COMPARISONS OF PLASMA VITAMIN E CONCENTRATIONS IN MATERNAL SMOKERS AND NON-SMOKERS AND MATCHING UMBILICAL BLOOD CORD PAIRS

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A THESIS

Presented to the Department of Graduate Programs in Human Nutrition

and the Oregon Health & Science University

in partial fulfillment of

the requirements for the degree of

Master of Science in Clinical Nutrition

November 2012

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
ApoB100	Apo-lipoprotein B100, the primary apolipoprotein in LDL
α-CEHC	Alpha-carboxyethyl hydroxychroman
α-T	Alpha-tocopherol
α-TTP	Alpha-tocopherol transfer protein
BMI	Body mass index
CEHC	Carboxyethyl hydroxychroman
COPD	Chronic obstructive pulmonary disorder
c-section	Caesarian section
DNA	Deoxyribonucleic acid
DRI	Dietary reference intake
EPIC	Computer system for medical records
EtOH	Ethanol
FFQ	Food Frequency Questionnaire
g	Gram
γ-CEHC	Gamma carboxyethyl hydroxychroman
γ-T	Gamma-tocopherol
HDL	High density lipoproteins
HPLC	High performance liquid chromatography
IRB	International Review Board
IU	International units
kcal	Kilocalorie
kg	Kilogram
L	Liter
lbs	Pounds
LDL	Low density lipoproteins
LMP	Last menstrual period
LPL	Lipoprotein lipase
MDA	Malondialdehyde, marker of lipid peroxidation
μmol	Micromole
mg	Milligram
ml	Milliliter
mmol	Millimole
NDSR	Nutrition Data System for Research
ng	Nanogram
nmol	Nanomole
OHSU	Oregon Health & Science University
PCP	Primary care physician

PLTP	Phospholipid transfer protein
pmol	Picomole
RDA	Recommended dietary allowance
RRR--α-tocopherol	Natural form of α-tocopherol
SD	Standard deviation
SGA	Small for gestational age
SPSS	Statistical Package for the Social Sciences
tGy	Triglycerides
VLDL	Very low density lipoproteins
vs.	Versus
WHO	World Health Organization

Acknowledgements

I would like to thank my committee, Cynthia McEvoy, M.D., Melanie Gillingham, Ph.D., R.D., and Maret Traber, Ph.D., for their guidance, interest, and support in performing this research study. I would also like to thank Scott Leonard of the Linus Pauling Institute for teaching me analysis methods and analyzing samples for me, and to the Linus Pauling Institute for allowing me to use their supplies and instruments. I also owe a great debt of gratitude to Shelley Winn, Ph.D. of Oregon Health & Science University who helped with lipid analysis and allowed us to use his equipment. I would like to recognize Nakia Clay, "Vitamin C" study coordinator, and Brittany Vuylsteke for collecting the data used for my project. Finally, I would like to thank Mike Lasarav for assisting with the statistical analysis and interpretation during this research study.

ABSTRACT

Background:

Vitamin E is an antioxidant that protects cell membranes from lipid peroxidation. Vitamin C, a water soluble antioxidant, helps replenish vitamin E in the body thus enhancing the action of vitamin E as an antioxidant. The recommended dietary allowance (RDA) of vitamin E increases for smokers because of increased oxidant damage from cigarette smoking. Research has linked low levels of vitamin E in mothers to an increased risk of asthma, wheezing, and other pulmonary problems in the neonate, suggesting that neonates of maternal smokers are at an increased risk of having inadequate plasma concentrations of vitamin E and developing a pulmonary disorder.

Objective:

This study aimed to determine if greater vitamin C intake in mothers who smoked during pregnancy increased their vitamin E concentrations similar to mothers who did not smoke during pregnancy. It further aimed to determine if there were differences in plasma vitamin E and vitamin E metabolite carboxyethyl hydroxychroman (CHEC) concentrations between pregnant smokers and non-smokers and their matched cord pairs.

Methodology:

This study was a sub-study of a larger ongoing study of vitamin C supplementation in pregnant smoking women being conducted at Oregon Health & Science University (OHSU) by Dr. Cynthia McEvoy. In this study 159 pregnant smokers and 76 non-smokers were consented to the study and were followed through their pregnancy to parturition with serial collections of

blood, dietary intake information, and smoking history. From this cohort, 28 pregnant smokers and 34 pregnant non smokers were also included in the sub-study evaluating available samples for vitamin E and lipid levels. Maternal plasma samples were collected before or at delivery, from weeks 28-36 for vitamin C samples, and weeks 30-42 for vitamin E and lipid samples. Cord plasma samples were collected immediately post-partum. Dietary intake was determined by up to three, 24-hour dietary recalls, and analyzed using the computer software program Nutrient Data System for Research (NDSR). Blood samples used to determine vitamin E and CEHC concentrations were analyzed in Dr. Maret Traber's lab at the Linus Pauling Institute at Oregon State University in Corvallis, Oregon via high performance liquid chromatography (HPLC), and samples for determining lipid concentration were analyzed at OHSU by colorimetric assay.

Statistical Analysis:

Study groups were compared via independent paired t-tests followed by correlation analysis to determine the relationships between intakes and concentrations of vitamin E in smokers, non-smokers, and their neonates. Correlation analyses were also used to determine relationships between plasma vitamin C, dietary intake of calories, fat, vitamin E, and vitamin C, and vitamin E concentrations in both mothers and neonates. Pearson's correlations were conducted to determine if pre-pregnancy body mass index (BMI) and/or weight gain during pregnancy were related to vitamin E concentrations. We also performed a multiple linear regression analysis to determine the effect of BMI, weight gain during pregnancy, caloric intake, fat intake, and vitamin C concentration on vitamin E and CEHC concentrations.

Results:

Plasma vitamin C did not differ between our groups therefore we could not analyze the differences in vitamin E concentrations between smokers receiving the vitamin C supplement and smokers receiving the placebo. Thus, we compared only two groups: smokers and non-smokers. Maternal plasma α- and γ-tocopherol concentrations were not statistically different between smokers and non-smokers. Neonatal plasma α-tocopherol concentrations did not differ between smoking and non-smoking groups. However, γ-tocopherol concentrations were lower in neonates of mothers who smoked during pregnancy than neonates of non-smokers. Maternal and neonatal α-CEHC concentrations did not differ between our groups, but γ-CEHC concentrations were lower in mothers who smoked during pregnancy and their neonates than in non-smokers.

Dietary vitamin E intakes and serum vitamin E concentration were not correlated in either group of mothers and neonates. Positive correlations existed between corresponding maternal and neonatal α-tocopherol, γ-tocopherol, α-CEHCs, and γ-CEHCs in both smokers and non-smokers. There were also positive correlations between maternal and neonatal α- and γ-tocopherol concentrations normalized to lipids in non-smokers, but not in smokers. Linear regression analysis showed that there was no significant effect of BMI, weight gain during pregnancy, caloric intake, fat intake, and vitamin C concentration on vitamin E and CEHC concentrations in any of our groups.

Conclusion:

Results of this study were contradictory to our hypotheses. We were unable to determine if providing a vitamin C supplement to smokers would increase plasma tocopherol concentration since plasma vitamin C concentrations were not affected by supplementation in our subset of

patients. Second, we concluded that tocopherol concentrations did not differ between smokers and non-smokers except for neonatal γ-tocopherol concentrations which were lower in our smoking group. We also noted decreased concentrations of γ-CEHCs in our group of smokers perhaps because of increased utilization and, therefore, less degradation in the liver. Our results also suggest that fetal α- and γ-tocopherol concentrations are proportional to maternal concentrations. Also, in cases of smoke exposure during pregnancy, γ-tocopherol may be used as a reactive nitrogen species scavenger thus decreasing γ-tocopherol available to the fetus and reducing the amount of γ-tocopherol bound for catabolism.

CHAPTER 1

Specific Aims

Smoking during pregnancy increases disease risk in offspring (1). Children born to mothers who smoked during pregnancy are at increased risk for developing pulmonary disorders including asthma (1). Infants born to smoking mothers are more likely to have a lower birth weight, a premature birth, and have an increased risk for sudden infant death syndrome (2). Aggressive anti-smoking programs have lowered the incidence of smoking during pregnancy but despite warnings from doctors, about 14% of pregnant women in the United States smoke every year (2). Continued smoking during pregnancy is due to complex socio-economic factors, the addictive nature of nicotine, aggressive advertising by cigarette companies, and certain genotypes that make it more difficult for women to stop smoking cigarettes. These factors make the complete elimination of smoking during pregnancy unlikely. Therefore, there is a need for further research to determine how to reduce disease risk in offspring born to women who cannot quit smoking during pregnancy.

One mechanism by which smoking might damage tissues is by increasing oxidative damage in the body that could potentially transfer to the fetus during pregnancy. Smokers appear to utilize vitamin E more rapidly than non-smokers (3) and in some studies have been seen to have lower serum antioxidant vitamin E and vitamin C concentrations (3-5). Supplementing mothers with vitamin E and C could potentially decrease oxidative damage in the mother and thus lower oxidative stress to which the fetus may be exposed. It had been suggested by several researchers that providing supplementary antioxidants during pregnancy to mothers who continue to smoke may reduce bronchial airway oxidation and the risk of subsequent childhood asthma, but further research needs to be conducted to verify this hypothesis (4, 6).

Previous studies have reported maternal and cord plasma α-tocopherol concentrations in smoking mothers which demonstrated that vitamin E concentrations were lower in smoking mothers compared to non-smoking mothers (4, 7, 8). However, there were no differences between fetal α-tocopherol concentrations in these two groups (7). We recently investigated plasma α-tocopherol and its water soluble metabolites carboxyethyl hydroxychromans (CEHCs) in normal, non-smoking pregnant mothers and found that the α-tocopherol concentrations were lower in the fetus compared to the maternal circulation (7). The CEHC metabolites, which are a biomarker of vitamin E status since vitamin E intake is positively correlated with the amount of CEHC excreted (9), were the same between the mother and the baby (7). There was a correlation between increased vitamin E intake in the mother and increases in CEHC metabolite concentration in the fetus (7). In contrast, we did not observe increased α-tocopherol in the fetus with increased maternal vitamin E intake (7). Understanding the ability of supplemental vitamin E to cross the placenta and enter fetal circulation is critical to determine the potential effects of maternal supplementation on fetal outcomes (7).

Vitamin C utilization in maternal smokers is higher than in non-smoking mothers (8). Cigarette smoke rapidly oxidizes vitamin C in vitro and once oxidized, vitamin E radicals are no longer reduced, thus lipid peroxidation continues unabated (10). Largely, the function of vitamin E is to scavenge peroxyl radicals, but it does not inhibit the initial generation of radicals (11). Lower concentrations of vitamin C may contribute to decreased α-tocopherol concentrations in mothers and therefore reduced serum concentrations in the fetus. By supplementing smoking mothers with vitamin C, we expect an increase in serum α-tocopherol concentrations in both the mother and fetus.

To date no study has reported the concentrations of vitamin E metabolites in maternal and cord plasma of smoking mothers. We propose evaluating plasma vitamin E and its metabolites in a cohort of smoking pregnant women and their infants. In addition, smoking mothers were randomized to supplemental vitamin C or placebo as part of the "In-Utero Smoke: Vitamin C and Newborn Lung Function" study. We propose to evaluate the effects of supplemental vitamin C on tocopherol concentrations in these mothers and infants. The goal of this study is to evaluate for the first time the maternal and cord plasma concentrations of vitamin E and its metabolites CEHCs in smoking mothers and to determine if supplemental vitamin C alters vitamin E concentrations in the context of maternal smoke exposure.

Specific Aims:

1. To determine if vitamin C supplementation increases maternal and cord plasma vitamin E concentrations in cases where the mother smoked during pregnancy.
    - Hypothesis:

      Supplementation of vitamin C in maternal smokers will increase both maternal and cord plasma vitamin E concentrations and decrease the concentration of plasma vitamin E metabolites.

2. To evaluate the difference in maternal and cord plasma vitamin E and its metabolites between maternal smokers and non-smokers with similar dietary intake of vitamin E.
    - Hypothesis:

      Vitamin E and vitamin E metabolite concentrations will be lower in maternal smokers and their neonates compared to non-smokers and their neonates.

CHAPTER 2

Background

Introduction

The metabolism and transfer of vitamin E from mother to fetus is poorly understood. While we know that maternal serum concentrations of vitamin E increase during pregnancy, we do not understand the impact increased serum vitamin E in mother has on the fetus. We also know that vitamin E requirements increase with smoking but there are virtually no studies on vitamin E requirements in maternal smokers. This study seeks to learn more about vitamin E status in smoking mothers and their infants.

Vitamin E

Vitamin E exists in 2 subgroups: tocopherols and tocotrienols (12). Each form has four subsets termed α-, β-, γ-, and δ-. All forms consist of a chormanol ring and a phytyl tail which is either saturated, in the case of tocopherols, or unsaturated, in the case of tocotrienols (12). Differing numbers of methyl groups on the head define the form of the subgroup as α-, β-, γ-, or δ- (12). The stereochemistry of the phytyl tail is indicated by either R- or S at three positions in the tail (2, 4 prime, and 8 prime). The 2R-alpha tocopherol is the form required by humans (13).

Dietary sources of vitamin E include wheat germ, nut oils, corn oil, superfortified cereals, and broccoli (14). All forms of the vitamin are found in these sources although the most abundant form of vitamin E in the typical American diet is γ-tocopherol (15). Tocopherols are absorbed and metabolized in the human body, but the form that has the highest biologic activity is α-tocopherol, or more specifically, *RRR*--α-tocopherol (13, 15, 16). The specificity for α-tocopherol within humans appears to be a direct result of the α-tocopherol transfer protein (α-TTP) found in the liver (17, 18).

Figure 1:

Eight Forms of Vitamin E (19)

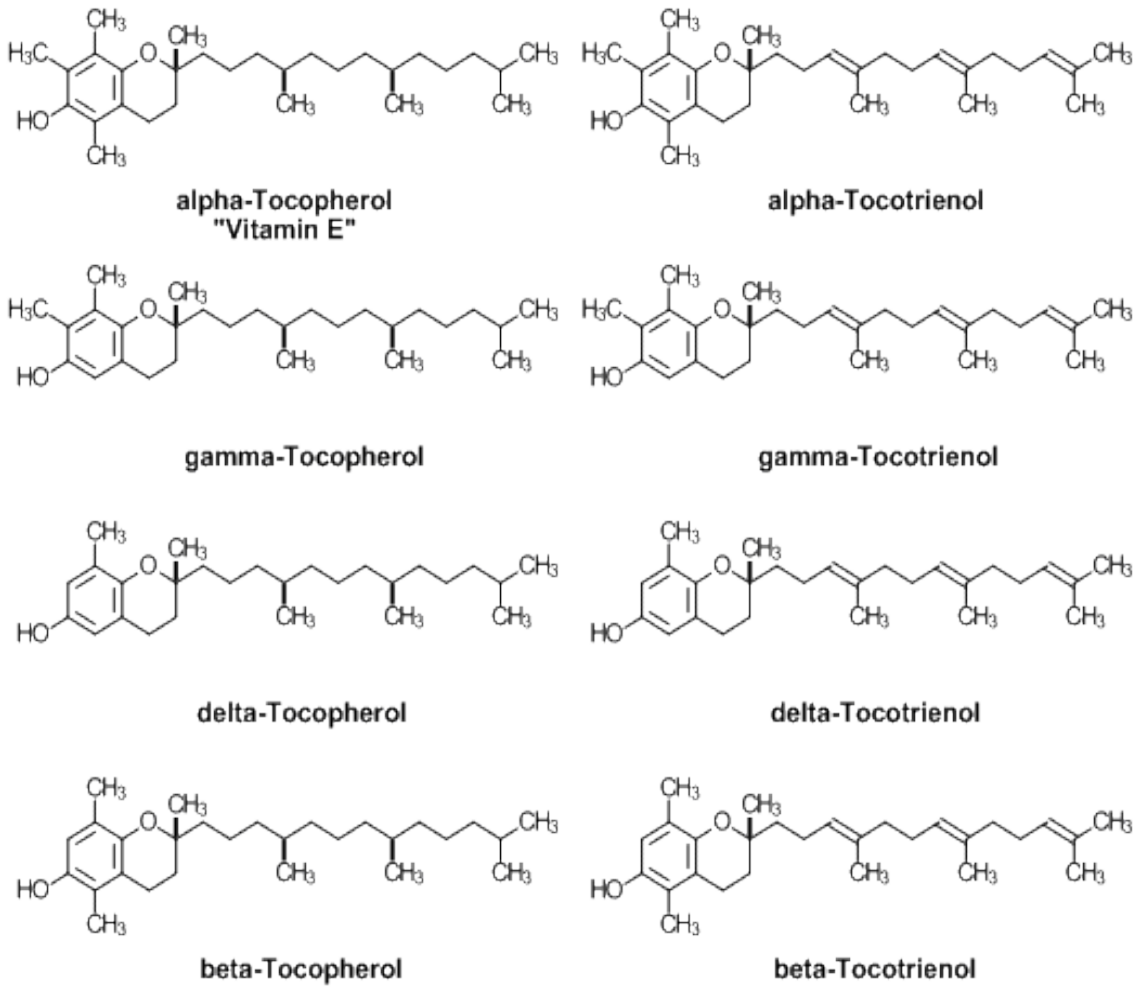
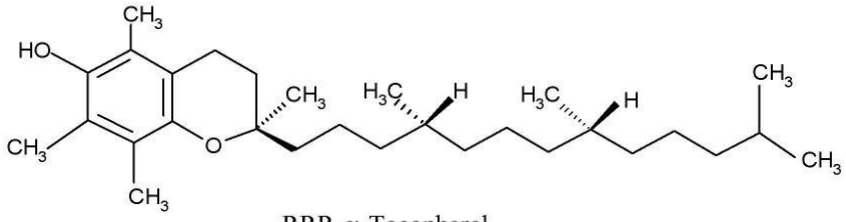
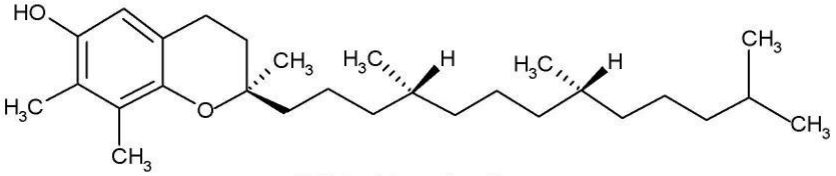


Figure 2:

Stereoisometric Forms of Vitamin E (19)



RRR-α-Tocopherol



RRR-γ-Tocopherol

Vitamin E Absorption

A fat-soluble vitamin, absorption of vitamin E follows the absorption of lipids requiring bile acids to form mixed micelles (20). Vitamin E incorporated into mixed micelles is transported to the small intestine where it is absorbed into enterocytes. Up to 70% of ingested vitamin E is absorbed and the rest is excreted in the feces (12). From the enterocyte, vitamin E is packaged into chylomicrons and transported into the lymph and ultimately into circulation (21). Once in circulation, lipoprotein lipase (LPL) acts to break down triglycerides within chylomicrons releasing free fatty acids and vitamin E to enable its transfer to high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs) (21). The transfer of α-tocopherol between lipoproteins is facilitated by phospholipid transfer protein (PLTP), which equilibrates the concentration of tocopherol among circulating lipoproteins (17). During this process, α-tocopherols are transferred to cells including adipose, muscle, and possibly brain cells via binding of LDLs (12). Tocopherols remaining in circulation are carried to the liver in chylomicron remnants.

Chylomicron remnants containing residual vitamin E are transported to the liver and taken up into hepatocytes. α-Tocopherol is repackaged into very low-density lipoproteins (VLDLs) and secreted from the liver to circulate through extrahepatic tissues (22). Packaging of vitamin E in the liver is regulated by the α-TTP (16). Synthesized in the liver, this protein preferentially incorporates *RRR*--α-tocopherol into plasma. Other tocopherols, and excess α-tocopherol, are targeted for cytochrome P450 enzyme-mediated degradation and excretion.

While in higher concentrations in the liver, α-TTP messenger ribonucleic acid (mRNA) has also been detected in the human placental trophoblast cells and the fetal capillaries endothelium (18, 23). Müller-Schmehl et al. (23) found that α-TTP mRNA concentration in the

human placenta was 1/40th that in the liver, also the second highest concentration in mammalian tissue. Other tissues that contain this protein mRNA include the spleen, lung, kidney, brain, and adrenals (24). Although we know that α-TTP mRNA can be found in the placenta, we do not know if it functions to transfer vitamin E to the fetus or its affinity for doing so. The protein has been described as having a hinge and cover mechanism that traps α-tocopherol causing the protein to fold thus allowing its transport within the liver and facilitating the incorporation of α-tocopherol into nascent VLDLs (17, 24). α-TTP is thought to be the primary determinant of plasma α-tocopherol in both maternal and fetal models as this protein correlates positively with α-tocopherol concentrations (23, 24).

Vitamin E Metabolism and Excretion

Metabolism of vitamin E occurs in the liver to form carboxyethyl hydroxychromans (CEHCs) (25). All forms of tocopherols are found catabolized in urine, and the highest concentrations of CEHCs are the α- and γ- forms (7). The concentration of metabolites increases with an increase in vitamin E supplementation both in maternal and fetal circulation (7). Metabolism of vitamin E occurs through ω- oxidation by cytochrome P450 enzymes present in the liver followed by β-oxidation (18, 25). Cytochrome P450 enzymes are involved in degradation of drugs in the liver, but also in degrading all forms of vitamin E into CEHCs. The first step in the cytochrome P450 catabolism pathway is catalyzed by the CYP4F2/CYP3A enzyme, which omega-hydroxylates the phytyl tail (26). Subsequently, the hydroxyl group on the phytyl tail is converted to a carboxyl, which then can participate in ß-oxidation (27). Two more rounds of β-oxidation occur converting vitamin E to carboxymethylhexyl hydroxychroman, carboxylmethylbutyl hydroxychroman, and finally to CEHC (25). Metabolites exist both in

maternal and fetal circulation, but it is still unknown whether these metabolites are transported across the placenta from the mother's circulation or if the fetus metabolizes vitamin E either in the placenta or the fetal liver. Metabolites, CEHCs, are water soluble and are excreted by the kidneys in urine (28).

Figure 3:

Metabolism of Vitamin E to CEHCs (25)

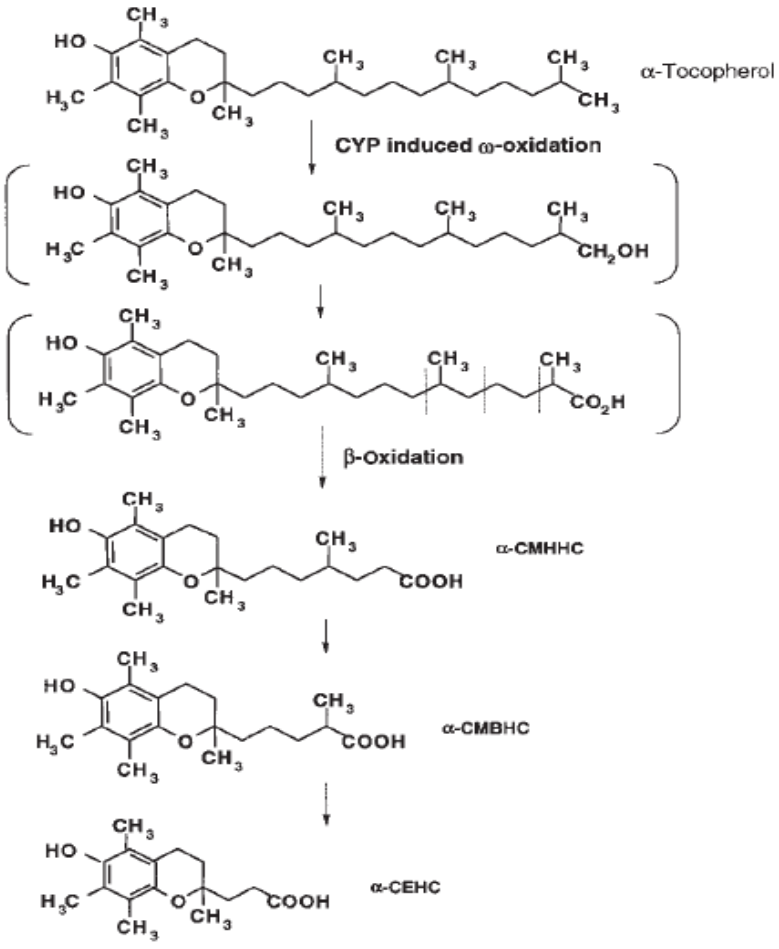


Figure 3 Metabolism of vitamin E (α-tocopherol) to α-carboxyethyl hydroxychromans. This cascade occurs in the liver and is triggered by the cytochrome P450 (CYP) enzyme CYP4F2 in humans. Intermediate forms of this process include α-carboxymethylhexyl hydroxychroman (α-CMHHC) and α-carboxylmethylbutyl hydroxychroman (α-CMBHC). The final form of tocopherol metabolism is carboxyethyl hydroxychroman.

Vitamin E Storage

Vitamin E is stored primarily in adipose tissue but is also present in the liver, lung, heart, muscle, adrenal glands, spleen, and brain (29). Most organs store a small amount of vitamin E but tissue storage is dependent on the lipid content of the organ. In contrast, adipose cells are capable of storing larger quantities depending on the amount of vitamin E ingested, the lipid content of the adipose tissue, and the availability of adipose cells.

Vitamin E Function

The antioxidant effects of vitamin E were described in the 1950s (30). Vitamin E's ability to scavenge and stop the chain reaction of lipid peroxidation is what classifies it as an antioxidant. Vitamin E is present in cell membranes where peroxyl radicals can abstract the hydrogen from the chromanol ring generating an unpaired electron known as a tocopheryl radical. The tocopheryl radical prevents further lipid peroxidation because the peroxyl radical has become a lipid hydroperoxide. Vitamin C can regenerate α-tocopherol from the α-tocopheryl radical and thereby restore its antioxidant capability (3).

Figure 4:

Vitamin C Accepts Electron from Vitamin E and Donates its Hydrogen to Rebuild Active Vitamin E (31)

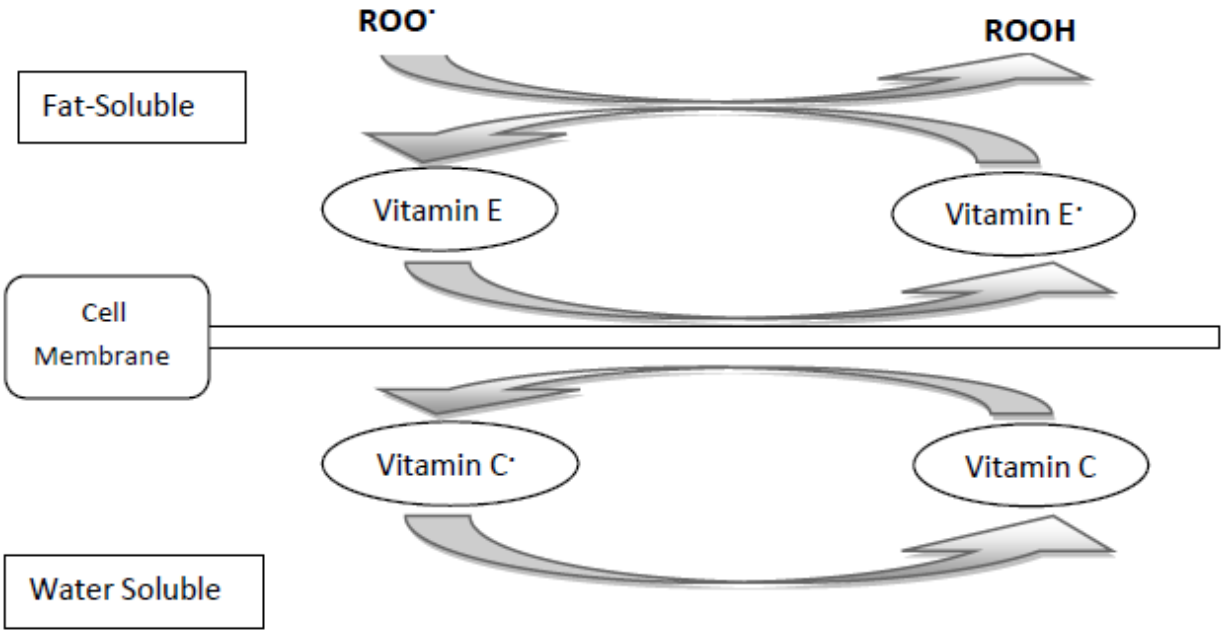


Figure 4 Vitamin E's role in preventing lipid peroxidation and the recycling of vitamin E by vitamin C. A dot by a molecule represents a radical. The presence of a peroxyl radical (ROO˙) from a cell membrane triggers the donation of an electron from vitamin E, creating a stable organic molecule (ROOH). Each vitamin E radical is recycled to active vitamin E by vitamin C, creating a vitamin C radical. In the presence of free iron or copper, the peroxyl radical can generate hydroxyl radicals, considered one of the most dangerous radicals because of its quick reaction with any nearby molecule.

Vitamin E Requirements in Pregnancy and Infancy

The Institute of Medicine has established recommended vitamin E intakes for Americans across the life cycle that provide adequate vitamin E. Current recommendations for individuals over the age of 14 are 15 mg/day or 22 IU/day of 2*R*-α-tocopherol (14). The recommended intake of this antioxidant during pregnancy is the same as what is recommended for males and females over the age of 14 and it is only during lactation that suggested intake of vitamin E increases to 19 mg/day (13). These recommendations are for healthy non-smoking mothers. The vitamin E needs for smoking mothers have not been investigated although it is theorized that smoking mothers may need higher intakes of vitamin E than non-smokers due to the increased oxidative damage and inflammation caused by smoking. In studies looking at vitamin E concentrations in smokers, researchers noted a rapid decrease over 6 hours in serum α- and γ-tocopherol concentration after cigarette smoke exposure (3). The decrease in serum concentration was attributed to an increase in whole body oxidation and not by increased liver mediated α-tocopherol degradation (5). When exposed to cigarette smoke for 6 hours, γ-tocopherol concentrations in human plasma decreased by 60% in vitro demonstrating that cigarette smoke has a significant effect on vitamin E levels (5).

Table 1:

Recommended Intake of Vitamin E, as 2*R*-α-Tocopherol in Different Age Groups and Genders

(13, 14)

Age	Males	Females	Pregnancy	Lactation
Birth to 6 months	4 mg (6 IU)	4 mg (6 IU)		
7 – 12 months	5 mg (7.5 IU)	5 mg (7.5 IU)		
1 – 3 years	6 mg (9 IU)	6 mg (9 IU)		
4 – 8 years	7 mg (10.4 IU)	7 mg (10.4 IU)		
9 – 13 years	11 mg (16.4 IU)	11 mg (16.4 IU)		
14+ years	15 mg (22.4 IU)	15 mg (22.4 IU)	15 mg (22.4 IU)	19 mg (28.4 IU)

Supplementation of vitamin E and placental transfer

Optimal serum concentrations of vitamin E in neonates are unknown. The current recommendation for adequate intake of vitamin E in neonates is 4 mg/day (6 IU/day), which can be achieved from breast milk in the first 6 months of life (32). The ideal concentrations of vitamin E and its metabolites at birth that are associated with protection from oxidant damage are still unknown. It is still unknown how vitamin E is transported across the placenta to the fetus. Recent studies have focused on exploring the mechanism behind transport of vitamin E through the placenta to the fetus to aid in the determination of the both the recommended maternal and neonatal intake of vitamin E for disease prevention (7).

The majority of studies found that there is no correlation between the amount of maternal vitamin E supplementation and fetal serum concentrations of vitamin E. Didenco et al. (7) studied 19 pregnant mothers and their neonates. Participants were counseled to take a prenatal vitamin, but the vitamin was not provided to them. Surveys were conducted to determine what prenatal vitamin participants consumed and how often they were taken. Dietary assessments were conducted by collecting at least one 24-hour recall per participant and entering the data into the dietary analysis computer program Nutrition Data System for Research (NDSR). Researchers observed a positive correlation between dietary and supplemental vitamin E intakes and serum α-tocopherol concentrations in the mother (7). This correlation remained when adjusting for serum lipid concentrations. However, increasing intake of vitamin E did not correlate with increased serum α-tocopherol in neonates, but did predict higher concentrations of cord blood CEHCs (7). Didenco et al. (7) suggest that transport of vitamin E from mother to fetus is either limited or that vitamin E is metabolized before being transported into fetal circulation or in the fetal liver.

Supplementing mothers with vitamin E during pregnancy does not appear to increase fetal serum vitamin E concentrations.

Hågå et al. (33) performed a similar study and observed no correlation between increased vitamin E intake in mothers and increased serum concentration of vitamin E in the neonate. This observational study conducted in Norway followed 40 mothers through pregnancy to evaluate changes in serum concentration of vitamin E in mothers and their neonates (33). Blood was drawn every month from participants and at delivery. A sample of cord blood was also collected at delivery and used to assess neonatal concentration of vitamin E. Participants did not receive any supplementary vitamin E and dietary analyses were not conducted. Hågå et al. (33) observed a steady increase in plasma vitamin E concentration throughout pregnancy as compared to non-pregnant women. The increase in vitamin E had a strong correlation with increases in β-lipoproteins, a marker of VLDL and LDL concentrations. In fact, Hågå et al. (33) observed that when accounting for lipoprotein concentrations, the ratio of vitamin E concentration to lipoprotein concentrations were similar among non-pregnant women, women throughout pregnancy, and in neonates. Hågå (33) thus noted that vitamin E concentrations in plasma increased with increasing β-lipoproteins in maternal circulation. Lipoprotein concentrations are very low in neonates; vitamin E concentrations in fetal serum are also low. This study suggests that increasing intake of vitamin E in pregnant mothers will not increase fetal α-tocopherol concentrations due to the low concentrations of β-lipoprotein observed in neonates.

Another study that aimed to determine the correlation between maternal intake of vitamin E and the concentration of neonatal vitamin E was conducted by Léger et al. (34) with a population of 10 pregnant mothers and their neonates. Léger et al. (34) collected a blood sample from participants 1 week prior to delivery (all planned caesarian sections) and began

supplementing participants with 1 gram of α-tocopheryl acetate 3 days pre-partum. Blood samples were collected at parturition from mother and neonate. After comparing samples, Léger et al. (34) observed a significant increase in maternal serum concentration of vitamin E, finding that it was twice as high as concentrations before supplementation (33.43 ± 3.26 mg/l vs. 18.57 ± 2.05 mg/l). Neonatal serum concentrations of vitamin E were very low (3.51 ± 0.38 mg/l) compared to mothers concentrations taken before and after supplementation. When compared to total concentration and lipid-corrected concentrations in the mother, the neonatal vitamin E concentrations remained very low (34). Researchers concluded that vitamin E transport across the placenta must be limited and may be affected by the concentration of lipids in fetal circulation, a macronutrient already known to have limited transfer across the fetus.

Two studies by Léger (34) and Baker (35) provided a vitamin E supplement to pregnant women and evaluated the vitamin E concentration in the mothers and their neonates. In the 1998 study conducted by Léger et al. (34), subjects were given a 1 g α-tocopheryl acetate supplementation 3 days prior to delivery and found no significant increase in neonatal serum concentration of vitamin E. Baker et al. (35) had similar findings in a 1975 study including 174 mothers and their neonates taking place over the full term of pregnancy. 133 mothers took 15-30 mg of vitamin E and were compared with 41 controls. Upon delivery, neonatal concentrations were ¼ the concentration of mothers suggesting that supplementation of the mother with vitamin E does not correlate with increases in neonatal concentrations (35). The placenta, therefore, is reasoned to block the transport of α-tocopherol from mother to fetus. However, it is also possible that the limited ability of the liver to secrete lipoproteins stimulates the metabolism of vitamin E to CEHC.

The majority of researchers found no correlation between intake of vitamin E and serum concentration of vitamin E in the neonate. Three studies carried out by Leonard et al. (36), Kiely et al. (37), and Sanchez-Vera et al. (38) observed a positive correlation between maternal and neonatal vitamin E concentrations. One major difference between these three studies and others is the timing for collection of blood samples and the sample size. Leonard et al. (36), Kiely et al. (37), and Sanchez-Vera et al. (38) collected blood samples on the day of delivery and compared concentrations only at that time. None of these studies recorded maternal supplementation use or measured serum CEHC metabolite concentration in the mother and neonate pair. Despite having much lower total vitamin E concentrations (36, 39), these studies found that increasing maternal serum concentration of vitamin E correlates with an increase in neonatal serum vitamin E concentration.

Three studies evaluated vitamin E concentration among smoking mothers and their babies (4, 37, 40). Bolisetty et al. (4) followed 32 pregnant women, 14 of whom were smokers, from 30 weeks gestation to 4 days post-partum. During this time, they conducted dietary analyses to estimate total fat and vitamin E intakes and collected blood samples of mothers at delivery and 4 days post-partum (4). They found that smoking and non-smoking mothers had similar concentrations of serum vitamin E during the timeline of this study (4). They also found a significantly lower concentration of vitamin E in neonates born to smokers (4.7 ± 0.73 μmol/l) compared to non-smokers (6.52 ± 0.66 μmol/l) at birth (4). However, when neonatal serum concentrations were compared on the fourth day after delivery, no statistical differences were found. Bolisetty et al. (4) suggested that vitamin E was oxidized more readily in fetuses of smoking mothers because it was found in lower concentrations in neonates of this group, and thus plays an important role in defending against oxidative stress. One limitation of this study

was the lack of control over the amount of smoking in the mothers. Urine cotinine measurements can be used for correlation analysis in smoking mothers and their neonates to control for the amount of cigarette smoking. A correlation analysis of cotinine and vitamin E concentration in neonates would provide strong evidence that vitamin E was used more readily in fetuses of smoking mothers relating to the amount of nicotine exposure.

Scaife et al. (40) included smoking mothers in a study conducted in the United Kingdom. Following mothers from 12 weeks gestation to parturition, they collected blood samples from 747 mothers at recruitment and from mother and neonates at birth (40). Dietary analysis of mothers was conducted using a Food Frequency Questionnaire (FFQ) and a 4-day diet log. When a t-test analysis was conducted, no significant differences were found between vitamin E concentrations in mothers and their neonates between smokers and non-smokers (40). Neonatal plasma vitamin E concentrations were found to be similar between smoking and non-smoking groups and no correlation could be drawn between intake of vitamin E and neonatal cord concentration of the vitamin (40). Scaife et al. (40) stated that the population in this study was well-nourished and these results may not be the same in an undernourished population.

Another study that found no relationship between maternal and neonatal serum concentrations of α-tocopherol involved 66 women (31 smokers and 35 non-smokers) and their neonates in Ireland. Kiely et al. (37) recruited pregnant women at 10-20 weeks gestation and followed them to parturition. Blood samples from mothers were taken at recruitment and a cord blood sample was taken at delivery (37). A FFQ was conducted at recruitment with each mother. Kiely et al. (37) did not find any significant correlations between maternal and neonatal serum α-tocopherol concentrations among smokers and non-smokers even when they adjusted the data for lipid concentrations. This study had weaknesses in its design because researchers were not able

to draw a second sample of maternal blood at parturition thus were unable to correlate neonatal serum concentrations with maternal serum concentrations of vitamin E at delivery. Researchers compared serum concentrations of mother and baby at different times without accounting for the impact of diet or supplement on maternal and perhaps fetal serum concentrations of vitamin E. Their dietary assessment methods were also weak in that the FFQ they used was not designed to estimate total energy intake and the questionnaire was administered only once in the beginning of pregnancy. A more useful assessment providing details on total energy, total fat, and vitamin E and C intake from diet would have included a FFQ and at least 2 or 3, 24-hour recalls (41).

Vitamin E and C Supplementation in Preeclampsia

Preeclampsia is theorized to be a result of oxidant damage occurring during pregnancy (42). Lipid peroxidation occurs during pregnancy and normally increases throughout pregnancy noted by increasing malondialdehyde (MDA) levels. However, in preeclampsia, lipid peroxidation directly impacts the placenta causing premature birth and small for gestational age neonates (43). Also, significantly lower concentrations of vitamin E and C have been observed in preeclamptic women (44). Supplementation of the mother with vitamin E and vitamin C has been studied as one approach to reduce risk of developing preeclampsia and perhaps to reduce the side effects of preeclampsia. Studies in vitamin E and C supplementation in pregnant mothers at risk of preeclampsia indicate that maternal vitamin supplementation can be effective in increasing maternal serum concentrations of tocopherols and vitamin C. Neonatal serum concentrations of vitamin E and C were not measured in these studies, but an increase in maternal concentrations were suggested to increase fetal concentrations, but this is unlikely, as discussed above.

In a 1999 study Chappel et al. (45) found that by supplementing pregnant mothers daily with 1000 mg vitamin C and 400 IU vitamin E, serum concentrations of ascorbic acid increased by 32% and serum α-tocopherol by 54% (45). This study included 283 women considered to be at high risk of preeclampsia in the United Kingdom. Supplementation began at week 16-22 of gestation. Serum samples were taken at recruitment and at delivery and were used to determine maternal concentration of vitamin E and C (46).

Another study that considered supplementation on serum concentrations of vitamins E and C was conducted by Gulmezoglu et al. (47). They included 56 women diagnosed with preeclampsia recruited at 24-32 weeks gestation. At recruitment, 27 women began daily supplementation of 800 IU vitamin E, 1000 mg vitamin C, and 200 mg allopurinol, while 29 women received a placebo (47). At parturition and day 4 postpartum, a significant increase in serum vitamin E concentration in mothers was shown (45 vs. 23.6 μmol/L) compared with the placebo group (28.5 vs. 22.8 μmol/L), indicating that supplementation was effective in raising serum antioxidant concentrations in pregnant women and it continued to remain elevated after delivery ($p = 0.003$) (47).

Multiple other studies have investigated supplemental vitamins C and E to prevent preeclampsia but very few, such as those cited above, include serum measurements of vitamin C and E concentrations.

Effect of Antioxidant Supplementation on Risk of Preeclampsia

Although increases in vitamin E concentrations were seen in several studies, none of the studies found a reduction in risk of preeclampsia (46-50). A study conducted by The World Health Organization (WHO) agrees that supplementation of vitamin E and C in women at risk of preeclampsia has been ineffective in reducing the relative risk of the disease (51).

Roberts et al. (48) included 9969 women and supplemented 4993 of them with 1000 mg of vitamin C and 400 IU of vitamin E and found that the rates of preeclampsia were not statistically significantly changed by supplementation (7.2% vs. 6.7%, p=0.33). Serum concentrations of vitamins E and C were not measured in this study either to test compliance or to measure if an effect on serum concentrations was observed.

Beazley et al. (50) recruited 100 women who were also at risk of preeclampsia for a study on vitamin E and vitamin C supplementation and risk of preeclampsia. Researchers concluded that there were no differences between the treatment (1000 mg vitamin C and 400 IU vitamin E) and placebo groups with respect to risk of preeclampsia (17.3% vs. 18.8%, RR = 0.92) (50). Beazley et al. (50) concluded that in order to see a potential benefit from vitamin E and C supplementation, study sample size would need to exceed 200, but as discussed below, studies using larger sample sizes have not seen protection from preeclampsia in women given vitamin E and C supplements.

Poston et al. (49) conducted a study of 2395 participants and was also unable to find a benefit in preeclampsia risk from supplementation of vitamin E and C. To achieve this sample size, researchers identified women at risk of preeclampsia from 25 hospitals and randomized them to supplement of 1000 mg vitamin C and 400 IU of vitamin E (49). The rate of

preeclampsia in the supplemented group was found to be 15%, which was similar to the rate in the placebo group of 16% (RR = 0.97) (49).

The WHO study conducted by Villar et al. (51) included 1356 participants randomized to either supplement or placebo and concluded that vitamin E and C supplementation at 1000 mg vitamin C and 400 IU vitamin E did not prevent preeclampsia in high-risk women. Rates of preeclampsia development for the supplemented group were 24.1% versus 23.3% in the control group (51).

In studies focusing on decreasing risks of preeclampsia, it appears that supplementation with vitamin E and vitamin C does not significantly change risk of development of preeclampsia. However, studies in the field of preeclampsia do suggest that increasing dietary or supplemental vitamin E in the mothers can increase serum concentrations of total vitamin E in maternal circulation (46, 47).

Vitamin E Concentrations in Relation to Smokers

If the mother is a smoker, the Recommended Daily Allowance (RDA) of vitamin E may need to be increased to account for the increased level of oxidant damage. In studies evaluating vitamin E status in smokers using measures of vitamin E pharmacokinetics, a higher rate of disappearance of circulating vitamin E was observed in smokers compared to non-smokers, suggesting that vitamin E requirements in smokers may need to be increased (5, 52). When exposed in vitro to cigarette smoke for 6 hours, γ-tocopherol concentrations in human plasma decreased by 60% (5).

Bruno et al. (53) also found higher rates of α-tocopherol fractional disappearance in smokers compared to non-smokers. Not only were α-tocopherol fractional disappearance rates

13% higher in smokers than non-smokers, but α-tocopherol half-life was seen to be shortened by 10 hours (52). The increased disappearance in tocopherols was coupled with a decrease in CEHC concentrations, which are generated in response to excess tocopherol (53).This study suggests that cigarette smoking creates oxidant damage that is reduced by systemic vitamin E (52).

Smoking Cigarettes and its Detrimental Effects

Cigarette smoke has long been known for its harmful effects on lungs and the carcinogenic effects chemicals contained in cigarettes pose on the human body. Cigarettes contain over 7,000 chemicals and compounds of which at least 69 are considered carcinogenic (54). These chemicals and toxins are known to damage cell DNA and weaken the immune system causing an increase in pulmonary disease (54). Cigarette smoking is not condoned during pregnancy because of these potentially harmful effects to the fetus and the increased incidence of miscarriage, premature birth, and low birth weight (11, 54). Babies born to smoking mothers are at higher risk of pulmonary dysfunction, asthma, and wheezing than babies born to maternal non-smokers, reinforcing the idea that toxins in cigarettes do impact neonatal health (11).

The mechanism associated with the damage to pulmonary function caused by smoking begins with increased oxidant exposure from cigarette smoking. Oxidants produced during the gas phase of cigarette smoking lead to lipid peroxidation of somatic cell membranes which in turn can lead to decreased pulmonary function (11). The breakdown product of lipid peroxidation, malondialdehyde (MDA) is detected in normal pregnancy, but is increased in smokers and in the plasma of maternal smokers (55). In a study on maternal smokers and their infants, Chelchowska et al. (55) found that MDA levels in smokers were higher than in non-smokers, and children of smokers experienced higher MDA levels than children of their non-

smoking counterparts showing that both mother and baby are affected by increased lipid peroxidation caused by smoking cigarettes.

Damage to the human body caused by cigarettes may damage the offspring of smokers in other ways besides increased MDA. Nicotine crosses the placenta into the fetus and can be measured in neonatal nails (56). Research on the short term effect of nicotine itself on the baby is currently being conducted, but long term effects of cigarette smoking on the fetus already noted include decreased pulmonary function, wheezing, asthma, increased episodes of illness, emphysema, and chronic obstructive pulmonary disorder (COPD) (11, 54, 57). Maternal smoking has also been associated with slight impairment of mental and physical growth in children (58).

Smoking cessation before pregnancy is the obvious solution to decreasing or completely eradicating potential harm to the fetus. A study conducted in 1965 found that women who smoked before but not during pregnancy had similar outcomes with women who had never smoked (59).

Figure 5:

Vitamin E and Antioxidant Effect on Cigarette Smoke: The Vitamin E Cycle (5)

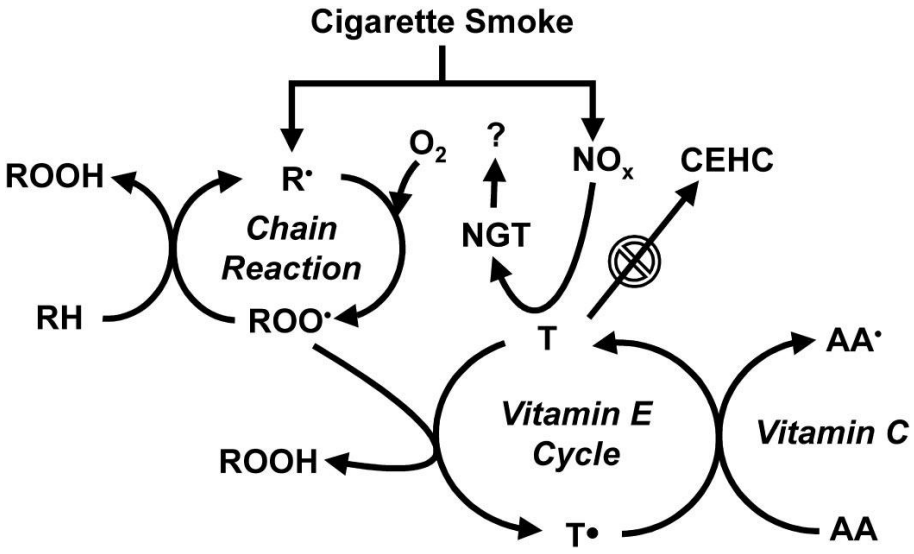


Figure 5 The vitamin E cycle and its relation to cigarette smoking. Cigarette smoke creates carbon-centered radicals (R˙) which combine with oxygen to form peroxyl radicals (ROO˙). Vitamin E (T) donates an electron, creating lipid hyperperoxide and tocopheryl radical (T˙). Ascorbic acid (AA) donates an electron to the tocopheryl radical reestablishing active vitamin E and an ascorbyl radical (AA˙). With increased utilization of vitamin E for scavenging peroxyl radicals, less vitamin E is bound for metabolism to carboxylethyl hydroxychromans (CEHCs).

Figure 6:

Transport of Vitamin E through the Body and into the Placenta (60)

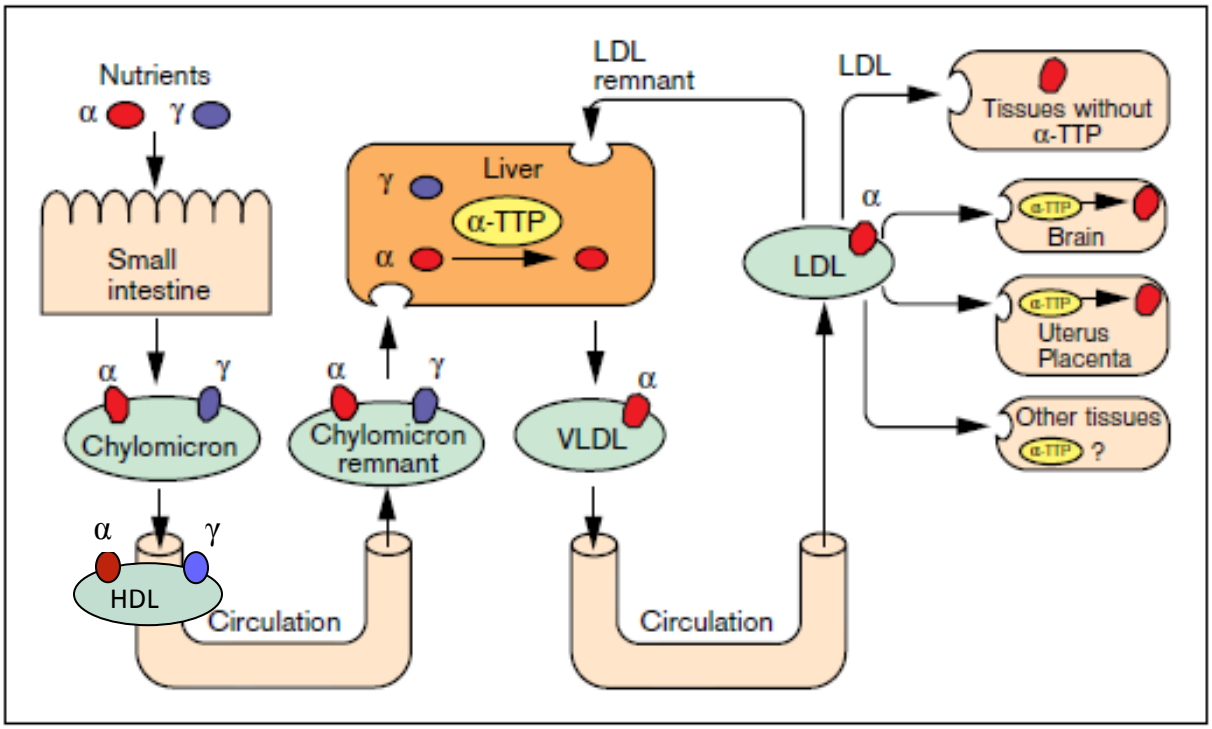


Figure 6 Transport of the alpha and gamma forms of vitamin E (α- and γ-) through the body and into the placenta and other tissues. Abbreviations: α-TTP, alpha-tocopherol transfer protein; VLDL, very low density lipoproteins; LDL, low density lipoproteins.

Smoking and Breast Milk Vitamin E Concentrations

The effects of smoking on breast milk vitamin E concentrations have not been extensively investigated. Bolisetty et al. (4) measured umbilical cord concentrations of vitamin E in infants born to smoking mothers. They found vitamin E concentrations were lower in neonates of smoking mothers than in neonates of non-smoking mothers (4). Interestingly, concentrations of neonatal vitamin E were no longer significantly different between smokers and non-smokers at day 4 post partum. The authors suggest the data indicate there is a high concentration of vitamin E in colostrum that rapidly increases infant vitamin E concentrations regardless of maternal smoking (4). One study on breast milk composition observed a difference in vitamin E concentrations in breast milk 40 days postpartum (8). Breast milk vitamin E concentrations in maternal non-smokers were significantly higher than maternal smokers (8). This study was not carried on long enough to determine trending of vitamin E status during the full term of breast feeding nor did it measure concentrations in neonatal serum from birth to 40 days postpartum and therefore no indication of neonatal needs was made. In this study, cord serum at term for both maternal smokers and non-smokers did not contain significantly different concentrations of vitamin E suggesting that smoking mothers are providing equal amounts of vitamin E to the baby (8).

Conclusion

Potential consequences of low antioxidant levels and oxidative stress from smoking in newborns include decreased pulmonary function, wheezing, asthma, low birth weight, increased risk of neonate classified as small for gestational age (SGA), and decreased mental capacity (6, 55, 57). Smoking can potentially lower antioxidant concentrations in the newborn. Further connections between smoking during pregnancy and health of the child are still being investigated with findings including increased episodes of childhood illness (57). Other risks are listed as part of the Surgeon General's warning against cigarette smoking. Smoking cessation is highly encouraged, especially during pregnancy. However, some pregnant women continue to smoke whether by choice or by inability to quit, leaving children at risk of detrimental health defects. Very few studies have investigated the serum antioxidant status among women who smoke during pregnancy and their offspring. This study seeks to understand the effects of maternal smoke on vitamin E concentration in mothers and their neonates compared to a control cohort of women who do not smoke. It further aims to determine the effects of supplemental vitamin C on vitamin E concentrations in smoking mothers and their neonates.

CHAPTER 3

Methods

General Study Design

This study is an observational prospective cohort sub-study done within the larger randomized controlled trial entitled "In-Utero Smoke, Vitamin C and Newborn Lung Function" being conducted by Dr. Cynthia McEvoy at Oregon Health & Science University (OHSU). Study groups included a reference group of non-smoking pregnant women and their neonates and a group of women (and their neonates) who could not quit smoking during pregnancy and were randomized to daily supplemental vitamin C versus placebo.

All participants, except those added from our previously published data (7) were provided the same prenatal vitamin through our clinic containing a standard vitamin complex including 20 mg/30 IU of vitamin E (dl-alpha-tocopheryl acetate) and 60 mg of vitamin C (the recommended minimum daily requirement). Pregnant women who could not quit smoking during pregnancy were randomized to daily supplemental vitamin C (500 mg) or placebo. This study was double-blinded with randomization done through the research pharmacy and data was blinded to the primary investigators until all data through one year of life was collected in the infants. The OHSU Institutional Review Board (IRB) has approved this sub-study of the "Vitamin C" study. Participation in this study was voluntary and written consent was obtained from each participant.

Participants

Participants were recruited at less than 20 weeks of gestation during routine prenatal appointments at OHSU obstetrical clinics, and if inclusion criteria were met, were consented into the study. Follow up occurred at each monthly prenatal appointment from consent through

delivery with monitoring of interval medical and smoking histories, study supplement pill counts, and the collection of the serial study biomarkers including fasting serum ascorbic acid and vitamin E concentrations. Inclusion and exclusion criteria are outlined in Tables 2 and 3. Subjects were eligible to participate if they were smokers, had a singleton gestation, were over the age of 15, and did not have a history of diabetes or kidney stones. Criteria were designed to exclude individuals whose personal characteristics, medications, obstetric condition, and medical conditions would significantly alter newborn lung function, vitamin C, vitamin E, and their metabolism, or compliance to the study protocol.

Included in the data were 17 participants from our previously published study (7). Recruitment, follow up, and analysis of data with these participants was the same as this study, but only included non-smokers. The difference between this study and the previous study (7) was that participants did not take the same prenatal vitamin. Participants were asked to take the prenatal provided by study coordinators, but were reluctant to change from their own prenatal vitamin. Consequently, vitamin E and C intake differed among participants. Also, vitamin C intakes were not recorded because this was not a primary focus of the "Vitamin C" study.

Table 2:

Inclusion and Exclusion Criteria for Non-Smoking Participants

Inclusion Criteria	**Exclusion Criteria**
1. Maternal age of at least 15 years old. 2. Pregnant with consent enrollment and randomization prior to 22 weeks gestation by last menstrual period (LMP) and confirmed by ultrasound when available. 3. Gives history of never smoking with confirmatory urine cotinine. 4. Singleton gestation. 5. Informed consent signed.	1. Multiple gestation. 2. Documental major fetal abnormalities. 3. Current maternal use of heroin, cocaine, crack, LSD, or methamphetamines. 4. Recent history of alcohol abuse: ≥ 3 drinks on ≥ 5 days / week since LMP, hospitalization for alcohol abuse. 5. Continuous daily use of high dose vitamin C and / or vitamin E supplements since LMP or refusal to abstain from using vitamins other than those provided through study. 6. History of kidney stones. 7. Current participation in other research projects that may interfere with vitamin C study. 8. Maternal unstable psychiatric illness or inability to confirm stable residence. 9. Insulin dependent diabetes. 10. Initial ascorbic acid level > 100 μmol/L when available. 11. Urine cotinine of > 0.50 ng/ml.

Table 3:

Inclusion and Exclusion Criteria for Smoking Participants

Inclusion Criteria	Exclusion Criteria
1. Maternal age of at least 15 years old. 2. Pregnant with consent enrollment and randomization prior to 22 weeks gestation by last menstrual period (LMP) and confirmed by ultrasound when available. 3. Smoker by history confirmed with urine cotinine level > 200 ng/mL. 4. Singleton gestation. 5. Appropriate smoking intervention offered and documented by PCP and / or study investigator (smoking cessation offered but declined). 6. Informed consent signed.	1. Multiple gestation. 2. Documented major fetal congenital anomalities. 3. Current maternal use of heroin, cocaine, crack, LSD, or methamphetamines. 4. Recent history of alcohol abuse: ≥ 3 drinks on ≥ 5 days / week since LMP, hospitalization for alcohol abuse. 5. Continuous daily use of high dose vitamin C and / or vitamin E supplements since LMP or refusal to abstain from using vitamins other than those provided through study. 6. History of kidney stones. 7. Current participation in other research projects that may interfere with vitamin C study. 8. Maternal unstable psychiatric illness or inability to confirm stable residence. 9. Less than 75% of placebo taken during 1-2 week initial compliance screening. 10. Insulin dependent diabetes. 11. Initial ascorbic acid level > 100 μmol/L when available.

Data Collection

Blood Sample and Dietary Recall Collection

Maternal fasting blood samples were collected at about 22 and 28 weeks gestation and at delivery. In addition, study personnel were present at the time of delivery and collected an umbilical cord blood sample for analysis. During these blood draws, at least one-half cc of blood was collected into a tube containing 1 mg/ml EDTA. Blood samples were centrifuged at 2500 x g, 4°C for five minutes using a refrigerated centrifuge within 2 hours of collection. Plasma was removed and stored at -80°C until analysis. Samples were transported to each analysis site on dry ice keeping samples frozen during this process.

Twenty-four-hour dietary recalls were also performed with consented mothers at about 22 and 28 weeks gestation. These recalls were unannounced and most often performed at obstetrical visits. The results of dietary recalls were entered into the computer database system Nutrition Data System for Research (NDSR).

Demographics, Anthropometrics, and Delivery Indices

Screening demographics to assess patient status were obtained via EPIC, the OHSU electronic medical record. If the patient appeared to meet the inclusion criteria, research personnel interviewed and consented the patient and obtained face-to-face detailed demographics and medical histories using IRB approved questionnaires that included age, ethnicity, profession, marital status, parity, and pre-pregnancy BMI. Any important changes in medical history were updated at each visit such as development of preeclampsia, gestational diabetes, etc. At delivery, research personnel documented important details about the delivery history including mode of

delivery, date of delivery and gestational age and newborn anthropometrics including birth weight, birth length, and head circumference.

Supplement

Prenatal vitamins (Prenavite, Rugby Laboratories, Duluth, GA, U.S.A.) were administered to all participants in this study containing 20 mg/30 IU dl-alpha-tocopheryl acetate and 60 mg vitamin C. Participants randomized to receive supplemental vitamin C were given 500 mg extra vitamin C per day. Assessment of compliance with prenatal vitamin intake was assessed by counting pills left in prenatal pill bottles and/or by follow up questionnaires. 17 participants from a previously published study did not receive the same prenatal vitamin and their vitamin E intake differed (7). The different intakes of vitamin E in these mothers were taken into consideration in statistical analyses.

Dietary Assessment

Nutrient intakes of mothers were analyzed via the computer database system NDSR (2006 and 2007, University of Minnesota). Dietary intakes were assessed up to three times per participant at about week 22, week 28, and at delivery using a 24-hour recall taken during clinic visits or during unannounced phone calls to participant's home. From this data, intake of vitamin E (α-tocopherol), vitamin C, total fat, total cholesterol, and energy were estimated.

Blood sample analysis

Serum lipids including total triglycerides and total cholesterol were measured by autoanalyzer at the OHSU lipid clinic or by colorimetric assay in the laboratory of Dr. Gillingham at OHSU. Serum vitamin E and vitamin E metabolites were analyzed at the Linus Pauling Institute, Oregon State University (Corvallis, Oregon) in the laboratory of Dr. Traber. Plasma vitamin C concentrations were measured at the Linus Pauling Institute in the laboratory of Dr. Balz Frei.

Tocopherol analysis

For the analysis of α- and γ-tocopherols, a modification of the method by Podda et al. was used (61). Briefly, about 100 µL of plasma was saponified with alcoholic KOH, extracted with hexane, dried under nitrogen, resuspended in 1:1 ethanol:methanol, then injected into an HPLC system. The HPLC system consisted of a Shimadzu LC-10ADvp controller (Kyoto, Japan) and a SIL-10ADvp auto injector with a 50 µl sample loop. Tocopherols were detected using a LC-4B amperometric electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN, U.S.A.) with a glassy carbon working electrode and a silver chloride reference electrode. The column used was a Waters Spherosorb ODS2 C-18 column, 100 x 4.6 mm, 3 µm particle size with a Waters Spherisorb ODS precolumn, 10 x 4.6 mm, 5 µm. An isocratic mobile phase delivery system was used, with a total run time of 6 minutes. The mobile phase used was 99:1 (v:v) methanol:water containing 0.1% (w:v) lithium perchlorate. The electrochemical detector was in the oxidizing mode, potential 500 mV, full recorder scale at 500 nA. Peak areas were integrated using Shimadzu Scientific 4.2 Class *VP* software package and tocopherols were quantified using authentic standards.

Metabolite CEHC analysis

CEHCs were extracted using a modified method of Leonard et al. (62). Briefly, plasma was added to a 10 ml screw-cap tube containing 0.8 ml Milli-Q® water and 0.5 mL 2% ascorbic acid solution. Samples were acidified with 0.5 mL HCl and incubated for 1 hour at 60°C. CEHCs were extracted with 4 mL diethyl ether and an aliquot of the ether fraction was collected and dried under nitrogen. The sample was resuspended in 1:1 (v/v) water:methanol containing trolox (Sigma) as the internal standard, and injected using liquid chromatography-mass spectrometry (LC-MS) with a Waters (Milford, MA) 2695 Separations Module and a Micromass ZQ2000 (Milford, MA). Instrument control and acquisition was performed with Waters Masslynx version 4.0 software. The column used was a SymmetryShield™ RP-18 column (3.0x150 mm, 3.5 m particle; Waters) with a Symmetry-Shield™ Sentry™ RP-18 precolumn (3.9x20 mm, 3.5 m particle; Waters). Single-ion recording mass-to-charge ratio (*m/z*) data were obtained for α-CEHC (*m/z* 277), γ-CEHC (*m/z* 263), and trolox (*m/z* 249). Typical retention times were 14.2, 14.6, and 15.4 min for trolox, γ-CEHC, and α-CEHC, respectively. Sample CEHC concentrations were calculated from the peak area of the corresponding ion to that of the trolox (internal standard) peak.

Lipid analysis

Standard lipid panels including total cholesterol and total triglycerides were measured in the OHSU lipid lab via the clinical autoanalyzer for the initial 17 non-smoking subjects included in an earlier publication (7). For all other samples, colorimetric assay kits were purchased from BioAssay Systems that were used to measure total triglyceride and cholesterol concentrations. Instructions for these assays can be found in Appendix B. HDLs and LDLs were not a part of

these lipid assays and were not evaluated in our samples. To calculate lipid standardized vitamin E concentration, tocopherol was divided by total lipid (cholesterol and triglyceride concentrations) to obtain a ratio vitamin E (μmol/L) / [cholesterol (mmol/L) + TG (mmol/L)].

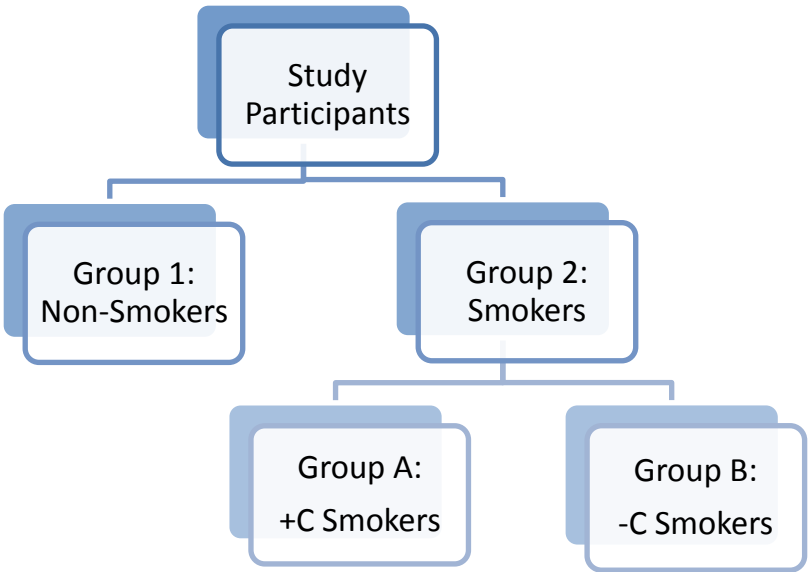
Table 4:

Outcome Variables

Demographics: Age Gravida Smoker Non-Smoker Cigarettes per day Height Weight Pre-Pregnancy BMI Weight gain during pregnancy Diet: Vitamin E (α-tocopherol) Vitamin C Total calories Total fat Total cholesterol Total saturated fat Percent calories from fat	Blood: (in mother and cord / neonate) Cholesterol Triglycerides α-tocopherol γ-tocopherol α-CEHC γ-CEHC Vitamin C concentration

Figure 7:

Grouping of Participants



Statistical Analysis

In a recent study conducted by Bolisetty et al. (4), serum concentrations of vitamin E in cord blood were determined to be 4.7 μmol/l for neonates of smoking mothers versus 6.52 μmol/l for neonates of non-smoking mothers with a difference of a 1.8 μmol/l between groups. Based on an effect size of 1.8 μmol/l between plasma vitamin E concentrations in neonates of non-smokers compared to smokers, a sample size of 21 per group of mother and neonate pairs was needed to achieve a power of 80% in this study. For all tests, $p < 0.05$ was considered significant.

Question 1: Does supplemental vitamin C increase plasma vitamin E in smoking mother and / or neonate?

Differences between plasma vitamin E and metabolites in maternal smokers randomized to placebo were compared to smokers randomized to vitamin C via independent t-test. We used correlation analysis to determine the relationship between plasma vitamin C and vitamin E intake with maternal and fetal plasma vitamin E concentrations among smokers. We also performed a multiple linear regression to determine the factors related to fetal α-tocopherol concentration. Factors included maternal plasma vitamin E concentration, maternal plasma vitamin C concentration, maternal dietary factors including estimated usual intake of fats and total caloric intake, pre-pregnancy body mass index (BMI), and weight gain during pregnancy.

Question 2: Does smoking decrease plasma vitamin E and its metabolites during pregnancy?

The difference in plasma vitamin E (α- and γ-) and ratio of vitamin E / total lipids were compared by independent t-test between non-smokers and smokers. Plasma vitamin E and metabolites were correlated to dietary vitamin E intake in both maternal and fetal plasma. The linear regression lines of the correlations were compared to determine if the relationship between dietary vitamin E and circulating vitamin E differs between maternal non-smokers and maternal smokers and their offspring.

CHAPTER 4

Results

Samples from 74 mothers and neonatal pairs were initially analyzed for this study. Twelve pairs were eliminated due to evident contamination of neonatal samples with maternal blood at the time of blood draw. Reasons for determining that samples were contaminated included mismatching sample labeling suggesting that sample was taken a few days post-partum, irregular appearance of sample during analysis, and notes stating that samples were difficult to obtain from the umbilical cord vein and likely contaminated. During analysis of vitamin E, we noted several plasma samples appeared cloudy, a sign of maternal protein contamination. Data from a total of 62 participants was used for this study including 28 non-smokers and 34 smokers, which includes 16 smokers receiving the placebo (-C Smokers) and 18 smokers receiving the vitamin C supplement (+C Smokers). The results of 17 of the non-smoking participants have been previously published (7). Eleven additional non-smokers were included in this sample.

Aim 1:

Our first aim was to compare α- and γ-tocopherol concentrations in smoking mothers and their neonates between –C Smokers and +C Smokers. Our hypothesis was that increased plasma vitamin C due to vitamin C supplementation could increase tocopherol concentrations. The samples were selected from the much larger total population collected for the "In-Utero Smoke, Vitamin C and Newborn Lung Function" study (+C Smokers n=76; -C Smokers n=83) in a non-random manner based on availability of the appropriate EDTA blood sample for both the mother and neonate. Our final sample was limited to a small subset of the total study population (+C Smokers n=18; - C Smokers n=16). Reported intake of vitamin C was significantly higher in the

+C Smokers group compared to both the –C Smokers and non-smokers because of the 500 mg/day vitamin C supplement (Table 5). However, there were no differences in plasma vitamin C between the +C Smokers and –C Smokers (Table 10). Plasma vitamin C concentration in +C Smokers was 42.8 ± 18.6 μmol/l and 41.5 ± 21.4 μmol/l for –C Smokers ($p = 0.857$).

We have rechecked the data compared to the larger study population and found our small subset of the +C Smokers was not representative of the larger population. (In the larger population, the +C Smokers had significantly higher plasma vitamin C concentrations than the -C Smokers). This finding appears to be due to the small sample size and non-random selection of samples in this study. We were not able to proceed with the analysis comparing the effects of vitamin C supplementation on vitamin E metabolism since supplementation did not affect plasma concentration.

We performed several other analyses to confirm that +C Smokers were similar to –C Smokers so that the groups may be combined. When we analyzed tocopherol concentrations in these groups, there were no differences in either α- or γ-tocopherol or α- or γ-CEHC concentrations. Pearson's regression correlations were performed to determine if there was any correlation between reported dietary vitamin C intake or plasma vitamin C concentration with maternal or neonatal vitamin E (α- and γ-tocopherol) or vitamin E metabolites (α- and γ-CEHCs). Dietary vitamin C intake did not correlate with tocopherol or CEHC (either α- or γ-) concentrations in either mothers or neonates. Serum vitamin C concentrations also did not correlate with tocopherol or CEHC (either α- or γ-) concentrations in smoking mothers or neonates. Therefore, we have combined these two groups into "Smokers" for analysis of our second aim. The following results compare outcomes from 34 smokers and 28 non-smokers.

Table 5:

Dietary Parameters Including +C and –C Smokers

Dietary factor	Participants			Total (mean ± SD) (n = 49)
	Non-smoker (n = 24)	-C Smokers (n = 11)	+C Smokers (n = 14)	
Calories (kcal)	1984	2038	2059	2027 ± 569
Fat (g)	73	76	80	76 ± 28
% calories from fat (%)	32	33	35	33 ± 6
Cholesterol (mg)	300	207	346	284 ± 224
Saturated (g)	24.5	28	30	28 ± 11
Vitamin E (mg) ‡	26.7	26.5	26.5	26.6 ± 13.2
Vitamin C (mg) ‡	132.4	73	568	258 ± 12*†

*$p < 0.05$ when comparing non-smokers to smokers
†$p < 0.05$ when comparing +C Smokers to –C Smokers
‡ includes prenatal vitamin and any supplement

Aim 2:

Demographics: Tables 6 and 7 depict the demographic data of the subjects included in this study. The average maternal age at consent was 27.0 ± 5.8 years, and the median gravida was 2.8 ± 2.1. Five percent of our population described themselves as non-white and 95% as white. The average height of our participants was 64.6 ± 2.7 inches, and non-smokers were significantly taller than smokers ($p = 0.019$). The average pre-pregnancy weight and BMI of our participants was 162 ± 41 lbs. and 27.2 ± 6.9 kg/m^2, respectively. Pre-pregnancy BMI was higher in smokers than in non-smokers, although this difference was not statistically relevant (NS: 25.3 kg/m^2, S: 28.7 kg/m^2; $p = 0.059$). Based on the World Health Organization's adult weight classifications (63), 2% of participants were underweight (BMI range < 18.50 kg/m^2) 44% of participants were normal weight (BMI range 18.5 – 24.99 kg/m^2), 18% were overweight (BMI range 25.00 – 29.99 kg/m^2), and 36% of participants were obese (BMI range ≥ 30.00 kg/m^2) at the start of their pregnancy. Weight gain during pregnancy averaged 32.4 lbs. and ranged from a loss of 25 lbs. (11.4 kg) to a gain of 80 lbs. (37.9 kg). There were no significant differences between our populations in these BMI categories.

Table 6:

Demographic Indices of Participating Mothers

Characteristic	Participants (n = 62) Mean ± SD	Range
Maternal Age (years)	27.0 ± 5.8	17 – 40
Ethnicity (%)		
Caucasian	95	-
Non-white	5	-
Smokers (%)†	56.5	-
Preeclampsia (%)	8.1	-
Gravida†	2.8 ± 2.1	1 – 10
Height (inches) †	64.6 ± 2.7	58.7 – 69.5
Pre-pregnancy Weight (lbs.)	162 ± 41.4	88 – 295
Weight gain during pregnancy (lbs.)	32.4 ± 18.3	-25 – 80
Pre-pregnancy BMI (kg/m^2)	27.2 ± 6.9	17.9 – 47.7
Pre-pregnancy BMI* (%)		
Underweight	2.0	-
Normal	44.0	-
Overweight	18.0	-
Obese	36.0	-

*BMI: Body Mass Index classification from the World Health Organization, 1995.
† $p < 0.05$ for comparison between smokers and non-smokers

Sixteen percent of our patient population reported having asthma. The incidence of asthma was equal between smokers and non-smokers ($p = 0.352$). 8.1% of participants developed preeclampsia during this study, and the incidence did not differ between our groups ($p = 0.611$). In all of these cases, babies had normal births and normal birth outcomes. There were no significant differences in our study populations with the development of preeclampsia or with the previous diagnosis of asthma.

Delivery outcomes also did not differ significantly between groups. Table 8 shows the delivery outcomes of our participants by study group. Two of the neonates in our study were born prematurely (1 from each of our study groups), but none were born with complications. Seventy-nine percent of deliveries were vaginal and 21.0% were via caesarian section (c-section) (NS vs. S: $p = 0.937$). The average gestational age was 39.1 ± 1.3 weeks ($p = 0.264$). The average birth weight of neonates was $3{,}356 \pm 545$ grams ($p = 0.578$), average head circumference was 34.1 ± 1.6 cm ($p = 0.664$), and the average birth length was 50.7 ± 2.9 cm ($p = 0.431$). Two neonates, one in our group of smokers and the other in our group of non-smokers, were born small for gestational age (SGA) which is defined as a weight that is 2 standard deviations below the mean weight for gestational age (64). Neither neonate born SGA was also born prematurely. There were no significant differences between our groups and the number of premature births ($p = 0.873$).

Table 7:

Defining Characteristics of Participating Mothers Divided into Groups

Characteristics	Participants (n = 62)		
	Non-smokers (n = 28)	Smokers (n = 34)	Total (%)
Asthma (n)	5	5	16.1%
Insurance (n)*			
Medicaid	9	32	66.1%
Private	19	2	33.9%
Cigarettes per day (n)*			
0	28	0	45.2%
1 – 4	0	12	21.0%
5 – 9	0	13	22.6%
10 – 14	0	5	9.7%
15 – 19	0	3	4.8%
20	0	1	1.6%
30	0	0	0.0%

* $p < 0.05$ for differences between non-smokers and smokers.

Table 8:

Delivery Outcomes

Delivery outcomes	Participants (mean ± SD) (n = 62)
Delivery mode (%)	
Vaginal	79.0%
C-Section	21.0%
Gestational Age (weeks)	39.1 ± 1.3
Birth weight (g)	3356 ± 545
SGA (%)	3.2%
Birth length (cm)	50.7 ± 2.9
Head circumference (cm)	34.1 ± 1.6
Apgar score (mode)	
1 minute	9
5 minute	9
Surfactant needed (%)	0%

* $p < 0.05$ for differences between non-smokers and smokers

Table 9:

Dietary parameters

Dietary factor	Participants		
	Non-smoker (n = 24)	Smokers (n = 25)	Total (mean ± SD) (n = 49)
Calories (kcal)	1984	2050	2027 ± 569
Fat (g)	73	78	76 ± 28
% calories from fat (%)	32	34	33 ± 6
Cholesterol (mg)	300	285	284 ± 224
Saturated (g)	24.5	29.1	27.5 ± 11.4
Vitamin E (mg) †	26.7	26.5	26.6 ± 13.2

† includes diet and prenatal vitamin (α-tocopherol only)

*$p < 0.05$ when comparing non-smokers to smokers

There was a significant difference in the number of cigarettes smoked each day between smokers and non-smokers (5-9 cigarettes per day vs. none, $p < 0.001$), and in the type of insurance coverage (private vs. Medicaid, $p < 0.001$). 66.1% of smokers and 33.9% of non-smokers were covered by Medicaid. All other participants were covered by private insurance. There were no other significant differences in maternal characteristics or delivery indices between smokers and non-smokers.

Diet Records: Diet records were collected from a subset of 49 participants. Diet records for the remaining 13 participants in our study were either not collected or were incomplete and could not be analyzed. For our dietary analysis we examined total vitamin E, vitamin C, total calories, and fat intake. If multiple diet records were collected, an average of the records was used. The following data includes 24 non-smokers and 25 smokers.

Total calories consumed daily averaged 2027 ± 569 by our study groups (Table 9). There were no significant differences in total caloric intakes between smokers and non-smokers. There were also no statistical differences in fat intake or any of the subcategories of fat intake between study groups (% calories from fat, saturated fat, cholesterol). Total fat intake averaged 76 ± 28 grams per day which equated to approximately 33% of total calories. Saturated fat intake averaged to 27.5 ± 11.4 grams per day or 36% of total fat intake. Finally, cholesterol intake was 284 ± 224 mg per day among our groups, and intake did not differ significantly between groups.

Vitamin E intake in all groups was statistically similar. Non-smokers consumed 26.7 ± 18.8 mg and smokers consumed 26.5 ± 2.9 mg of vitamin E daily. Total vitamin E intake reported is a summation of α-tocopherol from the diet and *dl*-α-tocopheryl acetate provided by the daily prenatal vitamin consumed by participants.

Plasma lipids: Plasma concentrations of lipids (cholesterol + triglycerides) were compared via independent t-test in both mothers and their corresponding neonates (Table 10). There were no differences in either maternal or neonatal lipid concentrations between smokers and non-smokers. Mean lipid concentration for maternal smokers was 7.38 ± 2.26 mmol/l and for non-smokers was 7.60 ± 1.45 mmol/l ($p = 0.661$). Mean lipid concentration for neonates born to mothers who smoked was 1.79 ± 1.14 mmol/l and for neonates born to non-smokers was 2.03± 0.61 mmol/l ($p = 0.315$).

Plasma Vitamin E: Differences between plasma vitamin E, plasma vitamin E normalized for total plasma lipids (cholesterol + triglycerides), and vitamin E metabolites (CEHCs) between smokers and non-smokers were compared by independent t-test. Analysis was performed for maternal and for corresponding neonatal samples (Table 10). In maternal samples, α- ($p = 0.448$) and γ-tocopherol ($p = 0.414$) concentrations were not statistically different between smokers and non-smokers (Table 10, Figure 8). Mean α-tocopherol concentration in smoking mothers was 37.4 ± 11.9 μmol/l and 35.3 ± 9.1 μmol/l for non-smokers. Mean γ-tocopherol concentration for smokers was 1.89 ± 0.83 μmol/l and 1.73 ± 0.68 μmol/l for non-smokers. When normalized for total serum lipids, α- ($p=0.081$) and γ- ($p = 0.211$) tocopherol concentrations were not significantly different between smokers and non-smokers (Figure 9). The mean concentration of α-tocopherol normalized to lipids for smokers was 5.25 ± 1.20 μmol/mmol and 4.72 ± 1.15 μmol/mmol for non-smokers. Mean γ-tocopherol concentration normalized for lipids was 0.27 ± 0.11 μmol/mmol for smokers and 0.24 ± 0.09 μmol/mmol for non-smokers. However, the sum of the lipid ratios or total vitamin E to lipid ratio was higher in maternal plasma of smokers compared to non-smokers (5.36 μmol/mmol vs. 4.96 μmol/mmol, $p = 0.025$).

Maternal serum α-CEHC concentration in smoking mothers was 29.5 ± 21.1 nmol/l and 43.3 ± 42.5 nmol/l in maternal non-smokers, but this difference was not statistically significant ($p = 0.124$; Figure 10). Serum γ-CEHCs of smoking mothers was significantly lower (59.9 ± 36.4 nmol/l) compared to non-smoking mothers (106.9 ± 76.6 nmol/l; $p = 0.005$). Total metabolite concentration (α + γ) was significantly lower in smoking mothers compared to non-smoking mothers (89.48 vs. 150.23 nmol/l, $p = 0.011$; Table 10).

Neonatal concentrations were much lower than maternal samples for all parameters (Table 10). The mean concentration of α-tocopherol in neonates born to smoking mothers was not different from the concentration in neonates born to non-smoking mothers (8.03 ± 2.77 μmol/l compared to 7.25 ± 2.65 μmol/l; $p = 0.266$; Figure 8). γ-Tocopherol concentration was lower in neonates of mothers who smoked compared to neonates of mothers who did not smoke ($p = 0.045$; Figure 8). Mean γ-tocopherol concentration was 0.25 ± 0.09 μmol/l in neonates born to smokers and 0.33 ± 0.19 μmol/l in neonates born to non-smokers. When normalized for lipids, neither α- ($p = 0.093$) or γ- tocopherol ($p = 0.554$) concentrations were significantly different between groups of neonates born to smokers and those born to non-smokers (Figure 9).

Mean α-CEHC concentration was not different between neonates of smokers compared to neonates of non-smokers (35.2 ± 39.2 nmol/l and 33.2 ± 24.3 nmol/l respectively; $p = 0.800$; Figure 10). γ-CEHC concentration in neonates of smokers (37.0 ± 19.3 nmol/l) was significantly lower compared with neonates of non-smoking mothers (77.9 ± 58.6 nmol/l) ($p = 0.001$; Figure 10).

We also calculated ratios of metabolites to tocopherol concentration for both α- and γ-forms. The ratio for maternal α-CEHC to α-tocopherol was not different between smokers (0.76 ± 0.45) and non-smokers (1.3 ± 4.5; $p = 0.090$). However, the ratio for γ- forms was lower in

smoking mothers (36.4 ± 29.3) compared to non-smoking mothers (67.3 ± 55.2; p = 0.011; Table 10). The ratio of α-CEHC to α-tocopherol concentration in neonates was not statistically different between smokers (4.3 ± 3.7) and non-smokers (5.1 ± 4.5; p = 0.438). However, the ratio of γ-CEHC to γ-tocopherol concentration was significantly lower in neonates born to mothers who smoked during pregnancy (162.9 ± 93.5) compared to neonates born to non-smoking mothers (289.7 ± 226.0; p = 0.010; Table 10).

Table 10:

Concentration of Serum Vitamin E, Metabolites (CEHCs), and Lipids in Smokers and Non-Smokers

Non-smokers vs. Smokers	NS* (n = 28) Mean ± SD	S* (n = 34) Mean ± SD	p-value (2-sided)
Mothers			
α-tocopherol (μmol/l)	35.4 ± 9.08	37.4 ± 11.9	0.448
γ-tocopherol (μmol/l)	1.73 ± 0.68	1.89 ± 0.83	0.414
α-T: Lipids (μmol:mmol)	4.72 ± 1.15	5.25 ± 1.20	0.081
γ-T: Lipids (μmol:mmol)	0.24 ± 0.09	0.27 ± 0.11	0.211
α-CEHC (nmol/l)	43.3 ± 42.5	29.5 ± 21.1	0.124
γ-CEHC (nmol/l)	106.9 ± 76.6	59.9 ± 36.4	0.005
α-CEHC: α-tocopherol	1.3 ± 4.5	0.76 ± 0.45	0.090
γ-CEHC: γ-tocopherol	67.3 ± 55.2	36.4 ± 29.3	0.011
Lipids* (mmol/l)	7.60 ± 1.45	7.38 ± 2.26	0.661
Vitamin C (μmol/l)	58.7 ± 25.8	42.2 ± 19.7	0.010
Neonates			
α-tocopherol (μmol/l)	7.25 ± 2.65	8.03 ± 2.77	0.266
γ-tocopherol (μmol/l)	0.33 ± 0.19	0.25 ± 0.09	0.045
α-T: Lipids (μmol:mmol)	4.09 ± 2.30	5.08 ± 2.16	0.093
γ-T: Lipids (μmol:mmol)	0.17 ± 0.10	0.16 ± 0.08	0.554
α-CEHC (nmol/l)	33.2 ± 24.3	35.2 ± 39.2	0.800
γ-CEHC (nmol/l)	77.9 ± 58.6	37.0 ± 19.3	0.001
α-CEHC: α-tocopherol	5.1 ± 4.5	4.3 ± 3.7	0.438
γ-CEHC: γ-tocopherol	289.7 ± 226.0	162.9 ± 93.5	0.010
Lipids* (mmol/l)	2.03 ± 0.61	1.79 ± 1.14	0.315

*NS = non-smoker, S = smoker, Lipids = cholesterol + triglycerides
Data shown as mean ± the standard deviation (SD).

Figure 8:

Comparison of Study Group Vitamin E Concentrations in Mothers and Neonates

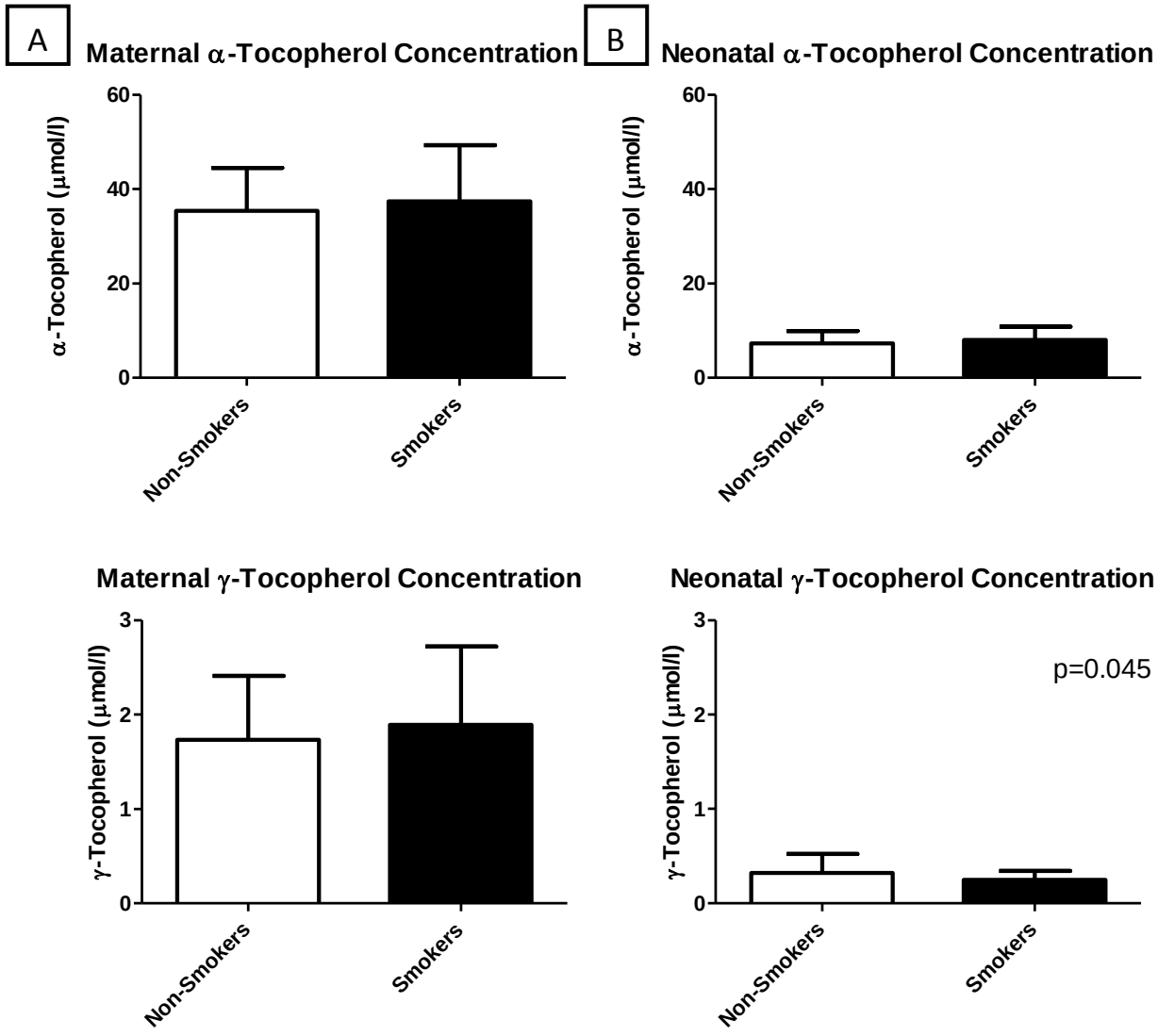


Figure 8 α- and γ-Tocopherol concentration of pregnant women who smoke during pregnancy (Smokers) compared to those who do not smoke (Non-Smokers) for plasma (A) maternal and (B) neonatal samples. The bars represent the mean +/- the standard deviation (SD). Significant p-values are noted on the figures.

Figure 9:

Tocopherol Concentration Normalized to Lipids

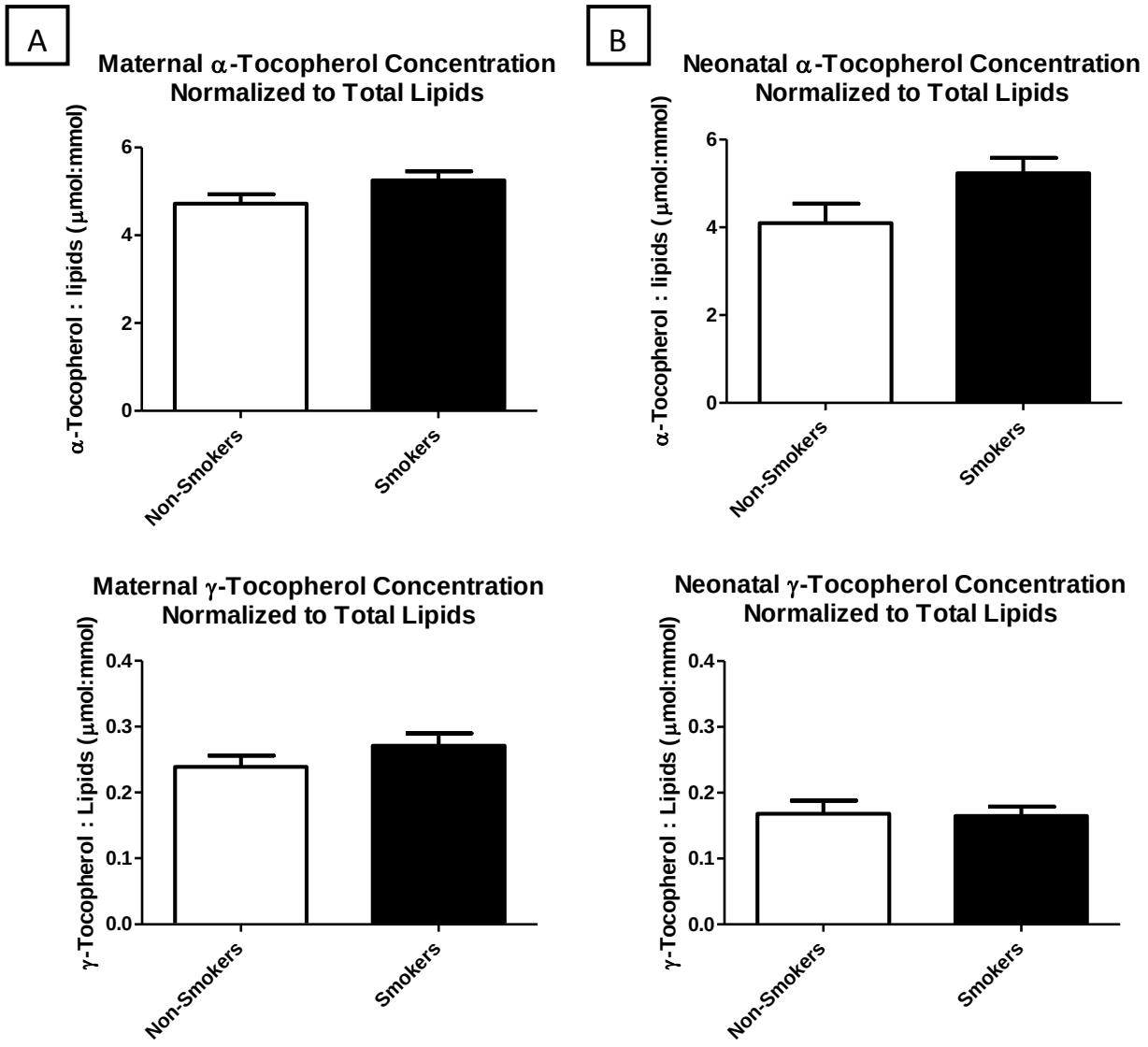


Figure 9 Vitamin E concentration normalized to total plasma lipids of women who smoke during pregnancy (Smokers) compared to those who do not smoke (Non-Smokers) for plasma (A) maternal and (B) neonatal samples. The bars represent the mean +/- the standard deviation (SD). Significant p-values are noted on the figures.

Figure 10:

Comparison of CEHC Concentration

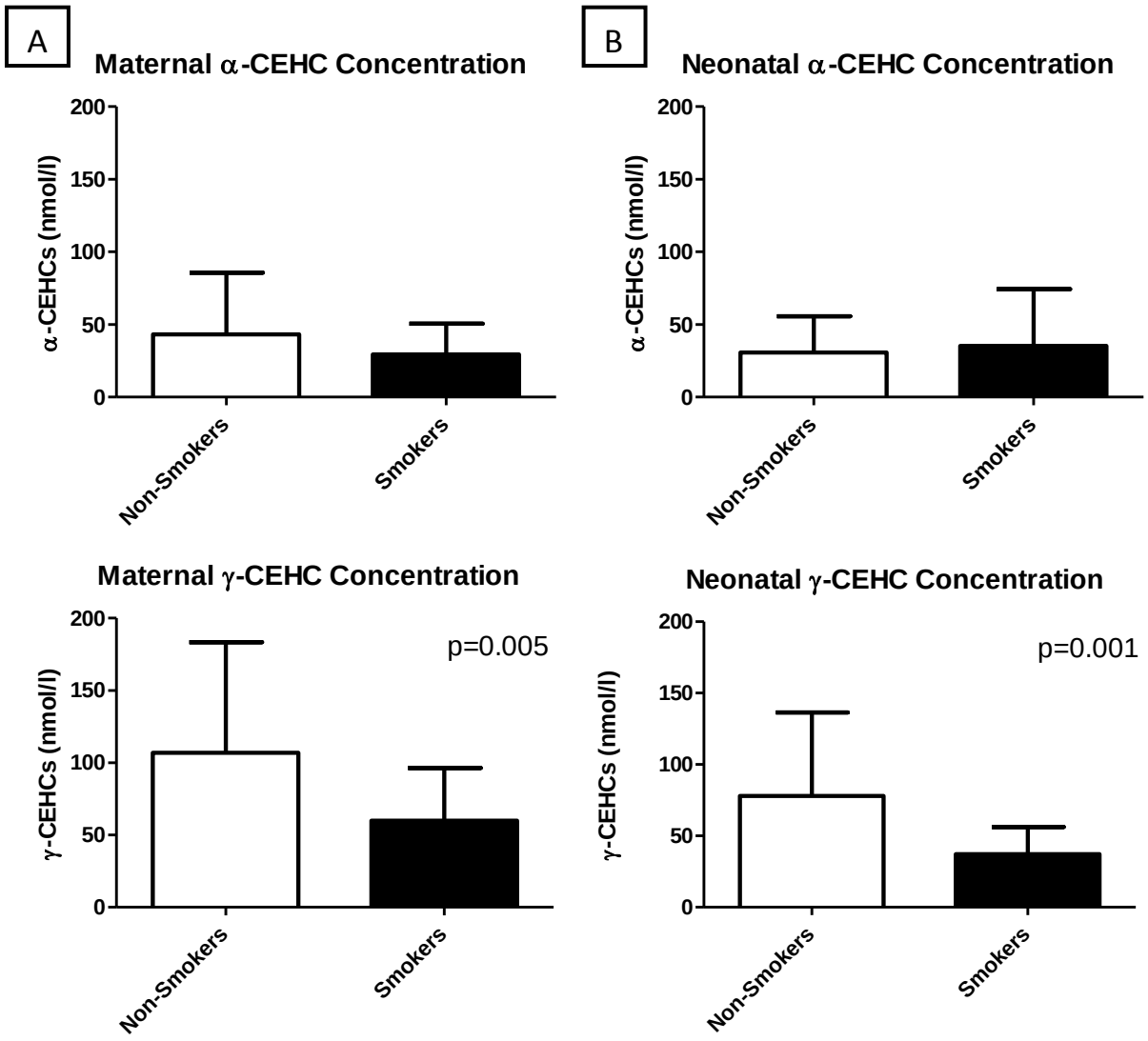


Figure 10 Vitamin E metabolite concentration (α- and γ-CEHC) of pregnant women who smoke during pregnancy (Smokers) compared to those who do not smoke (Non-Smokers) for plasma (A) maternal and (B) neonatal samples. The bars represent the mean +/- the standard deviation (SD). Significant p-values are noted on the figures.

Correlations: Pearson's regression correlations were performed to determine if any associations existed between dietary vitamin E, maternal, or neonatal vitamin E (α- and γ-, separately) and CEHCs (α- and γ-, separately). Correlations for non-smokers (n = 28) are shown in Table 11, and for smokers (n = 34) in Table 12. Contrary to our previous study (7), both smokers and non-smokers had little variation in vitamin E intake since all but 17 of the non-smoking participants previously reported received a prenatal vitamin containing 20mg *dl*-α-tocopheryl acetate. Similar to previous reports, there were no significant correlations between total vitamin E intake and plasma tocopherol or metabolite concentration in smokers (7). However, in non-smokers, there was a significant correlation between total dietary vitamin E intake and maternal and neonatal α-CEHCs (Maternal: $p = 0.015$, $R^2 = 0.240$, $\beta = 0.490$; Neonatal: $p = 0.007$, $R^2 = 0.164$, $\beta = 0.405$).

In both groups, maternal α-tocopherol concentrations were positively correlated with neonatal α-tocopherol concentrations, a finding which is consistent with our previous study (7) (S: $p = 0.010$, $R^2 = 0.191$, $\beta = 0.437$, NS: $p = 0.013$, $R^2 = 0.215$, $\beta = 0.464$). Further, when samples were normalized for lipids, maternal and neonatal α-tocopherol concentrations were significantly correlated for non-smokers ($p<0.001$) but not for smokers ($p = 0.037$). Maternal α-tocopherol concentrations were correlated with both maternal ($p <0.001$, $R^2 = 0.358$, $\beta = 0.599$) and neonatal α-CEHC concentrations among smokers ($p = 0.001$), but only with neonatal α-CEHC in non-smokers ($p = 0.026$). Neonatal α-tocopherol concentrations were also correlated with neonatal α-CEHC concentrations, but only in smokers ($p = 0.002$, $R^2 = 0.260$, $\beta = 0.509$). There was also a significant correlation between maternal and neonatal γ-tocopherol concentrations in both smokers and non-smokers (S: $p = 0.011$; NS: $p = 0.002$) and with

maternal and neonatal γ-CEHC concentrations (S: $p<0.001$, $p = 0.001$; NS: $p = 0.006$, $p = 0.002$). All of these correlations were positive.

Table 11:

Correlations between Maternal and Neonatal Vitamin E and Vitamin E Metabolite Concentrations for Non-Smokers

Non-Smokers Maternal	Neonatal α-tocopherol	γ-tocopherol	α-tocopherol: total lipids	γ-tocopherol: total lipids	α-CEHC	γ-CEHC
α-tocopherol	p=0.013 R=0.215 β=0.464	No correlation	No correlation	No correlation	p=0.026 R=0.190 β=0.436	No correlation
γ-tocopherol	No correlation	p=0.002 R=0.313 β=0.559	No correlation	No correlation	No correlation	No correlation
α-tocopherol: total lipids	p<0.001 R=0.380 β=0.616	No correlation	p=0.011 R=0.230 β=0.479	No correlation	No correlation	No correlation
γ-tocopherol: total lipids	No correlation	p=0.005 R=0.280 β=0.529	No correlation	p=0.029 R= 0.149 β=0.428	No correlation	No correlation
α-CEHC	No correlation	No correlation	No correlation	No correlation	p=0.006 R=0.277 β=0.526	No correlation
γ-CEHC	No correlation	No correlation	No correlation	No correlation	p=0.038 R=0.168 β=0.410	p=0.002 R=0.309 β=0.556

Table 12:

Correlations between Maternal and Neonatal Vitamin E and Vitamin E Metabolite Concentrations for Smokers

Smokers Maternal	Neonatal α-tocopherol	γ-tocopherol	α-tocopherol: total lipids	γ-tocopherol: total lipids	α-CEHC	γ-CEHC
α-tocopherol	p=0.010 R=0.191 β=0.437	No Correlation	No Correlation	No Correlation	p=0.001 R=0.311 β=0.558	No Correlation
γ-tocopherol	No Correlation	p=0.011 R=0.186 β=0.430	No Correlation	No Correlation	No Correlation	No Correlation
α-tocopherol: total lipids	p=0.037 R=0.132 β=0.364	No Correlation	No Correlation	No Correlation	p=0.005 R=0.228 β=0.478	No Correlation
γ-tocopherol: total lipids	No Correlation	No Correlation	No Correlation	No Correlation	No Correlation	No Correlation
α-CEHC	p=0.009 R=0.195 β=0.442	No Correlation	No Correlation	No Correlation	p<0.001 R=.485 β=0.697	No Correlation
γ-CEHC	No Correlation	No Correlation	No Correlation	No Correlation	No Correlation	p=0.001 R=0.279 β=0.528

Figure 11:

Correlations between Maternal and Neonatal Vitamin E Concentration

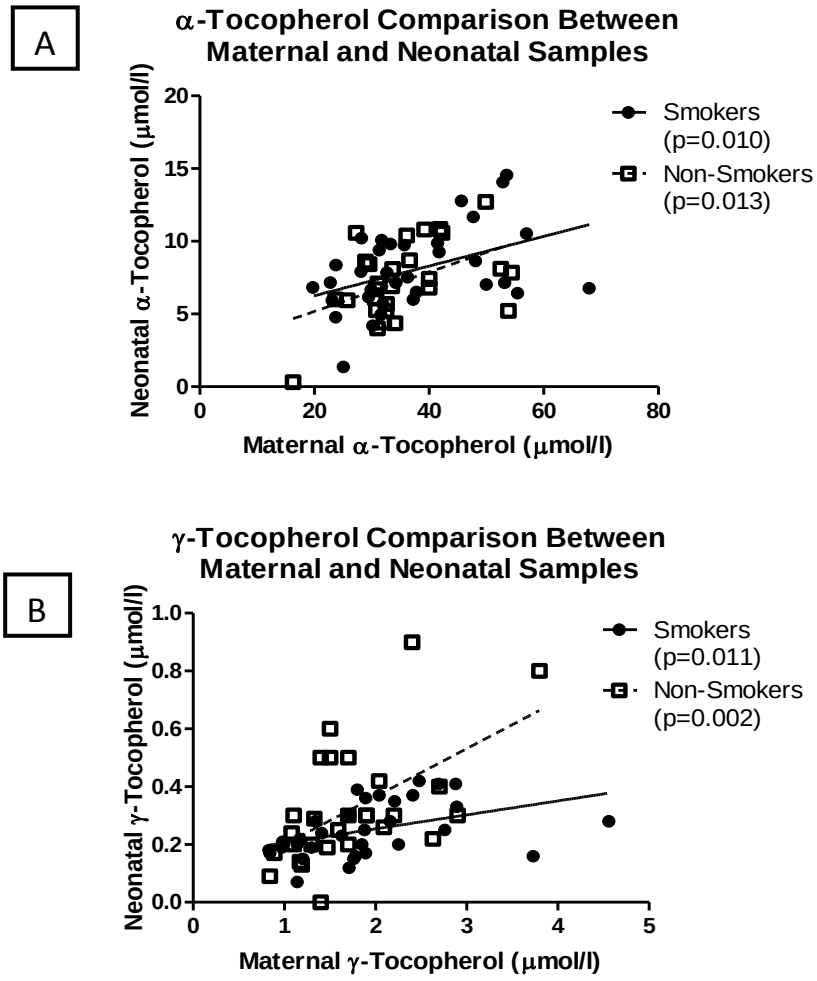


Figure 11 Correlation between maternal and neonatal plasma α-tocopoherol (A) and γ-tocopherol (B) of women who smoke during pregnancy (Smokers) compared to those who do not smoke (Non-Smokers). Smokers are shown with a solid dot and solid line. Non-Smokers are shown as an open square with dashed line. P-values (p) are shown to the right of each graph. R-squared values (R) and correlation coefficients (β) are shown in Tables 11 and 12.

Figure 12:

Correlations between Maternal and Neonatal Samples of Smokers and Non-Smokers

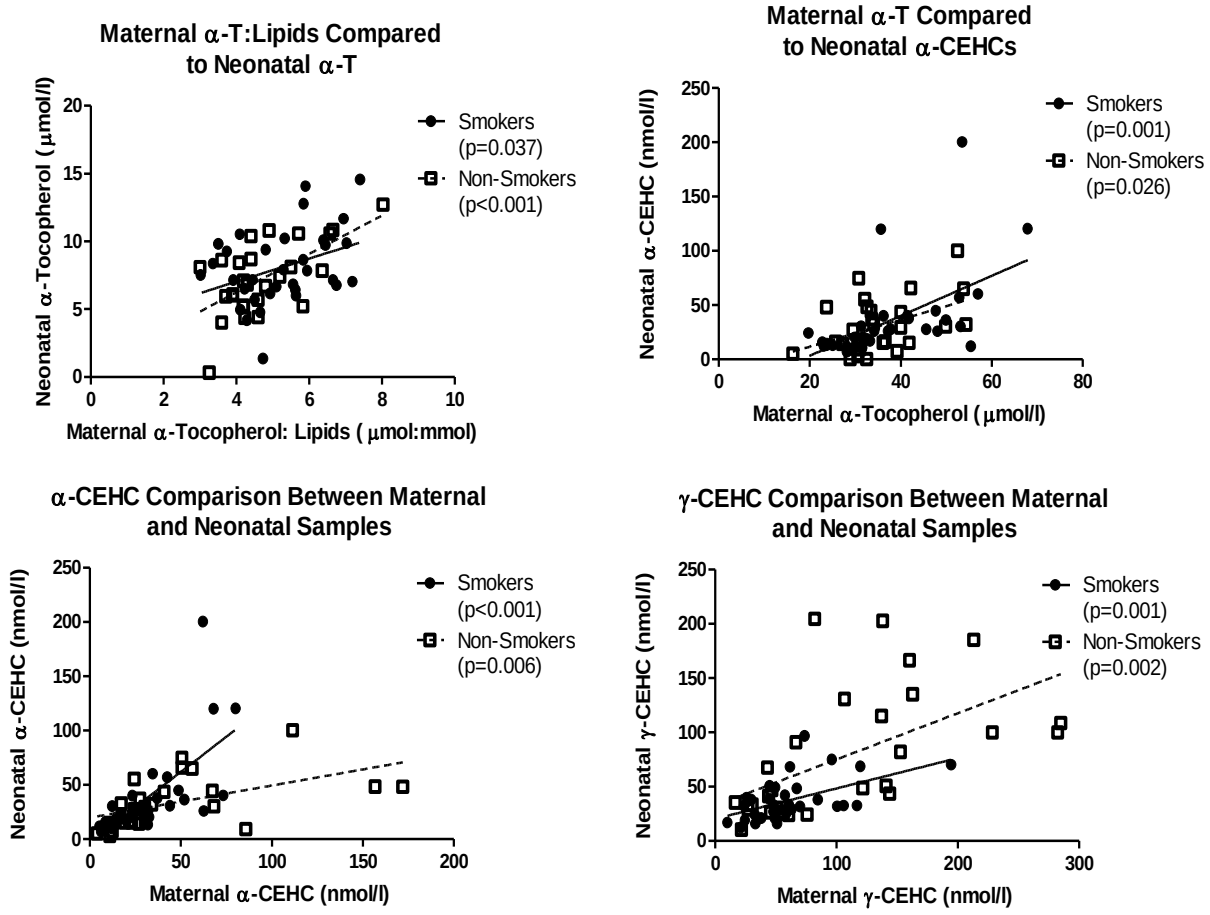


Figure 12 Correlation between maternal and neonatal plasma concentrations of women who smoke during pregnancy (Smokers) compared to those who do not smoke (Non-Smokers). Smokers are shown with a solid dot and solid line. Non-Smokers are shown as an open square with dashed line. P-values (p) are shown to the right of each graph. R-squared values (R) and correlation coefficients (β) are shown in Tables 11 and 12.

Multiple linear regressions

We proposed to test maternal factors that could explain maternal or neonatal vitamin E concentrations by multiple linear regression. These factors included maternal pre-pregnancy BMI, weight gain during pregnancy, total calorie intake, total fat intake, and maternal plasma vitamin C concentration. Because of limited data sets for the analysis, no clinically relevant or significant conclusions could be drawn from the models.

CHAPTER 5

Discussion

This prospective, observational study was conducted in a normal population of pregnant mothers and their neonates, comparing mothers who smoked during pregnancy to mothers who did not smoke. Our primary finding was that there were no statistical differences in plasma vitamin E concentrations, either α- or γ-tocopherol, between smokers and non-smokers in maternal samples. Previous studies examining vitamin E concentration in smokers and non-smokers have found either that there were no differences, or that smokers had lower concentrations of vitamin E (4, 37, 40, 55, 65-68).

Multiple studies have reported no difference in maternal α- or γ-tocopherol concentrations between smokers and non-smokers (4, 37, 65, 68-70). In all of these studies, plasma vitamin E concentration was assessed via HPLC. Supplementary vitamin E was not provided to participants in these studies. Dietary intake information was gathered in 5 studies and correlation analysis revealed that there were no associations between dietary vitamin E intakes and plasma concentrations. The report by Bruno et al. (3, 5) indicated that smokers had higher concentrations of γ-tocopherol, but not α-tocopherol compared to non-smokers but we saw no difference in either form.

In contrast, several studies have indicated that smoking increases α-tocopherol utilization thus reducing plasma concentration of α-tocopherol in smokers (11, 71, 72). Studies conducted in the pregnant population that have found lower vitamin E concentrations in smokers include those conducted by Fayol (67), Scaife (40), and Chelchowska et al. (55). At parturition, Fayol et al. (67) found that smoking mothers had significantly lower vitamin E concentrations, analyzed by HPLC, compared to non-smoking mothers ($p = 0.03$). Chelchowska et al. (55) also found

significantly lower vitamin E concentrations in mothers who smoked during pregnancy with a difference of 6.9 μmol/l ($p < 0.01$). In these studies, plasma vitamin E was not divided into α- and γ- forms of tocopherol, but was expressed as total vitamin E. Dietary intake information was only assessed by Scaife et al. (40) and showed that participants consumed less vitamin E than is recommended. A vitamin E supplement was not provided to participants in any of these studies. Thus, dietary intake of vitamin E from food and supplements may have been inadequate.

One potential explanation for the different observations in these studies compared with our results is that previous research did not measure and account for variations in dietary intakes of vitamin E. It is possible that differences between smokers and non-smokers were due to different dietary intakes and not due to the effects of smoking. Despite the inaccuracies associated with diet recalls, they do provide a range of intake which can be used to estimate excess, adequacy, or inadequacy of dietary vitamin E intake. Another probable explanation is that other studies did not provide a vitamin E-containing prenatal vitamin to participants or record vitamin E intake from supplements. In our study, we provided a prenatal supplement containing a fairly high level of α-tocopherol (20 mg) to our participants. The mean vitamin E intake for our participants was 26.6 of α-tocopherol, well above the RDA of 15 mg per day for pregnant women (13). Although the normal range of vitamin E concentration in pregnant women is unknown, previous studies have reported concentrations of 20.4 to 43.7 μmol/l (33, 37, 40, 65, 73, 74). The maternal concentrations noted here are on the higher end of that range suggesting that by increasing vitamin E intake to over the recommended dose, maternal concentrations increase.

We also found that α-CEHC concentrations and the ratio of α-CEHC to α-tocopherol concentration did not differ between smoking and non-smoking mothers. However, γ-CEHC

concentrations were lower among maternal smokers. This suggests increased utilization of γ-tocopherol in pregnant mothers exposed to cigarette smoke. We also saw significantly lower ratios of γ-CEHC to γ-tocopherol concentration in maternal smokers compared to non-smokers which suggests decreased γ-tocopherol catabolism and increased somatic γ-tocopherol utilization. Although α-tocopherol has been noted as the most potent antioxidant of all the tocopherols (12), our research suggests that γ-tocopherol may also be important, perhaps as a scavenger of reactive nitrogen species (72). Interestingly, when α- and γ-CEHC concentration were combined, maternal smokers had lower metabolite concentrations compared to non-smokers ($p = 0.011$) indicating decreased tocopherol catabolism in the liver. Vitamin E metabolites are thought to increase when excess vitamin E is available to the body (75). Our findings suggest that despite similar dietary intakes and plasma tocopherol concentrations, less tocopherol is metabolized by the liver in smokers, an observation that has been noted previously (52). This suggests that more of the tocopherols are used by the tissues and less "excess" reaches the liver for targeted degradation.

Similar to maternal samples, there was no difference between neonatal plasma α-tocopherol concentrations in smoking and non-smoking groups. Previous studies support this finding, although most results report total vitamin E and do not separate α- and γ-tocopherols (67). We observed lower γ-tocopherol concentrations in neonates of mothers who smoked during pregnancy compared to neonates of mothers who did not smoke, but the difference was attributed to differences in plasma lipids since statistical significance was lost when γ-tocopherol was normalized to plasma lipids. In studies conducted by Fayol et al. (67) and Orhon et al. (65), neonatal concentrations of vitamin E did not differ significantly between offspring of mothers

who smoked and offspring of non-smoking mothers. On the contrary, both Bolisetty et al. (4) and Chelchowska et al. (55) observed lower neonatal concentrations among infants of women who smoked during pregnancy compared to infants of non-smokers.

Normal concentrations of α- and γ-tocopherols in neonates are unknown. Previous studies have found a range of neonatal vitamin E concentrations that spans from 2.6 to 9.0 μmol/l (33, 37, 40, 65, 73, 74). The concentration of vitamin E in neonates of mothers who smoked during pregnancy are on the upper end of this range as are the concentrations of neonates of mothers who did not smoke during pregnancy. This suggests that appropriate neonatal tocopherol concentrations are reached when mothers are supplemented with vitamin E.

α-CEHC concentrations in neonates of smoking mothers did not differ from those of neonates of non-smoking mothers, nor did the ratio of α-CEHC to α-tocopherol. However, γ-CEHC concentrations in neonates of mothers who smoked were almost half the concentrations of those from neonates of non-smoking mothers. The differences we observed in γ-CEHC concentrations in the mother were also observed in neonatal circulation. The mechanism for how CEHCs cross the placenta from maternal to neonatal circulation is unknown but, unlike vitamin E, these molecules are water-soluble compounds. It is also unknown whether the fetal liver synthesizes CEHCs or if the placenta is capable of CEHC synthesis. If tocopherol degradation occurs in the placenta or in the fetal liver, CEHCs measured in the cord blood could be of neonatal and not maternal origin. We also do not know if CEHCs from the fetal circulation are shuttled back to the maternal circulation possibly as a means of excretion.

Our results suggest that γ-tocopherol catabolism was decreased in smokers. The neonatal ratio of γ-CEHC to γ-tocopherol concentration was significantly lower in subjects whose mothers smoked during pregnancy indicating that in-utero smoke exposure reduces γ-tocopherol

catabolism to γ-CEHC. Decreased catabolism in the liver could be due to a decrease in the efficiency of the cytochrome P450 CYP4F2 and/or CYP3A enzyme that mediates tocopherol conversion to CEHCs. If there was a decrease in the P450 enzyme system, you might expect to observe an increase in the plasma γ-tocopherol concentration. We did not observe a difference in tocopherol concentrations between smokers and non-smokers. Another possible reason for lower γ-CEHCs could be that less γ-tocopherol reaches the liver after ingestion. This might indicate increased use of γ-tocopherol as an antioxidant in the case of high exposure to oxidative stress due to smoking. Regardless of the exact molecular mechanism, our data suggests that smoking during pregnancy leads to decreased γ-tocopherol catabolism seen by lower concentrations of γ-CEHC in cases of cigarette smoke exposure during pregnancy.

The bioactivity of γ-tocopherol is different from that of α-tocopherol and is not completely understood (12). Compared with α-tocopherol, serum γ-tocopherol concentrations in mothers and neonates were low, which is consistent with previous literature (76-78). Previous studies indicate that γ-tocopherol concentrations in serum are usually about 10 times lower than α-tocopherol concentrations (79). It has been reported that supplementation with α-tocopherol decreases serum γ-tocopherol concentration and increases γ-tocopherol metabolism (30).

Maternal concentrations of α-tocopherol and α-CEHCs were reflected in neonatal circulation (38, 67). The placenta may transport α-tocopherol between mother and baby via the α-TTP, but it is also possible that α-TTP regulates α-tocopherol transfer back to maternal circulations. γ-Tocopherol also appears to be transferred to neonates, although in much lower concentration than α-tocopherol. The mechanism of γ-tocopherol transfer through the placenta is still unknown. Our results suggest that γ-tocopherol may be transferred to neonates disproportionately in cases where the mother smoked during pregnancy. We do not currently

know the mechanisms behind γ-tocopherol transport across the placenta, but cord γ-tocopherol does not appear to correlate with maternal concentrations in cases of cigarette smoke exposure during pregnancy.

Correlations

The correlations between dietary vitamin E intakes (α-tocopherol) and serum concentrations of vitamin E and CEHCs were not significant in any of our study groups. Previous research studies have found similar results, indicating that intake of vitamin E does not correlate well with serum concentrations in either mother or baby (33, 34, 37, 39, 40). However, there is a body of research that has shown a positive correlation between dietary vitamin E and serum concentrations (7, 40). In our previous study we observed a positive correlation between dietary vitamin E intake and maternal vitamin E concentration (7). However, when the population was expanded, this correlation was no longer significant. This may be a result of inaccurate dietary intake information or could be the result of supplementation which may have caused plasma concentrations of vitamin E to reach a maximum or threshold. In our previous study, three 24-hour recalls were collected for each participant, which was not continued throughout this study due to lack of time and materials. Scaife et al. (40) also observed a positive correlation between dietary vitamin E intake and maternal tocopherol concentration, but only after adjusting for total energy intake which we did not do in this study. The lack of correlation may be the result of inaccuracies in collecting dietary records, but our findings are corroborated by previous research. In this study, we provided the majority of our participants with the same vitamin E-containing prenatal vitamin which limited the range of total vitamin E intake in our participants. Providing vitamin E in higher doses than is considered adequate could have caused

plasma concentrations to reach a threshold. Therefore, we may not have seen a correlation between vitamin E intake and plasma concentration simply because we did not have enough variability in our data.

We found significant correlations between maternal and neonatal plasma tocopherol concentrations with both α- and γ- forms. In both smokers and non-smokers there was a significant positive correlation between maternal and neonatal α-tocopherol. There were also significant correlations between maternal and neonatal γ-tocopherol concentrations in both smokers and non-smokers. These results suggest that increasing tocopherol concentrations in the mother directly increases neonatal blood concentrations. Previous research has also noted a positive correlation between maternal plasma vitamin E concentration and neonatal vitamin E concentration, but has not looked at the α- and γ- forms of tocopherols and their relationship (38, 67).

However, at least one other report has not found a direct correlation between maternal and neonatal tocopherol concentrations suggesting that there are other factors involved in the transfer of tocopherols from mom to baby (37). Kiely et al. (37) did not find a correlation between maternal and neonatal vitamin E concentration explaining that factors such as lipoprotein content in mother and baby may limit the transfer of vitamin E across the placenta. The limited ability of lipids to pass through the placenta has been previously noted and may play a role in limiting tocopherol transfer to neonatal circulation (80).

In order to determine the relevance of lipid concentration with tocopherol transfer from mother to baby, we normalized tocopherols to total lipid concentration. Our analysis showed that when lipids were accounted for, maternal α- and γ-tocopherol concentrations were positively associated with the corresponding neonatal α-and γ-tocopherol in non-smokers. In our smoking

group, maternal α-tocopherol normalized to lipids were positively associated with neonatal α-tocopherol concentrations (not normalized to lipids), but there was no association between γ-tocopherol concentrations. Jain et al. (81) found a significant correlation between maternal and neonatal tocopherol normalized to total lipids. This study did not include smokers nor did it distinguish between forms of tocopherols. α-Tocopherol concentration in neonates was also seen to positively correlate with neonatal γ-tocopherol concentration, which was previously noted in a study by Handelman et al. (71).

We also noted significant correlations between maternal and neonatal α- and γ-CEHCs in both smoking and non-smoking groups. Since vitamin E metabolites are water soluble, perhaps some sort of equilibrium in metabolites between mothers and neonates is achieved during pregnancy. One reason that this may happen is to aid in excretion of metabolites. It may also suggest that the neonatal liver is capable of catabolizing vitamin E in the later stages of growth.

Limitations

This study was a sub-study of the "In Utero Smoke, Vitamin C and Newborn Lung Function" study. Limitations to this study included demanding and difficult data collection, incomplete dietary records for all participants, missing data points in maternal indices information, and bias associated with possible inconsistent maternal intake of supplements. We were also unable to address our initial hypothesis that supplementing smoking mothers with vitamin C would increase tocopherol concentration in both mother and neonates, due to selection bias of a non representative sub sample of the larger population in which the smokers randomized to vitamin C did not demonstrate a significantly increased ascorbic acid level compared to the smokers randomized to placebo.

Complete data collection was very challenging for this study. Deliveries and therefore sample collection was required twenty-four hours per day and it was not possible to control for timely data collection to ensure that there were no outside factors obscuring results. Collecting neonatal samples from the placenta can also be a very difficult task especially if the placental growth is decreased due to maternal smoking or study personnel were unable to be present specifically at the time of delivery prior to vasoconstriction of the umbilical vessels that occurs quickly after delivery. These factors resulted in difficult sample collections in some of our samples. Several neonatal samples were not able to be drawn from the placental artery and were "scooped" samples. These samples were interpreted as contaminated with maternal blood in the case of vitamin E analysis and any data from the contaminated sample was discarded.

Another limitation of this sub-study was the collection of dietary records, which was not one of the primary outcomes of the larger study. It was not always possible to record 24-hour recalls from patients within the limited time of appointments. Dietary analysis is typically difficult to analyze and interpret for "true" intake. In order to collect the most statistically true dietary information, a food frequency questionnaire (FFQ) plus 4-6, 24-hour recalls could have been collected (82). However, in this sub-study, there was only a subgroup of 49 participants that had dietary records collected, and most of the participants had only one diet record documented. Had more recalls been collected along with a FFQ, the analyses including dietary information would be more accurate or closer to the "true" intake of our participants.

Dietary analysis was performed using the Nutrition Data System for Research software (NDSR), but data collection was not performed using the NDSR multi-pass system. The multi-pass system allows the researcher to get a general overview of food intake and follow up with more detailed questions on how the food was prepared and exactly what ingredients were used.

Diet records were collected on paper at antenatal appointments and entered into NDSR after initial collection. Had diet records been recorded directly into NDSR using the multi-pass system, results would have been more accurate especially in the analysis of fats and total energy intake.

Another difficulty in analyzing dietary factors in this study was that diet often changes dramatically during pregnancy. Throughout pregnancy, caloric and other nutrient recommendations increase. Collection of just 1 dietary record cannot provide truly accurate data in a pregnant population. There is controversy in the research community on how to most accurately record and assess dietary intake in the pregnant population. Increasing the number of dietary records would improve the accuracy of our analysis, however was not feasible within the budget of the original study.

The primary study that we collected information from did not intend to collect data of participant characteristics such as pre-pregnancy BMI, weight gain during pregnancy, and height. In the majority of cases where maternal characteristics were not recorded by study personnel, we were able to find this data via the electronic medical record system, EPIC. Therefore there were a few missing data points.

There were also limitations resulting from the inability to ensure that all participants in the supplemented group were taking their supplements on a daily basis. Although it appears that participants consumed their prenatal supplements on a daily basis based on our results showing that tocopherol concentration did not differ between smokers and non-smokers, we did not have data available on study compliance. We also did not have compliance data on supplemental vitamin C intake which may have affected our ability to determine if vitamin C supplementation increases vitamin E plasma concentrations.

The study was not designed to investigate vitamin E metabolism, and our data is a sub-analysis of data collected for a different primary aim. The initial design of the study protocol was aimed at determining if maternal vitamin C supplementation in smokers resulted in significantly improved neonatal lung function. The specific patients in this sub-study did not have matched maternal samples of vitamin E and C collected at consent because we were not planning on comparing these concentrations to those at birth. Had those samples been collected, we could have determined if supplemental vitamin C did in fact increase maternal serum concentrations of both vitamin C and E throughout pregnancy and if mothers randomized to the vitamin C supplement had disproportionately lower levels of these antioxidants at the start of the study.

Vitamin C and vitamin E samples from mothers were also not collected concurrently with neonatal samples. Having maternal samples from different timelines may not show direct correlations between intake and serum concentration in mother or baby because of variable sample collection times.

Conclusion

In conclusion, it appears that with α-tocopherol intake that is slightly above the recommended dose, 26.5 mg per day, both α- and γ-tocopherol concentrations are similar among maternal smokers and non-smokers. Intakes of just 26.5 mg of α-tocopherol also appears to normalize α-tocopherol concentration of neonates born to mothers who smoke with that of neonates born to mothers who did not smoke during pregnancy. It does, however, seem to lower γ-tocopherol concentration in neonates of mothers who smoked during pregnancy, although the implications of this are not fully understood. While we could not examine our initial hypothesis that supplementing smoking mothers with vitamin C would increase tocopherol concentration in both mother and neonates, we found that by almost doubling the RDA for vitamin E intake, plasma concentrations of α- and γ-tocopherols in smoking mothers and their neonates were not different from concentrations of non-smokers.

We also found positive correlations between maternal and neonatal α- and γ-tocopherols indicating transport of both forms of the vitamin through the placenta. While α-tocopherol has previously been noted to be transported across the placenta via the α-TTP, the mechanism behind γ-tocopherol transport is not understood. Further study of γ-tocopherol transport across the placenta needs to be conducted. Finally, our results indicate that vitamin E α- and γ- metabolite concentrations in mothers and neonates correlate. However, we do not know if CEHCs are metabolized by the mother's liver and cross the placenta, or if the placenta and/or fetal liver have vitamin E catabolism capability.

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Appendix A

Study Identification	Participants	Duration	Design	Outcomes
Didenco S, et al. (2010) Increased vitamin E intake is associated with higher α-tocopherol concentration in the maternal circulation but higher α-carboxyethyl hydroxychroman concentration in the fetal circulation. *Am J Clin Nutr*	19 pregnant mothers and their neonates	9 months	Observational-followed during pregnancy. α-Tocopherol and α-CEHCs measured in mother and cord blood post-delivery	-α-T in mother increased -α-CEHC increased in neonate, but neonate not increased in α-T -No correlation between maternal intake and neonatal concentration of α-tocopherol -only metabolites in neonate correlated with maternal intake -maternal intake + correlation with maternal α-T concentration
Léger CL, et al. (1998) A short-term supplementation of pregnant women before delivery does not improve significantly the vitamin E status of neonates--low efficiency of the vitamin E placental transfer. *Int J Vitam Nutr Res.* Published in Switzerland	10 pregnant mothers and neonates	1 week prior to delivery to delivery via c-section.	Experimental: Supplemented women with 1 g α-T. Intervention began 3 days before delivery-delivery. Delivery by cesarean section. First blood sample taken from mom in the week prior to delivery. Results compared to previous study if non-supplemented mothers and neonates	-α-T concentration in mother increased significantly -no correlation in neonatal α-T did not increase significantly -Suggests low transfer of vitamin E through placenta perhaps from low lipid transfer through placenta
Bolisetty S et al. (2001) Postnatal changes in maternal and neonatal plasma antioxidant vitamins and the influence of smoking.	32 pregnant women: 14 smokers, 18 non-smokers	30 weeks gestation to 4 days post-delivery Other notes:	Prospective cohort. Compares dietary intake of fat and vitamin E in mother to plasma levels post-partum and	-Vitamin E levels in neonates were much lower than in mothers -both groups thought to be due to low lipophillic substances in cord blood.

		vitamin C levels higher in neonate than in adult 2/2 high transfer from mom to baby	compares plasma levels at birth to 4-day after delivery in both mother and neonate	Immediately after birth -vitamin E level in smoking group were significantly lower than in non-smoking (4.7 vs. 6.52 µmol/l), -not statistically different 4 days postpartum
Leonard PJ, Doyle E, and Harrington W. (1972). Levels of vitamin E in the plasma of newborn infants and of the mothers. *Am J Clin Nutr*.	554 mothers and 550 neonates	Day of delivery	Cohort observational study. No supplementation provided. Blood sample taken from mother and neonate at time of delivery and compared by parity, gender of neonate, and then by concentration of vitamin E.	-As plasma level of α-T increased in mother, it correlated with an increase in α-T in neonate -α-T levels in mother appears to increase naturally during pregnancy -Suggests should consider vitamin E supplementation of mothers with plasma concentration below 0.7 mg/ 100ml -Statistically significant results occurred in neonatal concentrations over 0.7 mg /100 ml.
Haga P, Ek J, and Kran S. (1982). Plasma tocopherol levels and vitamin E/β-lipoprotein relationships during pregnancy and in cord blood. *Am J Clin Nutr*.	40 mothers and neonates	9 months	Observational.	-No correlations-increased blood concentration of vitamin E in mother did not determine high level of α-T in neonate -differences in plasma transport capacity of the placenta and of the liver in the mother
Baker et al. (1975). Vitamin profile of 174 mothers and newborns at parturition. *Am J Clin Nutr*.	174 mothers and neonates	9 months	Observational. 133 took supplemental vitamin E from 15-30 mg, vitamin C from 100-500 mg.	-Vitamin E concentration in mother almost 2x that of non-pregnant woman -Vitamin C level in baby higher than in

			other 41 took no supplemental vitamins. Compares non-pregnant serum concentrations to pregnant and neonate's concentration.	mother by ratio of 1:2 and vitamin E lower in neonate than mother by ratio of 4:1 -Higher blood cholesterol concentration in mother may parallel higher vitamin E concentration -Other theories are that placenta blocks absorption of vitamin E or less transfer proteins
Sanchez-Vera I, Bonet B, Viana M, and Sanz C. (2004). Relationship between alpha-tocopherol content in the different lipoprotein fractions in term pregnant women and in umbilical cord blood. *Ann Nutr Metab*.	50 pregnant women	At delivery	Observational-cohort. Blood samples taken immediately post-partum from mother, placenta, and umbilical cord. Concentration of vitamin E and lipoproteins were compared between mother and neonate.	-Positive correlation between mother's vitamin E concentration and umbilical cord, between concentration vitamin E in maternal plasma and placenta -No correlation between vitamin E concentration in placenta and umbilical cord -Positive correlation between vitamin E concentration in LDL and VLDL with umbilical cord plasma levels -No correlation with maternal lipoproteins and vitamin E concentration in umbilical cord.
Kiely M, Cogan PF, Kearney PJ, and Morrissey PA. (1998). Concentrations of tocopherols and carotenoids in maternal and cord	40 pregnant women and neonatal pairs	9 months	Observational	-No association between concentrations of vitamin E in mother and neonate -Found correlation between γ forms

blood plasma. *Eu J Clin Nutr*.				
Scaife AR et al. (2006) Maternal intake of antioxidant vitamins in pregnancy in relation to maternal and fetal plasma levels at delivery. *Brit J Nutr*.	1149 women and neonates; 747 samples from neonates from these women; included smokers and non-smokers	9 months: recruited at about 12 weeks and followed through parturition	Observational cohort Dietary analysis: FFW and 4d food log Blood samples taken at recruitment and at delivery (mom and cord samples)	-No correlation found between vitamin E in mother and cord blood at delivery -Positive correlation found between intake of vitamin E and level of vitamin E in mother's plasma.
Roberts et al. (2010). Vitamins C and E to prevent complications of pregnancy-associated hypertension. *N Engl J Med.*	9,969 women. 5088 supplemented, 5066 control/placebo	Followed 9-16 weeks of pregnancy to parturition.	Multicenter double-blind intervention. Supplemented with 1000 mg vitamin C and 400 IU vitamin E daily and compared with placebo.	-No benefits from supplementation with vitamins E and C -No change in relative risk -No change in rates of preeclampsia or adverse peri-natal outcomes.
Rumbold A, and Crowther CA. (2010). Vitamin E supplementation in pregnancy. *Cochrane Database of Systematic Reviews* 2005(2).	566 women	Differs-explained below	Review of four trials	-No difference found with vitamin E supplementation and placebo with risk of stillbirth, neonatal death, peri-natal death, preterm birth, intrauterine growth restriction, or birth weight. -Decrease in risk of developing preeclampsia with vitamin E supplementation.
Beazley et al. (2002). Effects of vitamin C and E supplementation on total antioxidant status and 8-isoprostane levels in women at high risk of preeclampsia. *Amer J Obs and Gynec.*	109 women: 54 supplemented, 55 placebo	14-20 weeks gestation through parturition	Treatment received daily supplement of 1000 mg vitamin C and 400 IU vitamin E. Control received placebo	-No significant impact on study group -Power very low

Gulmezoglu et al. (1997). Antioxidants in the treatment of severe preeclampsia: an explanatory randomized controlled trial. *Brit J Obst and Gynec.*	56 women: 27 treatment, 29 control	24-32 weeks gestation through parturition in women diagnosed with preeclampsia	Treatment received 800 IU vitamin E, 1000 mg vitamin C, and 200 mg allopurinol	-Significant increase in serum vitamin E levels in supplemented group at parturition and 4 days postpartum -Could not determine a significant correlation between preeclampsia side effects and vitamin E, C supplementation (low power)
Chappel et al. (1999). Effect of antioxidants on the occurrence of preeclampsia in women at increased risk: a randomized trial. *Am J Obs and Gynec.*	283 women at high risk of preeclampsia	Week 16-22 gestation to parturition	Experimental observational cohort. Participants received 1000 mg vitamin C and 400 IU vitamin E daily or placebo. Blood samples taken monthly and at parturition	-Decrease ratio of PAI-1/PAI-2 during gestation -Vitamin supp associated with 21% reduction in PAI-1/PAI-2 -Decreased odds ratio of preeclampsia in supplemental group (17% vs. 8% in supplemented, OR = 0.39) -overall odds ratio = 0.24 -increased plasma α-T in supplemented group by 54% -increased vitamin C by 32%
Poston et al. (2006). Vitamin C and vitamin E in pregnant women at risk for preeclampsia (VIP trial): randomized placebo-controlled trial. *Lancet.*	2395 women from 24 facilities: 1196 in treatment group, 1199 placebo. All women had 1 risk factor for preeclampsia	16-21 weeks gestation to parturition	Experimental. Treatment group received 1000 mg vitamin C daily and 400 IU vitamin E daily.	-No significant difference in incidence of preeclampsia between groups -Supplemented group had lower birth weight babies

Appendix B

SWL
MGT Protocols
Updated 1/7/02

Vitamin E Assay - Amperometric Detection

Materials:
Water bath set at 70°C
1% ascorbic acid (AA) ethanol (1 g/100 mL) (takes approximately 10-20 minutes to dissolve using magnetic stir plate)
BHT (1mg/mL EtOH)
Milli-Q H_2O
Saturated KOH (dissolve 79.2 g KOH into 74.2 mL H_2O, on ice, very exothermic)
HPLC grade hexane (toxic-work in hood)
1% ascorbic acid (AA) water (1 g/mL)
EtOH/MeOH 1:1 mixture

Column: C18, 4.6 x 100mm, 3um, isocratic 1 mL/min

Vitamin E mobile Phase, 4 L:
80 mL Milli-Q H_2O
4.256 g $LiClO_4$
3920 mL HPLC grade Methanol (mix water and salt before added methanol)
Filter into a flask through a 0.2 μM filter. Pour into a brown bottle and sonicate 15 minutes

Sample Prep:
1. Add 100 uL plasma into a 10 mL screwcap glass tube containing: 2 mL EtOH/1% AA, and 900 μL Milli-Q H_2O. Then ass 0.3 mL saturated KOH and vortex briefly.
2. Place the sample rack into 70°C water bath for 30 minutes.
3. Cool on ice then ass 25 μL BHT (1 mg/mL EtOH) and 1 mL Milli-Q H_2O/1% AA. Then add 2 mL hexane.
4. Mix by hand inversion for ~1 minute. Allow to separate to partition, or centrifuge on low speed for 3 minutes.
5. Aliquot 1.6 mL into a new test tube.
6. Dry under nitrogen and re-suspend in 100 μL EtOH:MeOH mixture.
7. Vortex briefly and transfer to a HPLC injection vial with an insert in it.
8. Inject 20 μL into HPLC.

Standard Prep:
1. Dilute the stock standards so that your samples fall in the middle of your curves. For example: if your sample is human plasma the concentration will be ~20uM. This is the same as 20 pmol/μL. Using the above sample prep: 20 pmol/μL*100 μL/2 mL hexane*1.6 mL hexane/100 μL EtOH:MeOH*20 μL inj = 320 pmol inj. Injecting 10 μL = 150 pmol inj, and so on.
2. Take out 50 uL from the stock α-tocopherol standard and dilute to 5 mL with EtOH:MeOH. Also, add 50 μL γ-tocopherol stock solution to the same dilution, and any other standard you need. The gamma standard is 150 uM. Vortex and transfer to an HPLC injection CLEAN vial. Inject 5, 10, 20, 30, and 40 μL.
3. The software will make a standard curve by plotting pmol injected of the different tocopherols to the area for each concentration.

SWL
MGT Protocols

CEHC Assay, Plasma and Urine

Materials:
Dry incubator set at 37°C
Ascorbic Acid (AA)
Trolox stock solution
B-glucuronidase
Milli-Q H_2O
EtOH
HCl
Diethyl ether

Solution Prep:

- AA Solution – 10 mg Ascorbic acid into 1000 µL milli-Q H_2O
- B-G Solution – 10 mg β-glucuronidase into 1000 µL phosphate buffer
- Trolox Working Solution – 500 µL stock Tx diluted to 3000 µL EtOH (makes 67 µM Tx Solution)
- Trolox Internal Standard – 185 µL Trolox working solution (67 uM) diluted to 3000 µL EtOH
- 50% MeOH Solution – 50 µL glacial acetic acid added to 50 mL methanol and 50 mL milli-Q H_2O
- Standard Mix Solution – 20 µL 494 µM α-CEHC stock and 100 µL 180 µM γ-CEHC stock diluted to
  5000 µL 50 % MeOH solution

Sample Prep:

1. Add 0.5 mL plasma/urine to 10 mL screw cap tubes, add to each 10 µL AA solution, 20 µL Trolox internal standard (carefully), and 100 µL β-G solution.
2. Cap tubes, mix by tapping rack, and place in 37°C incubator for 30 minutes. Cool at room temperature.
3. In hood, add 10 µL 12 M HCl. Then add 5 mL diethyl ether to each tube.
4. Cap tightly and mix by inversion for ~2 minutes. Allow partition separation or centrifuge on low speed for 3 minutes.
5. Aliquot 4.2 mL into a new test tube.
6. Dry under nitrogen and resuspend in 100 µL 50% MeOH solution.
7. Vortex 10 seconds, transfer to an HPLC injection vial with an insert in it.
8. Inject 10 µL into HPLC/MS.

Standard Prep:

1. Using the Standard Mix Solution, make six 1:1 dilutions into 50% MeOH solution (using 1 mL of each solution). Remove 1 mL from the last dilution to make all volumes in the six standard tubes the same.
2. Add 10 µL Trolox Solution (67 mM) to each standard tube. Vortex each tube 5 seconds.
3. Transfer 200 µL of each standard to HPLC injection vials. Inject 10 µL into HPLC/MS.
4. Calculate peak area ratios of α- and γ-CEHC to Trolox. Plot standard curves with pmol injected vs. peak area ratio.

EnzyChrom™ Triglyceride Assay Kit (Cat# ETGA-200)

Quantitative Colorimetric Triglyceride Determination at 570nm

DESCRIPTION

TRIGLYCERIDE, also known as TRIACYLTRIGLYCERIDE or TRIACYLGLYCERIDE, is the main constituent in vegetable oil and animal fats. Triglycerides play an important role as energy sources and transporters of dietary fat. In the human body, high levels of triglycerides in the bloodstream have been linked to atherosclerosis, heart disease and pancreatitis. Simple, direct and automation-ready procedures for measuring triglyceride concentrations find wide applications in research and drug discovery. BioAssay Systems' triglyceride assay uses a single Working Reagent that combines triglyceride hydrolysis and glycerol determination in one step, in which a dye reagent is oxidized to form a colored product. The color intensity at 570nm is directly proportional to triglyceride concentration in the sample.

KEY FEATURES

Sensitive and accurate. Use as little as 10 µL samples. Linear detection range 0.01 mmol/L to 1.0 mmol/L (0.88 mg/dL to 88.5 mg/dL) triglyceride.
Simple and convenient. The procedure involves addition of a single working reagent and incubation for 30 min at room temperature, compatible for HTS assays.
Improved reagent stability. The optimized formulation has greatly enhanced the reagent and signal stability.

APPLICATIONS:

Direct Assays: triglyceride in biological samples (e.g. serum and plasma).
Drug Discovery/Pharmacology: effects of drugs on triglyceride metabolism.

KIT CONTENTS

Assay Buffer: 24 mL **ATP:** 250 µL **Dye Reagent:** 220 µL
Enzyme Mix: 500 µL **Lipase:** 1000 µL
Standard: 100 µL (equivalent to 100 mmol/L Triglyceride)

Storage conditions. The kit is shipped on dry ice. Store Assay Buffer at 4°C and other reagents at -20°C. Shelf life of three months after receipt.

Precautions: reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.

PROCEDURES

Note: (1) SH-group containing reagents (e.g. mercaptoethanol, DTT) may interfere with this assay and should be avoided in sample preparation; (2) If sample contains glycerol, use BioAssay Systems' EnzyChrom™ Glycerol Assay Kit (Cat# EGLY-200) to determine glycerol concentration and subtract the glycerol value to yield triglyceride concentration.

1. Equilibrate all components to room temperature. Keep thawed Lipase and Enzyme Mix in a refrigerator or on ice. Dilute Standard in distilled water as follows. Transfer 10 µL diluted standards into wells of a clear 96-well plate. Diluted standards can be used for future assays when stored refrigerated.

No	STD + H_2O	Vol (µL)	Triglyceride (mmol/L)
1	10µL + 990µL	1000	1.0
2	6µL + 994µL	1000	0.6
3	3µL + 997µL	1000	0.3
4	0µL + 1000µL	1000	0

Serum and plasma samples should be diluted 5-fold in dH_2O and are assayed directly. Cells and other solid samples can be solubilized in 5% Triton X-100 (*see Ref. 3*). Transfer 10 µL samples into separate wells of the 96-well plate.

2. Prepare Working Reagent for each well, by mixing 100 µL Assay Buffer, 2 µL Enzyme Mix, 5 µL Lipase, 1 µL ATP and 1 µL Dye Reagent in a clean tube. Transfer 100 µL Working Reagent into standards and sample wells. Tap plate to mix.
3. Incubate 30 min at room temperature. Read optical density at 570nm (550-585nm).

Note: 1. If the Sample OD is higher than the Standard OD at 1.0 mmole/L triglyceride, dilute sample in water and repeat the assay. Multiply by the dilution factor *n*.

CALCULATION

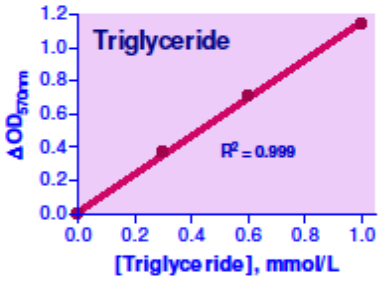
Subtract OD_{H2O} (water, #4) from the standard OD values and plot the OD against standard concentrations. Determine the slope using linear regression fitting. The triglyceride concentration of Sample is calculated as

$$[\text{Triglyceride}] = \frac{OD_{SAMPLE} - OD_{H2O}}{\text{Slope}} \times n \quad (\text{mmol/L})$$

OD_{SAMPLE} and OD_{H2O} are optical density values of the sample and the water blank (# 4). *n* is the dilution factor. For example serum or plasma samples are diluted 5-fold prior to assay, $n = 5$.
Conversions: 1 mmol/L triglyceride equals 88.5 mg/dL or 10 ppm.

MATERIALS REQUIRED, BUT NOT PROVIDED

Pipeting devices, centrifuge tubes, clear flat bottom 96-well plates (e.g. Corning Costar) and plate reader.



Standard Curve in 96-well plate assay

LITERATURE

1. Nägele U et al (1984). Reagent for the enzymatic determination of serum total triglycerides with improved lipolytic efficiency. J Clin Chem Clin Biochem. 22(2):165-74.
2. Bucolo, G. and David, H. (1973). Quantitative determination of serum triglycerides by the use of enzymes. Clin. Chem.19(5): 476-482.
3. Zhu Y et al (2000). Genomic interval engineering of mice identifies a novel modulator of triglyceride production. PNAS 97(3): 1137-1142.

EnzyChrom™ Cholesterol Assay Kit (ECCH-100)

Quantitative Colorimetric Determination of Cholesterol at 340 nm

DESCRIPTION

CHOLESTEROL is a sterol and lipid present in the cell membranes, and is transported in the bloodstream of all animals. It is used to form cell membranes and hormones, and plays important roles in cell signaling processes. Elevated levels (hypercholesterolemia) have been associated with cardiovascular diseases such as atherosclerosis; whereas, low levels (hypocholesterolemia) may be linked to depression, cancer and cerebral hemorrhage.

Simple, direct and automation-ready procedures for measuring cholesterol are very desirable. BioAssay Systems' EnzyChrom™ Cholesterol Assay is based on cholesterol esterase hydrolysis of cholesterol esters to form free cholesterol and cholesterol dehydrogenase catalyzed conversion of cholesterol to cholest-4-ene-3-one, in which NAD is reduced to NADH. The optical density of the formed NADH at 340 nm is directly proportionate to the cholesterol concentration in the sample.

APPLICATIONS

Direct Assays: cholesterol in serum, plasma, and other biological samples.
Pharmacology: effects of drugs on cholesterol metabolism.

KEY FEATURES

Sensitive and accurate. Detection limit of 5 mg/dL, linearity up to 300 mg/dL cholesterol in 96-well plate assay.
Convenient. Room temperature assay. No 37°C heater is needed.
High-throughput. Can be readily automated as a high-throughput 96-well plate assay for thousands of samples per day.

KIT CONTENTS (100 tests in 96-well plates)

Assay Buffer: 20 mL **Enzyme Mix: 120 uL**
NAD Solution: 2 x 1 mL **Standard: 1 mL 300mg/dL cholesterol**

Storage conditions. Store reagents at -20°C. Shelf life: 6 months after receipt.

Precautions: reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.

PROCEDURES

Important: bring all reagents to room temperature prior to assay. Serum and plasma samples should be clear and free of turbidity or precipitates. If present, precipitates should be removed by filtration or centrifugation in a table centrifuge. If not assayed immediately, samples can be stored at -20 to -80°C for at least one year.

1. *Standard Curve.* Prepare a 10-fold diluted standard (STD) by mixing 40 µL 300 mg/dL Standard and 360 µL Assay Buffer. Further dilute standard (STD) in Assay Buffer as shown below.

No	STD + Assay Buffer	Vol (µL)	10 x Conc. (mg/dL)
1	100µL + 0µL	100	300
2	80µL + 20µL	100	240
3	60µL + 40µL	100	180
4	40µL + 60µL	100	120
5	30µL + 70µL	100	90
6	20µL + 80µL	100	60
7	10µL + 90µL	100	30
8	0µL + 100µL	100	0

Transfer 50 µL diluted standards into wells of the 96-well plate.

Samples: dilute samples 10-fold (e.g. 10 µL sample with 90 µL Assay Buffer). Transfer 50 µL diluted sample in separate wells.

2. Prepare enough NAD solution in Assay Buffer as follows: for each reaction well, mix 40 µL Assay Buffer with 18 µL the provided NAD Solution.

   Add 50 µL of diluted NAD to standards and sample wells. Tap plate to mix well.

   Let stand 5 min at room temperature. Read background optical density at 340nm (OD_0).

3. Prepare enough enzyme mix as follows: for each reaction well, mix 10 µL Assay Buffer with 1 µL provided Enzyme Mix. Add 10 µL diluted enzyme mix per well. Tap plate to mix thoroughly. Note: the enzyme mix may appear to be turbid, but will be clear after mixing into the reaction mixture.

4. Incubate 30 min at room temperature. Read OD_{30} at 340nm.

5. *Calculation.* Subtract OD_0 from OD_{30} for the standard and sample wells. Use the ΔOD values to determine sample cholesterol concentration from the standard curve. ***Note: since both the standards and samples were diluted 10-fold, no dilution factor is required.***

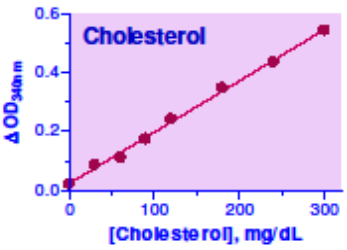
Note: if the sample OD value is higher than OD for the 300 mg/dL standard, dilute sample in water and repeat the assay. Multiply the results by the dilution factor.

MATERIALS REQUIRED, BUT NOT PROVIDED

Pipetting (multi-channel) devices, clear bottom 96-well plate and plate reader.

EXAMPLES

Samples were run in duplicate according to the standard procedure. The cholesterol concentrations (mg/dL) were 105 ± 3 for a human serum, 155 ± 11 for human plasma, 157 ± 2 for a bovine serum, 68 ± 2 for a rat serum, 129 ± 3 for a mouse serum, 123 ± 2 for a goat serum sample.



Standard Curve in 96-well plate assay

PUBLICATIONS

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