3)	937 (47.0, 1.8)	29 (81.9, 6.8)	437 (35.1, 2.0)	85 (59.0, 4.4)

[a] SE=standard error of estimated percent

Table 2- 3. Associations between concentrations of vitamins and nutrients and inflammation or between vitamins, nutrients, and CRP and type 2 diabetes in postmenopausal women, premenopausal women and men ≥50 years

	Odds Ratios (95% CI)[a]					
	Postmenopausal Women (n=1471)		Premenopausal Women (n=2494)		Men ≥50 Years (n=1442)	
	Unadjusted	Adjusted[b]	Unadjusted	Adjusted[b]	Unadjusted	Adjusted[b]
Inflamed/Non-inflamed[c]						
Lycopene	0.31 (0.16, 0.58)	0.44 (0.17, 1.14)	0.57 (0.27, 1.18)	5.24 (1.62, 17.02)	0.16 (0.06, 0.46)	0.03 (0.00, 0.42)
Trans beta carotene	0.11 (0.05, 0.24)	0.46 (0.10, 2.23)	0.21 (0.09, 0.45)	0.36 (0.07, 1.96)	0.39 (0.18, 0.85)	0.64 (0.22, 1.87)
Cis beta carotene	0.15 (0.07, 0.30)	0.57 (0.15, 2.14)	0.27 (0.13, 0.58)	0.86 (0.20, 3.70)	0.64 (0.23, 1.79)	0.42 (0.10, 1.78)
Alpha carotene	0.15 (0.08, 0.29)	0.51 (0.10, 2.50)	0.28 (0.16, 0.50)	0.55 (0.17, 1.76)	0.30 (0.12, 0.75)	0.17 (0.05, 0.59)
Vitamin C	0.35 (0.15, 0.82)	0.55 (0.10, 3.08)	0.43 (0.22, 0.82)	0.39 (0.12, 1.28)	0.16 (0.06, 1.28)	0.23 (0.04, 1.43)
Alpha tocopherol	0.34 (0.15, 0.79)	0.47 (0.14, 1.53)	0.86 (0.38, 1.94)	0.23 (0.04, 1.54)	0.42 (0.20, 0.90)	0.19 (0.02, 1.57)
Type 2 diabetic/Non-diabetic						
Lycopene	0.47 (0.20, 1.11)	0.75 (0.27, 2.08)	0.75 (0.24, 2.35)	1.24 (0.31, 4.98)	0.72 (0.37, 1.43)	1.40 (0.59, 3.32)
Trans beta carotene	0.39 (0.19, 0.84)	0.51 (0.14, 1.78)	0.29 (0.09, 0.90)	0.03 (0.00, 0.34)	0.54 (0.31, 0.93)	0.45 (0.21, 0.98)
Cis beta carotene	0.41 (0.19, 0.89)	0.32 (0.09, 1.17)	0.77 (0.20, 1.96)	0.09 (0.00, 4.44)	0.61 (0.31, 1.19)	0.68 (0.23, 2.04)
Alpha carotene	0.32 (0.17, 0.60)	0.24 (0.06, 1.01)	0.68 (0.22, 2.06)	0.20 (0.02, 1.93)	0.89 (0.41, 1.95)	0.68 (0.25, 1.91)
Vitamin C	0.68 (0.41, 1.12)	0.65 (0.27, 1.55)	0.30 (0.08, 1.06)	0.17 (0.01, 2.21)	0.38 (0.20, 0.73)	0.25 (0.09, 0.72)
Alpha tocopherol	1.47 (0.52, 4.14)	0.52 (0.10, 2.70)	2.34 (0.76, 7.26)	0.10 (0.01, 1.85)	2.08 (0.99, 4.37)	1.60 (0.41, 6.31)
CRP	3.59 (0.97, 13.21)	0.59 (0.15, 2.33)	---[d]	---[d]	2.41 (0.85, 6.86)	0.19 (0.03, 1.25)

[a] Odds ratio compares lowest quartile to highest quartile, as presented in Table 2.

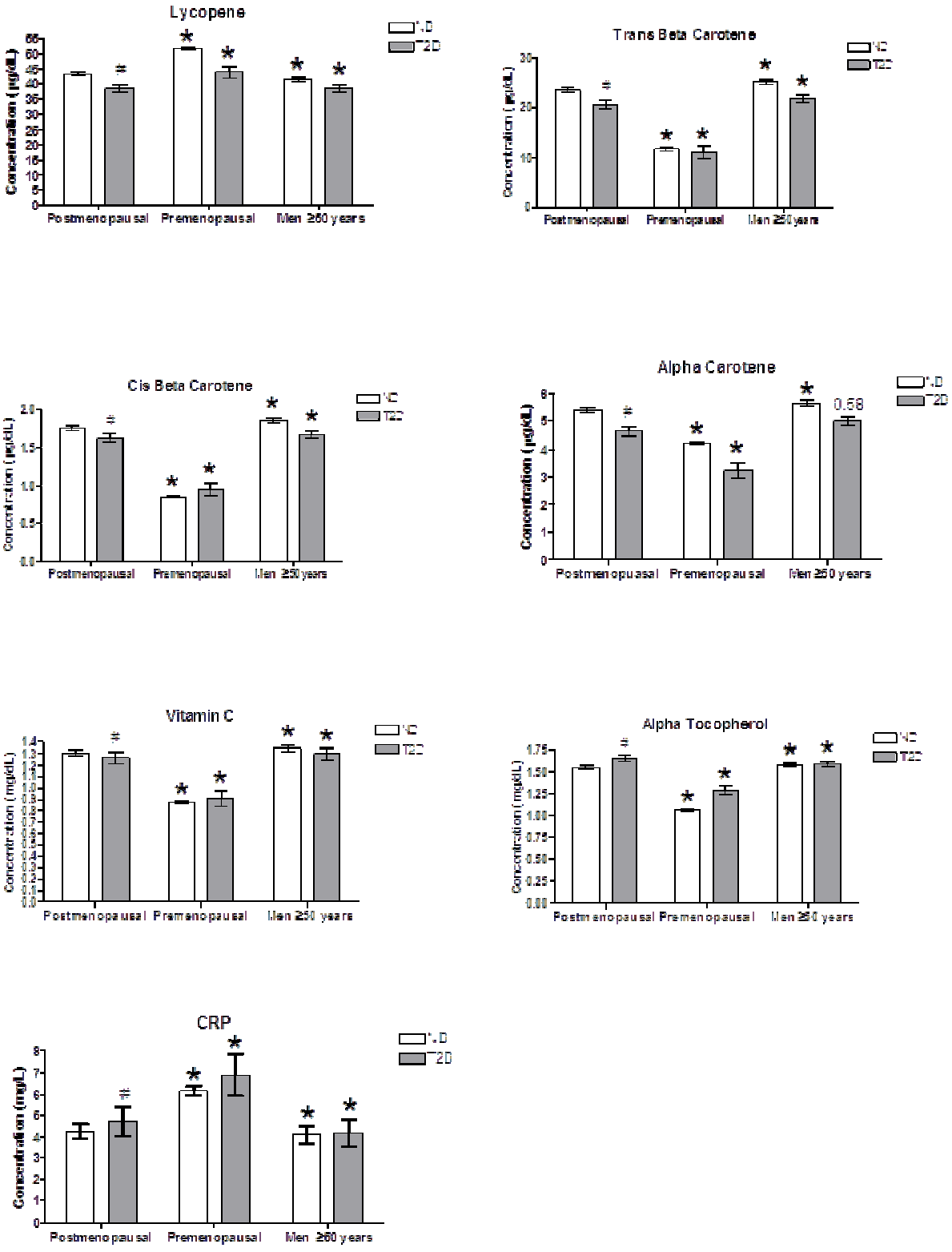
[b] Adjusted for BMI, age, race, physical activity, HRT with estrogen, LDL cholesterol, triglycerides, smoking, and family history of diabetes.

[c] Inflamed was CRP ≥1.3 mg/L and non-inflamed was CRP <1.3 mg/L.

[d] Undetermined due to small number of premenopausal women with type 2 diabetes.

2.8 *Figures*

Figure 2- 1. Adjusted serum vitamins, nutrients, or CRP concentrations by sex or menopausal status and health status (geometric mean and 95% confidence intervals)



Legend for Figure 2- 1: Concentrations were adjusted for BMI, age, race, physical activity, HRT with estrogen, smoking, LDL cholesterol, triglycerides, and family history of diabetes. ND=non-diabetic; T2D=type 2 diabetes. T-tests were conducted with log-transformed data. P-values for the differences between ND and T2D postmenopausal women are presented with "#" when $p<0.05$ or with the value. P-values for the differences between postmenopausal women and premenopausal women or men ≥50 years are presented with "*" when $p<0.05$ or with the value.

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CHAPTER 3

Dietary and supplemental nutrient consumption and inflammatory biomarkers in Type 2 diabetic and non-diabetic postmenopausal women

3.1 *Abstract*

Vitamins and nutrients inhibit oxidative stress which may reduce chronic inflammation and could prevent or delay the progression of type 2 diabetes (T2D). However this pathway remains controversial as many studies have not found an association between nutrients and T2D. We aimed to examine the separate relations of dietary or supplemental nutrients (alpha-tocopherol, vitamin C, alpha-carotene, beta-carotene, and lycopene) to biomarkers of inflammation [C-reactive protein (CRP), interleukin 6 (IL-6) tumor necrosis factor-α receptor II (TNF-R2)]. We examined 1,543 T2D cases and 2,170 matched controls nested within the Women's Health Initiative-Observational Study. Baseline plasma concentrations of CRP, IL-6, and TNF-R2 were measured. Baseline dietary and supplemental nutrient measurements were estimated using a validated semiquantitative food frequency questionnaire developed for this study.

After adjusting for age and BMI, dietary nutrients, including vitamin C (-0.08, $p<0.01$), beta-carotene (-0.08, $p<0.01$), and alpha-carotene (-0.07, $p<0.01$) were significantly inversely associated with IL-6. Additionally dietary beta-carotene was inversely associated with CRP (-0.09, $p=0.05$). Supplemental alpha tocopherol was inversely associated with CRP (-0.05, $p=0.02$) and IL-6 (-0.06, $p<0.01$). Supplemental beta-carotene was inversely associated with CRP (-0.07, $p=0.02$). Dietary vitamin C, beta-carotene, and alpha-carotene as well as supplemental vitamin E and beta-carotene demonstrate a modest inverse association with concentrations of systemic

inflammatory biomarkers among postmenopausal women and may contribute to a reduction in T2D risk.

3.2 *Introduction*

Oxidative stress and chronic subclinical inflammation are involved in the pathology of type 2 diabetes (T2D). (1-8) Higher levels of nutrients such as vitamin E, vitamin C, alpha-carotene, beta-carotene, and lycopene have been associated with reduced circulating concentrations of systemic inflammatory markers(9) and decreased risk of T2D(6;7) and may play a role in the prevention of metabolic disorders through reduced oxidative stress.(10) The antioxidants are believed to quench reactive oxygen species (ROS), the hallmark of oxidative stress, and reduce oxidative damage thereby reducing inflammation.(11)

Although this pathway seems biologically plausible, the nutrient-T2D association remains controversial. Previous studies of supplemental vitamin E (12;13), supplemental vitamin C (14), dietary and plasma lycopene (15;16), supplemental and plasma beta-carotene(17-19), and plasma alpha-carotene(20) and T2D have failed to detect an association.

Recent evidence suggests that nutrient supplements are not associated with reduced inflammation.(21) Therefore, we aimed to examine the separate cross-sectional associations of dietary and supplemental nutrients obtained by a semiquantitative food frequency questionnaire with plasma concentrations of inflammatory markers in a subset of postmenopausal women enrolled in the Women's Health Initiative Observational Study (WHI-OS).

3.3 *Subjects and Methods*

3.3.1 Study Population

The Women's Health Initiative Observational Study (WHI-OS) is a large prospective cohort study of ethnically diverse postmenopausal women living in the US (n=93,676). Details of the design and baseline characteristics of the WHI participants have previously been provided.(22-24)

For the current analysis, we included a total of 3,713 postmenopausal women (1,543 T2D cases and 2,170 matched controls) from a case-control study nested within the WHI-OS. All women included in the nested case-control study were free of cardiovascular disease, T2D, and cancers at baseline. A diabetes case was defined as a self-reported incident case on the annual follow-up form. Additional, cases were identified by questioning about the use of hypoglycemic medication or hospitalization for previously unreported diabetes. A complete description of case determination has been previously reported.(25) Cases were matched on race/ethnicity (white, black, Hispanic, and Asian/Pacific Islander), age, clinical center, time of blood draw, and follow-up time. White women were matched with a ratio of 1:1 cases and controls. Minority women were matched with a ratio of 1:2 cases and controls to increase statistical power in these populations because minorities comprised only 39 percent of the T2D cases identified.

3.3.2 Baseline Measurements

Dietary and supplemental nutrients, including alpha-tocopherol, vitamin C, alpha-carotene, beta-carotene, and lycopene, were measured at baseline with a validated semi-quantitative food frequency questionnaire (FFQ).(26) The FFQ was validated by examining 30 nutrients using 24-hour dietary recalls and 4-day food records. Most FFQ-estimated nutrients were within 10 percent of those estimated using the dietary recalls and food records.

Height and weight were measured at baseline by study personnel; body mass index (BMI) was calculated using these measurements. (27-29)

Inflammatory markers (tumor necrosis factor-α receptor II [TNF-R2], interleukin-6 [IL-6], and high-sensitivity C-reactive protein [hs-CRP]) were measured in blood specimens from selected participants in the WHI-OS. The methods have previously been described.(30) In brief, fasting blood samples were drawn at baseline. Samples were assayed in random order. TNF-R2 was assayed using an ELISA (R&D Systems, Minneapolis, Minnesota). IL-6 was assayed using an ultrasensitive ELISA (R&D Systems). CRP was assayed using an immunoturbidimetric assay (Denka Seiken Co Ltd, Niigata, Japan) on a chemistry analyzer (Hitachi 911; Roche Diagnostics, Indianapolis Indiana). The coefficients of variation for each analyte were 1.6% for hs-CRP, 7.6% for IL-6, 3.5% for TNF-R2.(31)

3.3.3 Statistical Analysis

The distributions of inflammatory biomarkers were skewed, and thus log transformations were performed to achieve normal distributions. Since we wanted to compare 2 different population samples (cases and controls), we used paired t-tests to compare continuous variables between the 2 samples. Cross-sectional correlations (multiple linear regression coefficients) between dietary nutrients and inflammatory biomarkers were assessed after adjusting for T2D and age or T2D, age and BMI. All statistical analyses were conducted using SAS (version 9.2; SAS Institute, Cary, NC).

3.4 *Results*

Demographic factors and associations of inflammatory biomarkers with T2D in this study population have previously been described.(32) The mean age of cases was 62.8 years and of non-cases was 62.5 years. Of the dietary nutrients examined, alpha-tocopherol and lycopene

were significantly higher in T2D cases than non-cases before adjustment (p=0.007 and p=0.003, respectively). (Table 3- 1) Dietary vitamin C, beta-carotene and alpha-carotene were significantly lower in T2D cases than non-cases before adjustment (p=0.01, p=0.001 and p=0.005, respectively). Conversely, no supplemental nutrients were significantly different while comparing T2D cases and non-cases. As previously reported, (33) concentrations of inflammatory biomarkers were significantly higher among T2D cases than non-cases (P<0.001).

The results of the age-adjusted cross-sectional correlations (multiple linear regression coefficients) between each individual nutrient and inflammatory biomarker suggest that dietary vitamin C (-0.09, p=0.01) and alpha-carotene (-0.09, p=0.00) were significantly inversely associated with IL-6. (Table 3- 2) Beta-carotene was inversely associated with CRP and IL-6 (-0.12, p=0.01 and 0.10, p=0.00, respectively). Lycopene had a positive, but small association with TNF-R2 (0.03, p=0.04). After further adjustment for BMI, these associations remained significant.

We also observed individual inverse, but small, associations between supplementary alpha-tocopherol and both CRP and IL-6 in both models (age-BMI model: -0.05, p=0.02 and -0.06, p=0.00, respectively).(Table 3- 2) Beta-carotene was inversely associated with IL-6 in the age-BMI model (-0.07, p=0.02).

3.5 *Discussion*

In this population of postmenopausal women, we observed that baseline dietary alpha-tocopherol was not significantly associated with baseline plasma concentrations of inflammatory biomarkers, however supplemental alpha-tocopherol was significantly and inversely associated with CRP and IL-6. While dietary lycopene had a positive, but small, association with only TNF-R2, supplemental beta carotene had an inverse association with CRP. Dietary vitamin C was

inversely associated with IL-6, but supplemental vitamin C was not associated with any of the inflammatory biomarkers. Additionally dietary alpha-carotene was inversely associated with IL-6 and dietary lycopene was positively associated with TNF-R2.

Oxidative stress is initiated by at least two pathways in T2D. First, ROS are a by-product of the conversion of glucose or fatty acids to energy (ATP) in the mitochondria of cells.(34) These ROS trigger the nuclear factor-kappa beta (NF-κB) and activator protein-1 transcription factors and initiate the production of pro-inflammatory cytokines. The cytokines activate the acute phase response of the immune system. In turn, this inflammatory response produces more ROS. Second, pro-inflammatory cytokines produced in the adipose tissue of overweight and obese individuals also activate the acute phase response of the immune system.(35) The NF-κB and activator protein-1 transcription factors are also sensitive to antioxidants and block NF-κB.(36) Additionally, antioxidants could quench ROS, rendering them inactive, unable to cause harm to the body.(37)

Despite such biological plausibility, it has been difficult to demonstrate the nutrient-T2D associations. Vitamin E supplementation was reported as having a relative risk (RR) of 0.95 ([95% CI: 0.87-1.05;], P = 0.31).for T2D in the Women's Health Study, a slight protective effect, which was not significant.(38) In contrast, in the Women's Antioxidant Cardiovascular Study (WACS), vitamin E treatment had a RR of 1.13 (95% CI: 0.99, 1.29; P = 0.07).(39) Vitamin C supplementation was reported as having a RR of 0.89 (95% CI: 0.78, 1.02; P = 0.09)] on T2D risk compared to placebo in the WACS.(40) Lycopene was reported as having an OR of 1.13 (95% CI: 0.60, 2.13) in a nested case-control study in US women aged 45 and older.(41) In a large prospective cohort study, lycopene was reported as having a RR of 1.07 (0.91-1.26) on T2D risk (first vs. fifth quintile).(42) Beta-carotene supplementation was reported as having a

RR of 0.97 (95% CI: 0.85, 1.11; P = 0.68) on T2D risk compared to placebo in the WACS.(43) In another nested case-control study, in US women aged 45 and older, beta-carotene was reported as having an OR of 1.10 (95% CI: 0.57, 2.13).(44) Beta-carotene supplementation was also reported as having a RR of 0.98 (95% CI:, 0.85-1.12) in the Physician's Health Study.(45) Alpha-carotene in a nested case-control study in US women aged 45 and older was reported as having an OR of 1.27 (95% CI: 0.63, 2.57).(46)

These non-significant reports from various studies range from a small protective to a small causal effect, but no consistent pattern was observed. Some studies evaluated supplements while others evaluated a combination of dietary and supplemental consumption of nutrients. Nonetheless this biological pathway remains plausible. Due to a recent review(21) of nutrients' effects on RA and osteoarthritis which concluded that supplemental vitamins A, C, and E did not demonstrate an improvement for these inflammatory conditions, we decided to examine whether it was the dietary nutrients alone, rather than the supplemental nutrients, which could produce the hypothesized benefit to systemic inflammation.

In our study population, a strong positive association of inflammatory biomarkers (IL-6: RR=3.08 [95% CI:, 2.25, -4.23] and CRP: RR=3.46 [95% CI:, 2.50, -4.80]) with T2D risk was previously reported (47), which is consistent with the biologic pathway previously described. We now report an inverse association of supplemental vitamin E (alpha-tocopherol), but not dietary vitamin E. We also found that dietary beta-carotene was inversely associated with CRP and IL-6, but supplemental beta-carotene was only inversely associated with CRP. Dietary vitamin C was inversely associated with IL-6, but supplemental vitamin C was not associated with any of the inflammatory biomarkers examined.

These results suggest that supplemental vitamin E and beta-carotene may reduce inflammation and subsequently T2D risk. Additionally, these results support a recommendation to increase dietary beta-carotene and vitamin C. Perhaps previous studies did not detect an association of nutrient consumption with T2D risk because each nutrient alone contributes a small but currently undetectable effect on T2D risk through reduced inflammation. Bias and residual confounding may have also contributed to the undetected associations.

We also report a small positive significant and association of dietary lycopene with the inflammatory biomarker TNF-R2, which is not consistent with the biologic pathway previously described. Thus, the effects of lycopene on inflammation remain unclear.

There are several strengths to this study including a validation of the food frequency questionnaire, baseline biomarker concentration measurements that were taken before the diagnosis of T2D. Nonetheless it cannot be discounted that this study suffers from the following inherent limitations of observational studies that merit consideration. Our findings may be due to measurement error and unmeasured confounding. First, cross-sectional associations cannot tease out the temporal relation between nutrient consumption and biomarker concentrations. Second, there may be some measurement error in the biomarkers with use only of single measurements, due to assay variability or degradation of the biomarkers; however, we expect that this bias should be non-differential between cases and non-cases. Third, our study did not account for multiple comparisons and some of the findings may be spurious. Fourth, FFQs may also produce some measurement error in assessment of nutrient consumption. Although a FFQ is useful for estimating various dietary factors and was validated in this study, it is not exact. Further, the nutrient calculations are a secondary variable estimated from other variables with measurement error. Many foods that are high in nutrients of interest were not assessed in the

FFQ for example blueberries, pomegranates, grapes and oranges. A participant may have been mislabeled as consuming low levels of nutrients if the types of foods high in nutrients that they were consumed were not included in the questionnaire.

Furthermore, a large body of evidence examining the associations of individual nutrients with T2D risk has not found any associations. Therefore, we may have detected a spurious association in this single study.

In summary, our cross-sectional findings suggest that dietary vitamin C, beta-carotene and alpha-carotene are inversely associated with systemic inflammation. The results also suggest that supplemental vitamin E and beta-carotene are inversely associated with systemic inflammation. Further prospective studies are needed to confirm these cross-sectional associations.

3.6 *Acknowledgements:*

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3.7 *Tables*

Table 3- 1. Dietary nutrient intake and concentration of inflammatory markers by diabetes case and control status among postmenopausal women (n=3,713)

Variable	n	Non-cases[a] Geometric Mean (95% CI) n=2170	n	T2D Cases Geometric Mean (95% CI) n=1543	p-value
Nutrients-Dietary					
Alpha-tocopherol (mg)	2156	4.84 (4.73, 4.96)	1532	5.10 (4.95, 5.25)	0.007
Vitamin C (mg)	2156	84.8 (82.4, 87.3)	1532	80.1 (77.4, 83.0)	0.01
Beta-carotene (mcg)	2156	2638 (2558, 2721)	1533	2429 (2337, 2526)	0.001
Alpha-carotene (mcg)	2156	473 (455, 491)	1533	433 (412, 454)	0.005
Lycopene (mcg)	2156	3326 (3210, 3446)	1532	3610 (3464, 3763)	0.003
Nutrients-Supplemental					
Alpha-tocopherol (mg)	1149	113 (104, 123)	759	116 (104,129)	0.70
Vitamin C (mg)	1161	235 (217, 254)	747	244 (222, 269)	0.54
Beta-carotene (mcg)	879	4060 (3937, 4186)	584	4510 (4338, 4688)	0.06
Inflammation biomarkers					
CRP (mg/dL)	1898	0.19 (0.18, 0.20)	1346	0.39 (0.37, 0.41)	<0.001
IL-6 (pg/mL)	1893	1.78 (1.72, 1.85)	1342	2.88 (2.75, 3.00)	<0.001
TNF R2 (ng/mL)	1888	2.36 (2.33, 2.39)	1337	2.68 (2.63, 2.72)	<0.001

All variables were log-transformed for t-test.

[a] Matched variables: ethnicity (white, black, Hispanic, and Asian/Pacific Islander), age, clinical center, time of blood draw, and follow-up time.

Table 3- 2. Cross-sectional correlations between dietary nutrient consumption and biomarkers of inflammation at baseline

	Inflammatory Biomarker[a] Multiple Regression Coefficient (p-value)					
	Age-Adjusted Model			Age- and BMI-Adjusted Model		
Nutrient	CRP	IL-6	TNF R2	CRP	IL-6	TNF R2
Dietary						
Alpha-tocopherol	0.038 (0.535)	-0.036 (0.388)	0.018 (0.290)	-0.023 (0.684)	-0.073 (0.059)	0.009 (0.613)
Vitamin C	-0.025 (0.628)	**-0.086 (0.012)**	-0.006 (0.682)	-0.021 (0.643)	**-0.084 (0.008)**	-0.005 (0.700)
Beta-carotene	**-0.116 (0.014)**	**-0.101 (0.002)**	-0.012 (0.367)	**-0.087 (0.045)**	**-0.084 (0.005)**	-0.007 (0.570)
Alpha-carotene	-0.070 (0.065)	**-0.088 (0.001)**	0.007 (0.489)	-0.037 (0.284)	**-0.069 (0.004)**	0.013 (0.222)
Lycopene	0.053 (0.217)	-0.008 (0.795)	**0.025 (0.039)**	0.056 (0.151)	-0.005 (0.840)	**0.025 (0.031)**
Supplemental						
Alpha-tocopherol	**-0.059 (0.010)**	**-0.063 (<0.001)**	0.001 (0.849)	**-0.050 (0.015)**	**-0.058 (<0.001)**	0.003 (0.679)
Vitamin C	**-0.054 (0.023)**	-0.026 (0.109)	0.001 (0.886)	-0.033 (0.135)	-0.013 (0.396)	0.004 (0.498)
Beta-carotene	-0.062 (0.064)	-0.016 (0.478)	0.003 (0.763)	**-0.072 (0.017)**	-0.022 (0.283)	0.001 (0.905)

[a]Nutrients and inflammatory biomarkers were log-transformed
Bolded values have a p-value≤0.05.

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CHAPTER 4

Interrelationship between alcohol intake and endogenous sex-steroid hormones on diabetes risk in postmenopausal women

4.1 *Abstract*

Previous prospective studies have documented an inverse association between moderate alcohol intake and type 2 diabetes (T2D). There is some evidence implicating alcohol in affecting plasma levels of various sex hormones. Additionally, sex hormones have been associated with T2D risk. We examined whether circulating concentrations of sex hormones, including estradiol, testosterone, sex hormone-binding globulin (SHBG), and dehydroepiandrosterone sulfate (DHEAS), were associated with alcohol intake or mediated the alcohol-T2D association. Among women not using hormone replacement therapy and free of baseline cardiovascular disease, cancer, and diabetes in the Women's Health Study, 359 incident cases of T2D and 359 matched controls were chosen during 10 years of follow-up.

Women with frequent alcohol intake (≥1 drink/day) were positively and significantly associated with higher plasma estradiol concentrations in an age-adjusted model (β=0.14, 95% CI, 0.03, 0.26), as compared with those who rarely/never drank alcohol. After adjusting for additional known covariates, this alcohol-estradiol association remained (β=0.19, 95% CI, 0.07, 0.30). Testosterone (β=0.13, 95% CI, -0.05, 0.31), SHBG (β=0.07, 95% CI, -0.07, 0.20), and DHEAS (β=0.14, 95% CI, -0.04, 0.31) showed positive associations without statistical significance. Estradiol alone or in combination with SHBG appeared to influence the observed protective association between frequent alcohol consumption and T2D risk, with a 12-21% reduction in OR in the multivariate-adjusted models. Our cross-sectional analysis showed positive associations between alcohol intake and endogenous estradiol concentrations. Our

prospective data suggested that baseline concentrations of estradiol and SHBG might influence the alcohol-T2D association in postmenopausal women.

4.2 *Introduction*

Recent data indicate that endogenous sex hormones play an important role in the pathogenesis of type 2 diabetes (T2D).(1) Sex hormones, including estrogen,(1;2) testosterone, (3-5) sex hormone-binding globulin (SHBG), (4;6;7) and dehydroepiandrosterone sulfate (DHEAS),(4;8) have been linked with insulin resistance, impaired glucose tolerance (IGT), and T2D risk. With the onset of menopause and the concurrent decrease in estrogen, T2D risk increases among postmenopausal women.(2)

There is some evidence suggesting that alcohol might be associated with increased concentrations of estrogen(9;10) and DHEAS.(9;11-13) Previous prospective studies have documented an inverse association between moderate alcohol consumption and the incidence of T2D;(14-23) however, the mechanisms underlying this potential benefit from alcohol intake are not completely understood. Given these interrelationships among alcohol intake, sex hormones, and T2D, it seems reasonable to hypothesize that sex hormones may, at least in part, explain the inverse relations between alcohol consumption and T2D risk. However, there is as yet no study directly testing these hormone-mediating pathways linking alcohol intake to T2D risk.

The objective of this study was to investigate whether alcohol consumption was associated with circulating concentrations of endogenous sex hormones. Also, we aimed to examine whether circulating levels of endogenous sex hormones mediate the association between alcohol consumption and T2D risk.

4.3 *Subjects and Methods*

4.3.1 Study Population

The Women's Health Study (WHS) is a randomized, double-blind placebo-controlled clinical trial of aspirin and vitamin E for the primary prevention of cardiovascular disease (CVD) and cancer. The participants are 39,876 female health professionals who were 45 years or older, had no history of CVD and cancer (except nonmelanoma skin cancer). Details of this trial have previously been described.(24-26) Among a total of 27,962 postmenopausal women in the WHS who had not used hormone replacement therapy (HRT) and were free of CVD, cancer, and diabetes at baseline, 359 incident cases of T2D and 359 matched controls were chosen using risk set sampling strategy during a median of 10 year follow-up.(3) Controls were matched on age, race, fasting status at time of blood draw, and follow-up time.

Written informed consent was obtained from all participants in the WHS. This study was approved by the Institutional Review Boards of Brigham and Women's Hospital, Harvard Medical School, and the University of California at Los Angeles (UCLA).

4.3.2 Assessment of alcohol consumption and other covariates

Baseline information on usual diet, including alcohol intake, was provided by 39,310 (99%) of the randomized participants, who completed a 131-item, validated, semiquantitative food-frequency questionnaire (SFFQ). A detailed description of the SFFQ and procedures used to calculate nutrient intake as well as data on its reproducibility and validity in a similar cohort was previously reported.(27) For each food, a commonly used unit or portion size was specified on the questionnaire, and the participants were asked how often on average within the previous year they had consumed that amount. The portion sizes for beverages containing alcohol were "1 glass, bottle, can" for beer and light beer, "4 oz. glass" for red wine and white wine, and "1 drink

or shot" for liquor. Nine responses were possible, ranging from "never or less than once per month" to "6 or more times per day." These 9 categories were condensed into 4 categories because of the few women who reported frequent alcohol consumption. The 4 categories are rarely/never, 1-3 drinks/month, 1-6 drinks/week, and 1+drinks/day. Frequent alcohol consumption is defined as the highest category, 1+ drinks/day. These 9 categories were also used to calculate alcohol consumption as a continuous variable in g/day. We scaled the continuous alcohol variable, measured in grams/day by the US standard for one alcoholic beverage, 14 g, according to the International Center for Alcohol Policies (ICAP). Women who did not respond to any of the alcohol questions were excluded. In brief, Spearman's correlation coefficient between total alcohol consumption as measured by four 1-week diet records and the SFFQ was 0.90.(4)

Other covariates, including body weight, height, family history of diabetes, smoking status, and physical activity, were assessed using questionnaires. Dietary variables were energy-adjusted using the residual method.(28) Body mass index (BMI) was calculated as weight (in kg)/height^2 (in m^2).

4.3.3 Biomarker measurements

Baseline blood samples were centrifuged and stored in liquid nitrogen freezers until the time of laboratory analysis. Matched case-control pairs were handled identically and assayed in random sample order in the same analytical run. Laboratory personnel were blinded to case-control status during all assays. Hankinson and colleagues(29;30) reported that a single measurement of plasma levels of sex hormones can reliably reflect average long-term hormone levels over a 3-year period, with correlations ranging from 0.66 to 0.92 for plasma levels of sex hormones, including estradiol, estrone, estrone sulfate, androstenedione, testosterone,

dehydroepiandrosterone (DHEA), DHEAS, and SHBG. In brief, Chemiluminescent immunoassays (Elecsys autoanalyzer 2010, Roche Diagnostics, Indianapolis, IN) were used to measure sex hormones and SHBG. As reported previously, the coefficients of variation from blinded quality control samples were 5.2% for estradiol, 7.4% for testosterone, 2.8% for DHEAS, and 2.8% for SHBG.(3;7;31;32) The limits of detection were

4.3.4 Statistical Analysis

Age-adjusted, age-and BMI-adjusted, and multivariate linear regression models were used to examine the associations between alcohol intake and biomarker concentrations. Adjusted geometric means of biomarkers were calculated using a multivariable adjusted regression model while controlling for age, smoking, BMI, physical activity, and family history of diabetes. Linear regression modeling was also conducted with alcohol as a continuous variable.

Conditional logistic regression was performed to assess the impact of sex hormone concentrations on the association of alcohol intake with T2D risk. We first adjusted for matching factors such as age, ethnicity, and fasting status at time of blood draw. In multivariate analyses, we adjusted for BMI (continuous), family history of diabetes (yes or no), smoking (never, past, and current smokers), and physical activity (continuous). To evaluate whether sex hormone concentrations mediate the association between alcohol intake and T2D risk, each sex hormone biomarker (estradiol, testosterone, SHBG, and DHEAS) was added individually to the alcohol-T2D model. A change in the OR towards the null (OR=1) in estimates of the parameter between the models was used to indicate how various sex hormone biomarkers may mediate the pathway between alcohol consumption and reduced diabetes risk.

All statistical analyses were conducted using SAS (version 9.2; SAS Institute, Cary, NC). All p-values were two-tailed (α=0.05). Figures were constructed using GraphPad Prism Software (version 4.0, GraphPad Software Inc., La Jolla, CA).

4.4 *Results*

The amount of alcohol consumption was significantly different between cases and controls. Cases tended to consume less alcohol than controls (Table 4- 1). Overall, there were linear trends towards positive associations of alcohol consumption with all sex hormone biomarker concentrations (Figure 4- 1). SHBG (Rarely/never: 24.0±0.8, 1-3 drinks/month: 26.8±1.7, 1-6 drinks/week: 26.0±1.3, ≥1 drinks/day: 29.3±2.1 nmol/L) and DHEAS (Rarely/never: 74.1±1.7, 1-3 drinks/month: 74.9±3.1, 1-6 drinks/week: 78.7±2.7, ≥1 drinks/day: 85.2±4.3 μg/dL) showed a generally consistent trend for increase with increasing alcohol consumption. Nonetheless, the estradiol linear trend (Rarely/never: 20.1±0.4, 1-3 drinks/month: 20.1±0.7, 1-6 drinks/week: 19.8±0.5, ≥1 drinks/day: 22.0±0.9 pg/mL) was largely influenced by the highest alcohol consumption category and the testosterone trend (Rarely/never: 0.24±0.005, 1-3 drinks/month: 0.26±0.01, 1-6 drinks/week: 0.23±0.0.006, ≥1 drinks/day: 0.27±0.01 ng/mL) was not linear, as we observed a bimodal trend. These linear trends were adjusted for age, case/control status, BMI, smoking physical activity, and family history of diabetes. Specifically, geometric means of estradiol (p-value=0.002), SHBG (p-value<0.001), and DHEAS (p-value<0.001) significantly increased across increasing alcohol consumption category.

In the linear regression models adjusting for age and case/control status (Model 1) or BMI, age and case/control status (Model 2), levels of estradiol were positively associated with alcohol consumption (β=0.14, 95% CI, 0.03, 0.26 from Model 1 and β=0.17, 95% CI, 0.06, 0.29 from Model 2) (Table 4- 2). Beta (β) indicates the amount that the sex hormone increases in

concentration (estradiol: pg/mL, testosterone: ng/mL, SHBG: nmol/L, DHEAS: μg/dL) with alcohol consumption compared to rarely/never consumption. Upon further adjustment for smoking, physical activity and family history of diabetes (Model 3), estradiol remained significantly associated with alcohol consumption (β=0.19, 95% CI, 0.07, 0.30). Testosterone, SHBG, and DHEAS were not significantly associated with frequent alcohol consumption, although each showed evidence of positive associations in the unadjusted model (Model 1). When alcohol consumption was evaluated as a continuous variable using the full model, women had significantly increased estradiol (β=0.07, 95% CI, 0.02, 0.12) and DHEAS (β=0.10, 95% CI, 0.03, 0.18). Beta (β) for the continuous variable indicates the amount that the sex hormone increases in concentration with each additional alcoholic beverage (14 g alcohol) consumed in a day.

We also examined associations between alcohol consumption and sex hormones by type of alcohol (data not shown). Each of the types of alcohol consumption, red wine, white wine, liquor and beer, demonstrated positive associations. After adjusting for covariates, only liquor consumption was positively associated with estradiol (β=0.32, 95% CI, 0.17, 0.48). Due to small numbers of subjects in each strata, the trend for a positive association with alcohol consumption for estradiol, testosterone and DHEAS and mixed results for the SHBG-alcohol consumption association were observed.

We examined and confirmed the inverse association between alcohol consumption and T2D risk in our population. As compared with women who rarely/never drank alcohol, women who drank alcohol ≥1 drink/day had reduced T2D risk (OR=0.43, 95% CI, 0.19, 0.99) (Table 4-3). Furthermore, we tested for mediation of the alcohol-diabetes association by sex steroid hormones. The addition of SHBG in the unadjusted model only suggested mediation between

alcohol consumption and T2D risk, however this difference was not observed in the full model. The addition of DHEAS in the full model did slightly shift the OR towards the null value (unadjusted: 3% change; full model: 5% change). Although estradiol did not seem to mediate the alcohol-T2D association with a shift in OR towards the null value, it did influence the association with a 12% reduction in the OR. In the model with both estradiol and SHBG, we observed a 21% reduction in the OR.

4.5 *Discussion*

Our cross-sectional analysis of 718 postmenopausal women suggested a positive association between alcohol intake and concentrations of endogenous estradiol, independent of age, BMI, physical activity, race, family history of diabetes and smoking. In our nested case-control study, we found that concentrations of endogenous estradiol alone or together with SHBG apparently influenced the prospective association of alcohol consumption and T2D.

Diabetes risk increases in middle-aged women when menopause occurs (with a pronounced decrease in estrogen).(2) As previously reported, circulating concentrations of estradiol and testosterone were higher in cases, while SHBG and DHEAS were lower in cases than matched controls in our study population.(3) Both DHEAS and estradiol increase insulin secretion through their effects on pancreatic β-cells (2;33) and DHEAS improves insulin sensitivity by inducing an increase in glucose transport activity.(8) Testosterone was reported higher in T2D women in cross-sectional studies and may increase risk for T2D in prospective studies.(1) Additionally, higher SHBG levels were reported to be more protective against diabetes risk in women than those with lower levels in contrast to men where lower SHBG levels were more protective.(1) These observations of T2D risk highlight the importance of the sex hormones in the T2D biologic pathway in postmenopausal women.

It has been suggested that alcohol, as a constituent in alcoholic beverages, may provide a protective effect on T2D risk.(23) The inverse association between alcohol consumption and type 2 diabetes risk observed in our study population is well established. (14-23) Correspondingly, alcohol has been suggested to influence the levels of sex hormones. Early reports had mixed results for the association of alcohol consumption and increased estradiol in postmenopausal women.(34) HRT was thought to modify this association; only those women taking HRT tended to have increased estradiol with alcohol consumption in a majority of the reports.(34) The increased estradiol was thought to be due to the inhibition of the conversion of estradiol to estrone.(34) More recently, results from a large cross-sectional study showed that postmenopausal women who drank more than 25 g alcohol per day had higher estradiol and DHEAS concentrations compared to nondrinkers independent of HRT.(35) Possible mechanisms for the increased estradiol include decreased catabolism of the sex hormones by the liver, increased aromatase activity, causing increased conversion of estradiol from androgens, or increased signaling of the adrenal gland to produce DHEAS (a precursor of estradiol).(35)

The beneficial effect of alcohol on type 2 diabetes risk is likely to occur through its influence on sex hormones as evidenced by the alcohol-sex hormone associations, alcohol-T2D association, and the sex hormones-T2D risk associations observed in our study population of postmenopausal women. Particularly estradiol and DHEAS have involvement in the insulin signaling pathway.

Experimental data indicate that estradiol increases insulin secretion through its effects on pancreatic β-cells.(2;33) Given the observed association between alcohol consumption and estradiol concentration in this study, it is possible that estradiol exerts its modulating effects on the protective effect of alcohol consumption on type 2 diabetes risk. Our study results provided

some suggestive evidence, but further mechanistic studies are warranted to elucidate the putative causal interrelationships. In addition, since SHBG binds estradiol and regulates the amount of available estradiol, (36) the interaction of these sex hormones may influence the association of alcohol with T2D risk.

DHEAS might be involved in the mechanisms underlying the effect of alcohol consumption on T2D risk as well. DHEAS has been shown to enhance insulin secretion in β-cells in vitro and in vivo (8) and improve insulin sensitivity by inducing an increase in glucose transport activity.(8) We observed an association between alcohol consumption and DHEAS concentration in this study. Similar to estradiol, it is conceivable that increased DHEAS induced by alcohol consumption may be one pathway by which alcohol exerts its protective effect on T2D risk. DHEAS is a precursor of estrogen,(37) DHEAS concentrations may also be influenced in this pathway. The mediation analysis provided a weak hint of mediation, suggesting that DHEAS may be a surrogate of estradiol or other sex hormone metabolisms in the pathway by which alcohol consumption reduces T2D risk.

Our study showed mixed results for SHBG, depending on which alcohol variable was used, categorical or continuous. This may be explained in part because of the "U-shaped" or "J-shaped" curve where risk beginning at rarely/never alcohol consumption decreases as alcohol consumption increases, reaches a nadir, then gradually increases again with increased heavy drinking. Another explanation could be SHBG's strong correlation with BMI (β=-0.51, p-value=0.008(38)). Due to this strong correlation, the effect of each constituent alone cannot be distinguished from the other. A third explanation for the mixed results could be due to low statistical power due to small numbers of subjects who consumed alcohol frequently.

At baseline BMI, physical activity and alcohol consumption were statistically significantly different between cases and controls. (Table 4- 1) These results were expected, as these variables are recognized risk factors for T2D. Since the primary comparisons were inflammation and alcohol consumption in our study, both measured at baseline before determination of case and control status, the alcohol consumption differences at baseline would not bias our results. Also since the BMI and physical activity measurements were made at baseline and incident cases of T2D were selected as cases, these differences would not bias the study results. Furthermore, we controlled for BMI, and physical activity in the final models.

This study is a risk-set sampled matched nested case-control study within the WHS. It has several strengths including a validation of the food frequency questionnaire, baseline biomarker concentration measurements, and prospectively collected disease diagnosis. However, there are some limitations that merit consideration. First, cross-sectional associations cannot tease out the causal relation between alcohol consumption and biomarker concentrations. Second, there may be some measurement error in the biomarkers with single measures, due to assay variability or degradation of the biomarkers. This bias should be non-differential among cases and controls, and therefore may cause bias to the null. Food frequency questionnaires, although validated in this study may also produce some measurement error in assessment of alcohol consumption. There remains the possibility of residual confounding due to unmeasured or poorly measured confounders, such as phytoestrogens. Finally, the results presented had wide confidence intervals, indicating some instability in the estimates, partially due to inadequate statistical power due to the small numbers of women who consumed alcohol frequently.

In conclusion, our cross-sectional analysis in this study suggested a positive association between alcohol intake and endogenous estradiol concentrations independent of covariates. Our

prospective results also suggested that baseline concentrations of estradiol alone or with SHBG may largely influence the alcohol-T2D association in postmenopausal women. Further investigation of our findings and possible mechanisms is warranted.

4.6 *Acknowledgements*

We would like to thank Sara Chacko for her editorial comments and Katie Chan for her review of the analysis and tables.

4.7 *Tables*

Table 4- 1. Baseline characteristics of 718 postmenopausal women participated in this prospective case-control study of type 2 diabetes

Variable	Cases (n=359)	Controls (n=359)	p-value
Mean±SD Age (years)	59.6 ± 6.1	59.6 ± 6.1	—
Race/Ethnicity			
White	332 [93]	332 [93]	—
Black	8 [2]	8 [2]	
Hispanic	5 [1]	5 [1]	
Asian/Pacific Islander	9 [3]	9 [3]	
Other/Unknown	5 [1]	5 [1]	
Mean±SD BMI (kg/m^2)	30.9 ± 6.1	26.0 ± 5.0	<0.001
Strenuous Physical Activity			
Rarely/Never	183 [51]	142 [40]	0.002[1]
<1 time/week	65 [18]	78 [22]	
1 time per week	28 [8]	28 [8]	
2-3 times/week	50 [14]	70 [19]	
4-6 times/week	23 [6]	27 [7]	
7+ times/week	9 [3]	14 [4]	
Smoking			
Never	52 [15]	49 [14]	0.57[1]
Past	136 [38]	132 [37]	
Current	170 [47]	178 [49]	
Total alcohol consumption			
Rarely/Never	218 [61]	169 [47]	<0.001[1]
1-3 drinks/month	49 [14]	55 [15]	
1-6 drinks/week	74 [20]	94 [26]	
≥ 1 drinks/day	18 [5]	41 [12]	

Presented as frequency [percent] unless otherwise noted as Mean ± SD. Age and Race/Ethnicity were matching variables and therefore were the same for cases and controls. [1] Mantel Haenszel Chi Square test for differences between cases and controls.

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Table 4- 2. Linear regression coefficients (95% CIs) for the increment in circulating concentrations of four endogenous sex hormone biomarkers according to each of the alcohol intake categories (as compared with never/rarely alcohol drinkers) among 718 postmenopausal women

N=718					
Model	Alcohol consumption	Estradiol	Testosterone	SHBG	DHEAS
Model 1	1-3 drinks/month	0.02 [-0.08, 0.11]	0.10 [-0.04, 0.24]	0.06 [-0.04, 0.17]	0.01 [-0.12, 0.14]
	1-6 drinks/week	0.02 [-0.06, 0.10]	0.01 [-0.12, 0.13]	0.02 [-0.07, 0.11]	0.03 [-0.03, 0.20]
	1+ drink/day	**0.13 [0.01, 0.25]**	0.14 [-0.05, 0.33]	0.08 [-0.06, 0.22]	0.11 [-0.06, 0.23]
Model 2	1-3 drinks/month	0.03 [-0.06, 0.12]	0.10 [-0.04, 0.24]	0.04 [-0.06, 0.14]	0.01 [-0.12, 0.14]
	1-6 drinks/week	0.04 [-0.04, 0.12]	0.01 [-0.12, 0.14]	-0.03 [-0.11, 0.06]	0.08 [-0.03, 0.20]
	1+ drink/day	**0.16 [0.04, 0.28]**	0.14 [-0.05, 0.33]	0.00 [-0.13, 0.14]	0.11 [-0.07, 0.28]
Model 3	1-3 drinks/month	0.04 [-0.05, 0.13]	0.12 [-0.03, 0.26]	0.04 [-0.06, 0.14]	0.03 [-0.11, 0.16]
	1-6 drinks/week	0.05 [-0.03, 0.13]	0.01 [-0.11, 0.14]	-0.02 [-0.11, 0.07]	0.03 [-0.02, 0.21]
	1+ drink/day	**0.17 [0.05, 0.29]**	0.12 [-0.07, 0.31]	0.02 [-0.11, 0.15]	0.11 [-0.02, 0.30]

Values presented are beta-coefficient [95% CIs]. Sex hormone concentrations were log-transformed. **Bolded** values have p-value<0.05.

Model 1: Adjusted for age

Model 2: Adjusted for case/control status, age, and BMI

Model 3: Adjusted for case/control status, age, BMI, race, family history of diabetes, physical activity, and smoking.

Table 4- 3. Prospective associations of alcohol intake with T2D risk and mediation by endogenous sex hormone concentrations

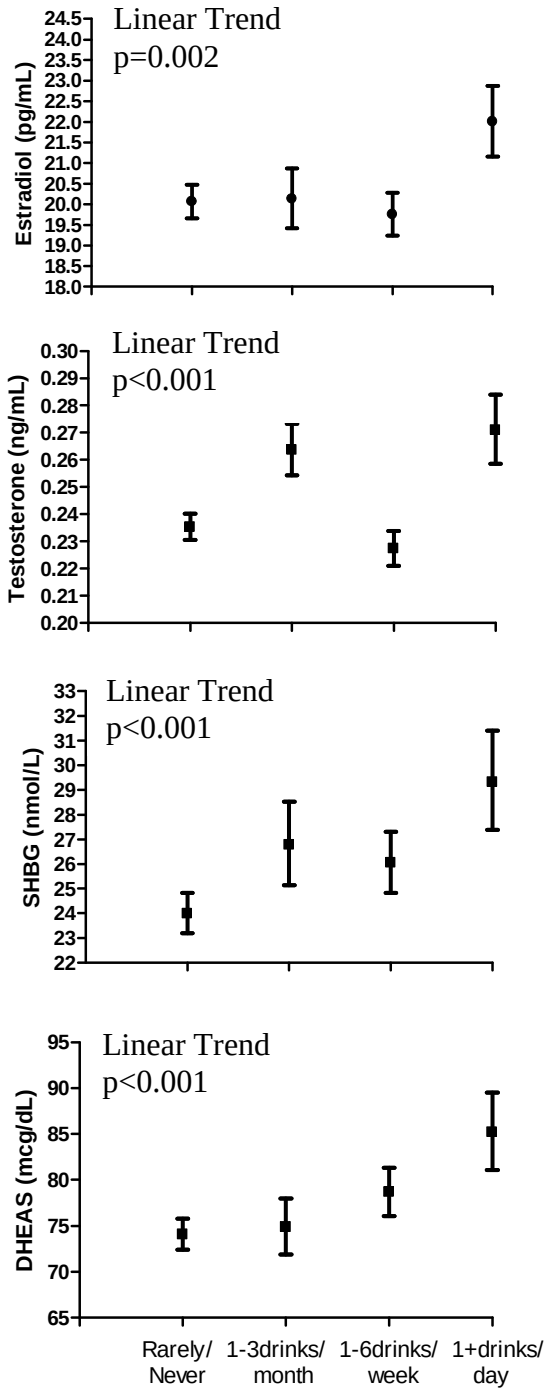
Alcohol Intake (Rarely/Never vs. ≥1 Drink/Day)	Un-adjusted[1]			Adjusted[2]		
Model	**OR**	**95% CI**	**Percent Difference**	**OR**	**95% CI**	**Percent Difference**
Model A	0.30	0.16, 0.58	--	0.43	0.19, 0.99	--
[Model A] + Estradiol	0.25	0.13, 0.50	-17	0.38	0.16, 0.89	-12
[Model A] + Testosterone	0.29	0.15, 0.57	-3	0.44	0.19, 1.01	2
[Model A] + SHBG	0.41	0.18, 0.93	37	0.44	0.18, 1.09	2
[Model A] + DHEAS	0.31	0.16, 0.59	3	0.45	0.20, 1.03	5
[Model A] + Estradiol + SHBG	0.35	0.15, 0.82	17	0.34	0.13, 0.90	-21
[Model A] + Testosterone + SHBG	0.40	0.18, 0.90	33	0.44	0.18, 1.09	2
[Model A] + Estradiol + Testosterone + SHBG	0.35	0.15, 0.83	17	0.34	0.13, 0.90	-21

[1] Adjusted only for matching

[2] Adjusted for age, BMI, smoking, physical activity, and family history of diabetes

4.8 *Figures*

Figure 4- 1. Adjusted baseline sex hormone concentrations across categories of alcohol consumption



Legend for Figure 4- 1: Geometric mean and 95% confidence intervals are presented. Geometric mean was adjusted for case/control status, age, BMI, smoking, physical activity, and family history of diabetes.

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