PLASMA VITAMIN E METABOLITE CONCENTRATIONS IN PREGNANT WOMEN AND UMBILICAL CORD BLOOD PAIRS

By

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List of Abbreviations

α-CEHC ..... 2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydrochroman
α-TTP ..... alpha-tocopherol transfer protein
γ-CEHC ..... 2,7,8-trimethyl-2-(2'-carboxyethyl)-6-hydrochroman
µmol ..... micromole
AGA ..... appropriate for gestational age
all-*rac*-α-tocopherol ..... synthetic form of α-tocopherol
BMI ..... Body Mass Index
cc ..... milligram
COX2 ..... cyclooxygenase 2
CV ..... coefficient of variation
CYP ..... cytochrome P450
DRI ..... Dietary Reference Intake
EAR ..... Estimated Average Requirement
EDTA ..... ethylenediaminetetraacetic acid
EtOH ..... ethanol
gm ..... gram
hCG ..... human chorionic gonadotropin
HDL ..... high-density lipoprotein
HPLC ..... high performance liquid chromatrography
IRB ..... Institutional Research Board
IU ..... Internation Unit
kcal ..... kilocalorie
L ..... liter
LDL ..... low-density lipoprotein
LGA ..... large for gestational age
LPL ..... lipoprotein lipase
mg ..... milligram
mmol ..... millimole
MRP ..... multi-drug resistance protein
NDSR ..... Nutrition Data System for Research

NHANES III .....Third National Health and Nutrition Examination Survey
nmol .....nanomole
OHSU..... Oregon Health & Science University
PUFA ..... polyunstaturated fatty acids
RDA ..... Recommended Daily Allowance
RNS.....reactive nitrogen species
RRR-α-tocopherol.....natural form of α-tocopherol
SD .....standard deviation
SGA..... small for gestational age
STATA.....Data Analysis and Statistical Software
TNF-α..... tumor necrosis factor-α
UL .....upper intake level
VLBW.....very low birth weight
VLDL.....very low-density lipoprotein

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Abstract

Previous studies have demonstrated low vitamin E concentrations in infants at birth, and deficient levels in preterm and low birth weight newborns. Low vitamin E has been associated with an increased risk of hemolytic anemia, detrimental fibroplasias, bronchopulmonary dysplasia, and thrombocytosis. It was suggested to supplement pregnant women with vitamin E in order to increase fetal stores, but studies have found that while supplementation during pregnancy increases maternal plasma concentrations, it does not increase the fetal vitamin E concentrations. Thus, the transfer of vitamin E across the placenta is limited. Studies of placental tissue demonstrate that the placenta expresses cytochrome P450 enzymes that are involved in vitamin E metabolism, suggesting that it might actively metabolize this nutrient, and thus prevent vitamin E accumulation in the fetal tissues. The purpose of this study was to 1) determine vitamin E metabolites, carboxyethyl hydroxychromans (α- and γ-CEHC), in umbilical cord plasma and maternal plasma, 2) to investigate possible correlations with α- and γ-tocopherol, 3) to evaluate associations with dietary parameters. The overall goal was to use indirect measures to assess whether the placenta actively metabolizes vitamin E.

A total of 15 maternal – cord blood pairs were analyzed for this sub-study. Healthy, pregnant women were enrolled from Oregon Health and Science University's obstetric clinic and at least one fasting blood sample was collected during their pregnancy. Umbilical cord blood samples were obtained from full-term, uncomplicated deliveries. Maternal and cord plasma samples were analyzed for α-tocopherol, γ-tocopherol, α-CEHC, and γ-CEHC concentrations. Total blood lipids were also measured to assess tocopherol/total lipids ratio between maternal and cord plasma.

Dietary and demographic information was analyzed for associations with biochemical parameters. Statistical differences were assessed using paired t-test and Pearson's correlation analysis.

Cord blood α- and γ-CEHC concentrations were 33.4 ± 29.4 nmol/L and 115.8 ± 55.8 nmol/L, and were not significantly different from maternal α- and γ-CEHC concentrations ($p = 0.07$ and $p = 0.08$, respectively). The metabolite to tocopherol ratio was significantly higher in cord blood. Cord blood α- and γ-tocopherol concentrations were significantly lower than maternal concentrations (6.8 ± 1.7 μmol/L vs. 34.2 ± 6.6 μmol/L α-tocopherol; and 0.4 ± 0.2 μmol/L vs. 1.9 ± 0.8 μmol/L γ-tocopherol; $p < 0.001$). Tocopherol/total lipids ratios were also significantly lower in cord blood for both forms, α and γ ($p < 0.001$ and $p < 0.05$, respectively). Of the tocopherols, only α-tocopherol/total lipids ratio was significantly correlated between maternal and cord concentrations ($r = 0.56$, $p < 0.05$). Furthermore, the higher dietary intake of vitamin E from prenatal vitamins was associated with the higher α-CEHC concentration in fetal circulation ($r = 0.66$, $p < 0.05$).

The present study did not find significant differences in vitamin E metabolite concentrations between maternal and cord plasma. However, higher dietary intake of vitamin E by the mother correlated with increased vitamin E metabolite concentration in the fetal circulation. This might imply that the higher level of supplemented vitamin E intake results in an increased metabolism of the vitamin, possibly by the placenta. Further insight into placental transfer of vitamin E is important to determine the safety and efficacy of administering supplemented vitamin E during pregnancy and its affect on the fetus.

CHAPTER 1

INTRODUCTION

Significance

Vitamin E is a generic term for a group of naturally occurring tocopherol and tocotrienol derivatives. It plays a major role as a lipid anti-oxidant by inhibiting the peroxidation of polyunsaturated fatty acids in cell membranes (1). Though all of the eight forms are equally absorbed from the diet, the α-tocopherol form is presently considered to be the most bioactive. Vitamin E is thought to be transformed to its metabolite-derivatives via cytochrome P450 enzymes (CYP) and β-oxidation. Carboxyethyl hydroxychromans (α- and γ-CEHC) are fairly new metabolites, and have not been previously investigated in the fetal circulation.

Previous studies have demonstrated lower vitamin E concentrations in fetal circulation compared to the maternal circulation. Deficiency is most often observed in preterm and low birth weight infants. In 2006, 12% of live births were preterm (before 37 weeks gestation) in the United States with a greater than 16% increase between 1996 and 2006, while low birth weight (less than 2500 gm or 5 ½ lb) was 8.3% of live births (2). Treating very low birth weight (VLBW) and preterm infants with pharmacologic doses of vitamin E has been proposed in order to prevent and limit associated vitamin E deficiency conditions such as retinopathy, intracranial hemorrhage, hemolytic anemia, and chronic lung disease. The 2003 Cochrane review on vitamin E supplementation of preterm infants (3) concluded that high dose supplementation greater than 30 IU/kg/day

or resulting in levels greater than 3.5 mg/dl (81 μmol/L) are associated with an increased incidence of sepsis, despite reducing the risk of intracranial hemorrhage, severe retinopathy and blindness among those examined. Since these complications in neonates were thought to be associated with oxidative stress, a prophylaxis with antioxidants for pregnant mothers was believed to prevent oxidative stress and thereby reduce perinatal complications in the infant and possibly reduce the risk of maternal preeclampsia (4). Thus, it has been suggested to supplement pregnant women with vitamin E to increase fetal stores.

Relatively little is known about the transfer of vitamin E by the placenta. Low fetal vitamin E concentrations suggest vitamin E transfer across the placenta is limited. It is possible that the placenta might restrict the passage of vitamin E to the fetus. The human fetal liver possesses cytochrome P450 enzymes that are involved in vitamin E metabolism, but its levels of expression and activity are lower than in adult tissue (5). Fetal liver would most likely have a limited capacity to metabolize vitamin E. Thus, the placenta may play a role in vitamin E metabolism to prevent toxic level of vitamin E reaching the fetus. Prenatal vitamin use is widespread in United States, with approximately 92% of married mothers reported taking vitamin supplements during pregnancy in the 1980 National Natality Survey (6). Most prenatal vitamins contain varying levels of vitamin E. A greater understanding of this nutrient's transport to the fetus is necessary to establish its safety and make a recommendation about supplementing pregnant women with the goal of increasing fetal stores.

Specific Aims

Study Objective: The purpose of this study was to determine vitamin E metabolites', carboxyethyl hydroxychromans (α- and γ-CEHC), concentrations in umbilical cord blood in comparison to maternal concentrations, and to investigate possible correlations with α- and γ-tocopherol, to evaluate associations with dietary parameters, and to indirectly assess whether the placenta metabolizes vitamin E.

Primary Aim #1: To measure concentrations of α/γ-CEHC, α/γ-tocopherol, and α/ γ-tocopherol/ total lipids ratio in fetal circulation and compare it to maternal concentrations. These results will be used to indirectly assess placental metabolism of vitamin E.

Hypothesis #1: Cord blood concentrations compared to maternal concentrations will have:

i. Higher α-CEHC and γ-CEHC concentrations
ii. Lower α-tocopherol and γ-tocopherol concentrations
iii. Lower α-tocopherol/total lipids and γ-tocopherol/total lipids ratios

Secondary Aim #1: To assess other factors that could be associated with maternal and fetal tocopherol and CEHC concentrations.

Hypothesis #2: Maternal and cord biochemical parameters will be significantly correlated with:

i. Demographic indices
ii. Delivery outcomes
iii. Dietary parameters: intake of vitamin E from food and prenatal vitamins

CHAPTER 2

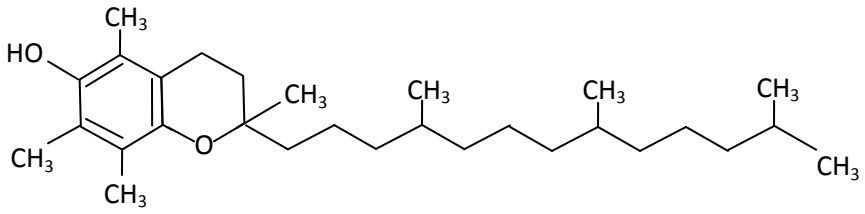
BACKGROUND INFORMATION

Vitamin E was discovered in 1922 when Evans & Bishops observed that rats fed a rancid fat diet resorbed their fetuses (7), which demonstrated the nutrient's essential role during pregnancy. It has also been shown that an adequate fetal storage is vital during birth to protect against oxygen toxicity and to stimulate the development of the immune system (8). Vitamin E deficiency is most often observed in premature neonates and low birth weight newborns. Low vitamin E has been associated with an increased risk of hemolytic anemia, detrimental fibroplasia, intraventricular hemorrhage, thrombocytosis, pulmonary oxygen toxicity, and bronchopulmonary dysplasia in newborns (8). Though supplementation of pregnant mothers increases maternal vitamin E concentrations, the same does not hold true for the fetal concentrations (9), and it is generally agreed that the plasma α-tocopherol levels are lower in the newborns as compared to the mothers (8, 10). The data is suggestive of a possible placental mechanism in restricting the passage of vitamin E to the fetus. Several possible explanations have been developed as to why the fetal concentrations tend to be lower. The goal of this project was to explore the possibility that the placenta actually metabolizes vitamin E, thus preventing any significant increase in fetal concentrations.

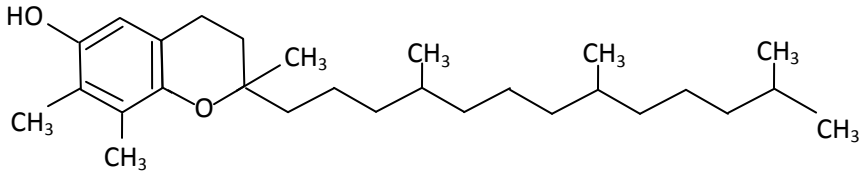
Vitamin E Structure and Metabolism

Vitamin E is a generic term for a group of naturally occurring tocopherol and tocotrienol derivatives (designated as α-, β-, γ-, and δ-) containing a chromanol head group attached to a 12-C hydrophobic side chain (11) (Figure 1). Though all of the eight forms are equally absorbed from the diet, the α-tocopherol form is presently considered to be the most bioactive and is the most abundant one in tissue (90%) (12). Since humans and animals cannot synthesize vitamin E, the primary dietary source is plant foods, such as vegetable oils, wheat germ, nuts and seeds. Gamma-tocopherol is the chief form in the American diet (13), but exhibits only 10-30% of the α-tocopherol activity (14). The synthetic form of vitamin E, all-*rac*-α-tocopherol, consists of a racemic mixture of all eight possible stereoisomers (RRR, RSR, RRS, RSS, SRR, SSR, SRS, SSS), but in nature only the RRR-α-tocopherol isomer is present. It was determined that the natural vitamin E has approximately twice the availability of synthetic vitamin E after supplementing with equimolar mixture of deuterated RRR-α-tocopheryl acetate and all-*rac*-α-tocopheryl acetate (15). This difference is mainly attributed to the action of α-tocopherol transfer protein (α-TTP) in the liver, preferentially transferring the 2R-α-tocopherol isomers to the nascent lipoproteins (16). There is a subsequent increase in metabolism of the 2S-α-tocopherol forms (17). Plasma concentration of RRR-α-tocopherol is relatively steady compared to other forms, with a half-life of 57 ± 19 hours compared to 13 ± 4 hours for γ-tocopherol (18) and 15.8 ± 5.7 hours for synthetic SRR-α-tocopherol form (19). The rate at which α-tocopherol enters plasma depends on its absorption, tissue delivery, metabolism, and excretion.

Figure 1. Structures of α- and γ-tocopherol.



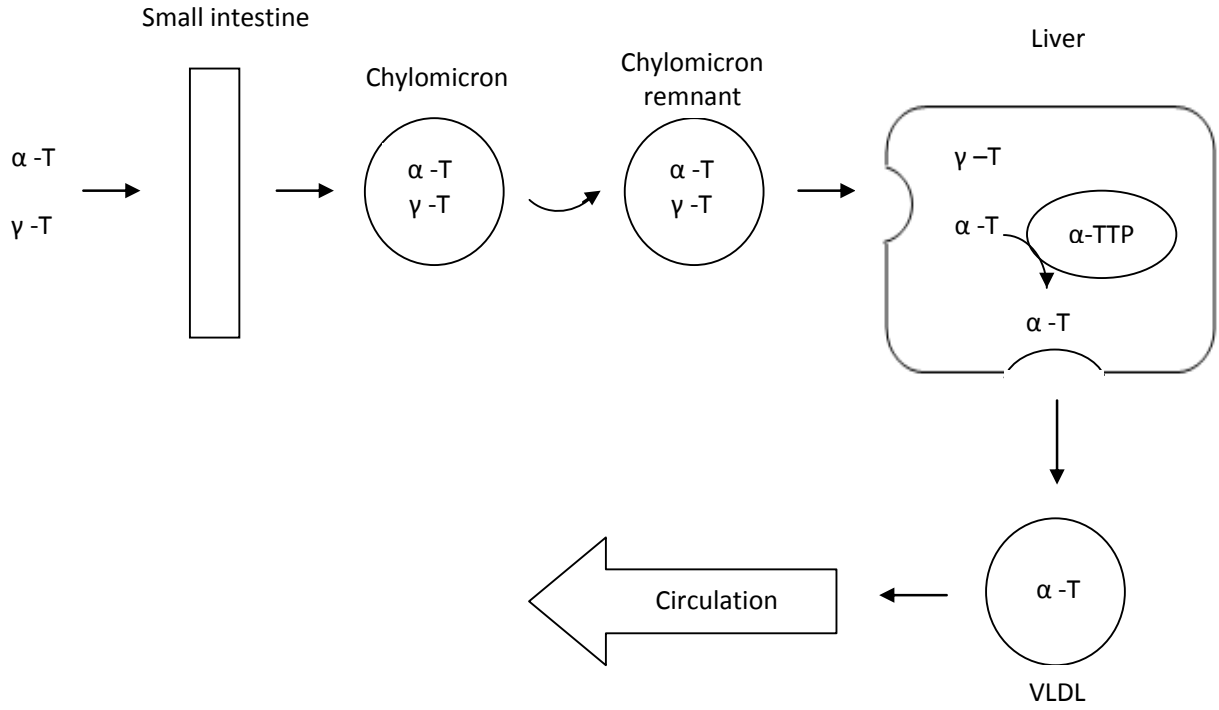
α-tocopherol



γ-tocopherol

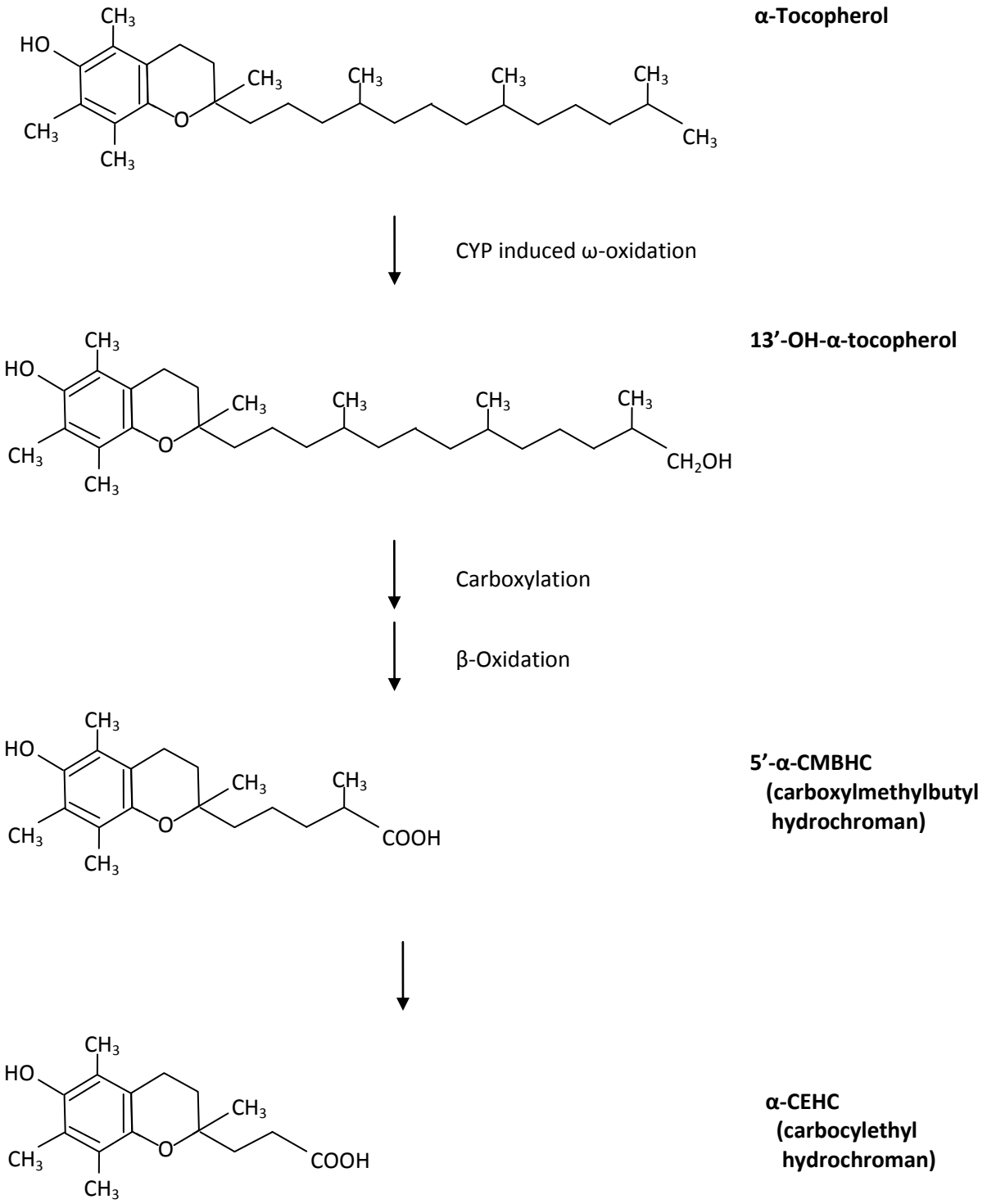
The mechanism of absorption is not fully understood, but it seems to follow that of lipid uptake. Intestinal absorption requires pancreatic esterases and bile acid secretion, which form micelles containing dietary fat and fat soluble vitamins. The micelles are taken up by intestinal enterocytes and subsequently vitamin E becomes incorporated into chylomicrons, which are secreted into the lymphatic system (20) (Figure 2). All forms of vitamin E seem to be equally absorbed across the intestine, and absorption is strongly correlated with the fat content of the meal (11). In the circulation, chylomicrons are hydrolyzed by lipoprotein lipases (LPL). The vitamin is released with fatty acids for uptake by peripheral tissue, or transferred to high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs). In the liver, chylomicron remnants are taken up and the contents are repackaged into very low density lipoproteins (VLDL) for plasma secretion. It has been shown that the RRR-α-tocopherol is preferentially incorporate into VLDLs by the action of α-TTP in the liver (21) while the other forms are primarily metabolized and excreted into the bile and urine. Studies determined that the affinity of α-TTP for different vitamin E forms varies, RRR-α-tocopherol = 100%, γ-tocopherol = 9%, β-tocopherol = 38%, δ-tocopherol = 2%, α-tocopheryl acetate = 2%, SRR-α-tocopherol = 11%, and α-tocotrienol = 12% (22). Thus, the protein is responsible for discriminating against non-alpha forms. The highest concentration of α-TTP is in the liver, but it has also been detected in the human brain (23) and human placenta (24); the function it has in those tissues is not presently understood.

Figure 2. Absorption and transport of vitamin E.



Unlike other fat-soluble vitamins, the liver plays an important role in the degradation of vitamin E, thus preventing its accumulation that could possible result in "toxic" effects (25). The first types of vitamin E metabolites identified, "Simon metabolites", have subsequently been shown to be the inevitable products of oxidation during sample preparation in vitro, and not the major in vivo vitamin E metabolites (26). In the 1980s, a different metabolite group was identified, 2-(2'-carboxyethyl)-6-hydroxychroman (CEHCs), from the urine of rats that were injected with δ-tocopherol (27). Metabolites of α- and γ- tocopherol, 2,5,7,8-tetramethyl-2-(2'carboxyethyl)-6-hydroxychroman (α-CEHC) and 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydrochrmon (γ-CEHC) are produced in the liver, and are also found in the plasma, bile, and urine. Though the exact process of metabolite formation is still under investigation, the proposed pathway involves an initial step of ω-oxidation by the cytochrome P450 (CYP) enzyme system, forming 13'-OH-tocopherol metabolites, followed by consecutive steps of β-oxidation, resulting in cleavage of the phytal tail and formation of CEHC metabolites (28) (Figure 3). The cytochrome P450 system of enzymes is involved in metabolism of different xenobiotics, such as medical drugs, toxic chemicals, vitamins, and also in synthesis and catabolism of steroid hormones (29). Cell culture studies demonstrate that the CYP 3A family may be involved in vitamin E ω-oxidation (30-32), and the CYP 4F2 isoform has been identified as the hydrolase of vitamin E in insect cells (33). In rats, supra-elevated hepatic α-tocopherol concentrations up-regulated hepatic proteins involved in metabolism of xenobiotics, CYP3A and CYP2B. Because vitamin E does not accumulate in the body to toxic levels, this suggests that up regulation of the CYP enzymes is a possible mechanism to regulate excess accumulation of the vitamin

Figure 3. Degradation of α-tocopherol by ω-and β-oxidation.



(34). Following CEHC formation, the metabolites are conjugated with sulfate or glucorinic acid and excreted in either urine or bile (28).

Vitamin E Function and Dietary Intake

The major role of vitamin E in the human body is that of a lipid antioxidant, which acts by inhibiting the peroxidation of polyunsaturated fatty acids (PUFA) in the cell membrane (35). Tocopherol reacts with the peroxyl radicals, breaking the auto-oxidation chain reaction of PUFAs. The subsequently formed tocopheroxyl radical reacts with vitamin C, regenerating it back to its reduced state (36). This fat-soluble antioxidant is believed to play a key role in delaying the pathogenesis of various degenerative diseases (i.e. cancer, inflammatory diseases, neurological disorders, chronic vascular diseases) through its function of preventing free radical-mediated tissue damage (8). Vitamin E is also important during pregnancy, both during gestation and at the time of birth. Several key stages have been identified that require vitamin E's involvement. Vitamin E is necessary for the development of early embryos in vitro (37), during implantation (38), for placental maturation (39), for protection of the fetus against oxidative damage (40), and at birth adequate stores of the nutrient are needed to prevent oxygen toxicity once the newborn leaves the womb.

The dietary reference intake (DRI) for adults and women during pregnancy is 15 mg of RRR-α-tocopherol per day (41). The requirements were determined based on studies done in vitamin E depleted human subjects and correlated hydrogen peroxide-induced erythrocyte lysis with their plasma α-tocopherol concentrations. Nineteen adult males followed a diet restriction of 2-4 mg of α-tocopherol and 55 gm of fat (30 gm from

vitamin E-free lard) for 2.5 years. Thereafter, lard was replaced with thermally oxidized corn oil, to increase the total intake of polyunsaturated fatty acids (PUFAs) and oxidant burden on vitamin E stores. Subjects followed a total of 6 years on this vitamin E depleting diet. The biomarker for Estimated Average Requirement (EAR) was established as the plasma α-tocopherol concentration that limited hydrogen peroxide-induced hemolysis to < 12%. Twelve μmol/L was chosen as the cutoff plasma concentration associated with normal in vitro hydrogen peroxide-induced hemolysis for 50% of the population. An α-tocopherol dietary intake of 12 mg per day was determined to generate a plasma levels correspondent to the biomarker criterion. Thus, the Recommended Dietary Allowance (RDA) was set at 12 mg plus twice the coefficient of variation (CV) to cover 97-98% of the population. Based on the NHANES III data (National Health and Nutrition Examination Survey, 1988-1994), less than 5% of the population surveyed was vitamin E deficient by this criterion.

During pregnancy, prenatal vitamins are prescribed as a standard of practice. The level of vitamin E in the prenatal vitamin varies from anywhere of 0 to 100 IU per tablet, typically 15 IU (personal observation). These preparations usually contain the all-*rac*-α-tocopheryl acetate form of the vitamin that is less biopotent. Some more "natural" types of supplements contain the RRR-α-tocopheryl acetate form. The upper tolerance level (UL) for α-tocopherol is set at 1000 mg/day by the Institute of Medicine's Food and Nutrition Board based on a possible increase in blood coagulation time, therefore, potentially increasing the risk of hemorrhage. Studies that looked at adult supplementation of vitamin E, found a dose-dependent kinetics of α-tocopherol, such that increases in vitamin E supplementation results in progressively smaller increases in

plasma concentrations. Regardless of the duration and the dose of vitamin E supplementation, the plasma concentrations in normal subjects are observed to raise only 3 to 4 fold (42).

γ-Tocopherol

Though γ-tocopherol is the most abundant form of vitamin E in the American diet, its plasma concentration is about 10-15 times lower than that of α-tocopherol (α-tocopherol 32 μmol/L vs. γ-tocopherol 1.9 μmol/L) (43). This difference is primarily due to the action of α-TTP preferentially transferring the alpha form into VLDLs (very low density lipoproteins). The liver metabolizes γ-tocopherol similarly to the α-tocopherol metabolism process; ω-oxidation through the action of CYP450, followed by β-oxidation, generating 2,7,8-trimethyl-2-(2'-carboxyethyl)-6-hydrochroman (γ-CEHC). It has been shown that α-tocopherol supplementation lowers the γ-tocopherol concentration in the blood and adipose tissue, but the significance of that is not yet identified. Huang and Appel showed a reduction in serum γ-tocopherol level by 58% after a 2 month supplementation with 296 mg/d of α-tocopheryl acetate (44). Hamdelman et al. analyzed vitamin E concentration in adipose tissue of 4 male subjects following a one-year supplementation with 800 mg/d of all-rac-α-tocopherol, and found a ≈50% decrease in γ-tocopherol adipose concentration, but no change in α-tocopherol concentration over the supplementation period (45). There is also data indicating that γ-tocopherol is preferentially metabolized with the higher α-tocopherol intake. A study performed in mice investigated the effects of supplementation with variable level of α- and γ-tocopherol on the protein expression of Cyp3a (46). A positive correlation was found

between liver α-tocopherol concentration and Cyp3a protein expression as well as γ-CEHC concentration. The authors suggested a possible mechanism where α-tocopherol supplementation increasing Cyp3a expression, leading to increased breakdown of the gamma form, while the alpha form is transferred into the plasma through α-TTP. The function of γ-tocopherol in the human body in not yet fully understood. It has been suggested that γ-tocopherol is superior in quenching oxidative damage from reactive nitrogen species (RNS), compared to α-tocopherol (47) and serves to provide an additional antioxidant effect to protect against cardiovascular diseases, cancer, and dementia (43). While supplementation with high levels of α-tocopherol might provide some health benefits, it could potentially diminish the affect of γ-tocopherol. The ideal ratio of α- and γ-tocopherol to promote human health is unknown at present.

Vitamin E deficiency in human adults is rare; no symptoms have ever been detected in healthy individuals consuming low amounts of vitamin E. Occurrences are noted in individuals with malabsorption syndromes, severe protein-energy malnutrition, and genetic defects in α-TTP. Symptoms include peripheral neuropathy, ataxia, skeletal myopathy, and pigmented retinopathy (48). Preterm and low birth weight infants are considered at risk for vitamin E deficiency. Accumulation of vitamin E typically occurs during the third trimester of pregnancy with the corresponding increase in fetal fat stores (8). Premature neonates have lower plasma α-tocopherol levels compared to term newborns (49), and it generally takes longer to replenish preterm infant's serum levels to normal. This difference could be attributed to a possible reduction in the ability to absorb vitamin E or a higher vitamin E requirement (50). Furthermore, a higher level of oxidative stress markers is observed in preterm infants compared to healthy term infants

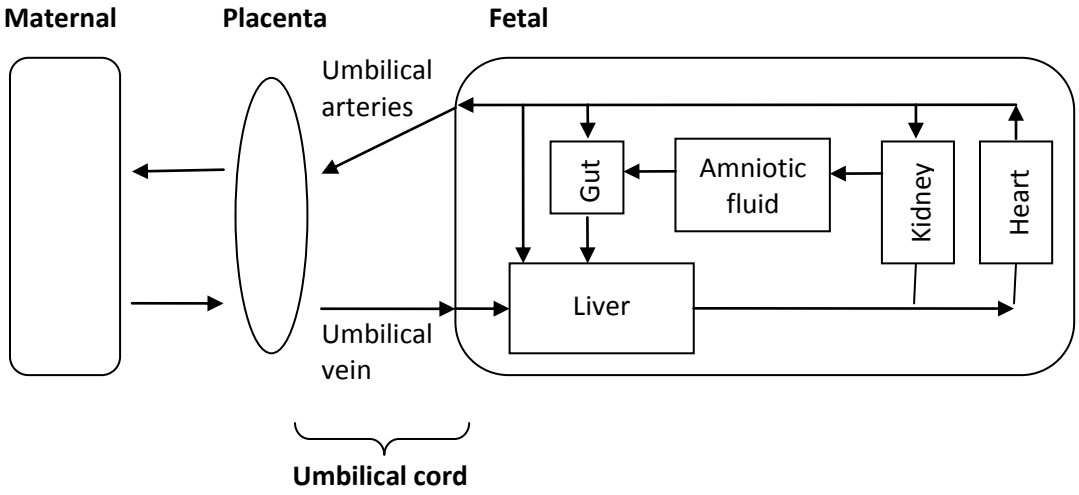
(49). Preterm infants exhibit immature antioxidant enzyme systems, increased oxidative stress during the birthing process, and increased levels of oxidative stress markers, suggesting they are at an increased risk for oxidative injury.

Placenta and Vitamin E

The placenta is an organ that, from the third month of pregnancy to the time of birth, serves to supply nutrients to, and remove wastes from the fetus (51). Composed of both maternal and fetal tissue, it includes the basal membrane (fetal-facing), which connects the umbilical cord, and the brush-border membrane (maternal-facing), which consists of chorionic villi, contributed by the fetus, and deciduas basalis, a region of the mother's endometrium. Fetal blood is delivered from the heart to the placenta by way of two arteries in the umbilical cord; it flows into the capillary beds of the villi for nutrient exchange, and returns to the fetus by way of a single umbilical vein (Figure 4). The chorionic villi are surrounded by the maternal blood pool in the inervillus space, but the two bloodstreams do not mix; maternal and fetal circulations remain separated by a thin barrier composed of capillary endothelium and trophoblasts that form the membrane covering of the villi. Nutrient transfer across this membrane occurs via different mechanisms; i.e. electrolytes, fatty acids, and steroids pass by simple diffusion; glucose by facilitated diffusion; amino acids by active transports; and insulin by receptor-mediated endocytosis (52).

The placenta also possesses different enzyme systems for the metabolism of xenobiotics, foreign substances to the body, and it acts as a barrier to reduce their transfer from the mother to the fetus. Similar to vitamin E, xenobiotics are metabolized by

Figure 4. Maternal-placental-fetal unit.



cytochrome P450 enzymes, which are thought to be located in the trophoblasts of the placenta (29). In the full-term placenta, mRNAs for CYP1A1, 1B1, 2E1, 2F1, 3A3-7 and 4B1 have been detected, but only CYP1A1, 2E1, 3A4, 3A5, 3A7, and 4B1 have been characterized at the protein level (53, 54). Relatively little is known about the individual forms of P450 enzymes in human placenta; even though mRNAs and proteins for CYP3A (the isoform thought to be involved in vitamin E metabolism) have been detected, no activities for the enzymes have been reported at present (55).

The transfer of vitamin E across the placenta is considerably slow, only 10% of the passively transferred marker L-glucose (56). The exact mechanisms are not fully understood, though it has been proposed to involve LPL and lipoprotein receptor pathways. One accepted explanation relates this to inefficient transfer of plasma lipids by the placenta (8). Cholesterol and triglyceride concentrations are found to be lower in cord versus maternal blood and to increase with fetal age. It is well documented that the fetal plasma α-tocopherol concentration is significantly lower than the maternal concentration and the fetal α-tocopherol concentration increases in correlation with the increase in lipids with gestational age (10). Due to this significant correlation, vitamin E is typically standardized with lipids in the plasma to represent a more accurate status (57). Another potential explanation involves the α-TTP, which is primarily expressed in the liver, and recently has been isolated from the human placental trophoblast cells (24, 58). The RRR-α-tocopherol form was shown to be preferentially transferred across the placenta compared to the all-*rac*-α-tocopherol form. This could be attributed to the presence of α-TTP stereoselectively transporting α-tocopherol from the maternal to the fetal circulation (59, 60). Since vitamin E's metabolism involves the CYP enzyme

system, it is also possible that placenta actively metabolizes the nutrient, and in this way prevents its accumulation in the fetus. The fetal liver is not mature enough to handle the whole flux of vitamin E, and therefore there is a possible mechanism emplaced to prevent fetal intoxication.

There seems to be no evident correlation between maternal and fetal blood concentrations of plasma α-tocopherol. Short-term supplementation with 1 g dl-α-tocopherol acetate (dl denotes a formerly used term for synthetic tocopherol = all-rac-tocopherol) for 3 days prior to delivery increased the maternal stores, but had no effect on the newborn vitamin E status (9). The lower concentrations of vitamin E in cord blood compared to maternal concentrations have been demonstrated in numerous studies (10, 61, 62), but standardization for plasma lipids results in varying conclusions among these studies. Jain et al. found a lower α-tocopherol/total lipids ratio in newborns compared to mothers (1.9 ± 0.1 nmol/μmol vs. 2.6 ± 0.1 nmol/μmol; $p = 0.0001$), with total lipids determined as a sum of triglycerides, cholesterol, and phospholipids concentrations (63). Dison et al. compared α-tocopherol/cholesterol ratio, and found no significant difference between fetal and maternal ratios (5.0 μmol/mmol vs. 6.0 μmol/mmol) (64). Herrera et al. investigated a relationship between the plasma fatty acid profile and the antioxidant vitamins. They found a lower α-tocopherol/plasma lipids (cholesterol plus triglycerides) ratio in the cord blood compared to the maternal ratio at delivery, but this difference did not reach significance (3.45 ± 0.19 μmol/mmol vs. 3.91 ± 0.21 μmol/mmol) (65).

The concentrations of vitamin E's metabolites (CEHCs) in cord blood have not been previously investigated. α-CEHC was first identified in human urine after a high intake of α-tocopherol (26), and it is thought to reflect the α-tocopherol that was neither

consumed in oxidation nor bound in the lipid phase, thus possibly indicating sufficient or higher-than-optimal intake of vitamin E. The observed range for α-CEHC in human serum is approximately 5 to 10 nmol/L, but can increase up to 200 nmol/L upon supplementation. Stahl et al. demonstrated a 15- to 30-fold increase in serum α-CEHC after 500 IU RRR-α-tocopherol supplementation over a period of 4-7 weeks, while α-tocopherol rose only by a factor of 1.5 to 3 (66). The γ-CEHC was first isolated from human urine while searching for a natriuretic hormone (67). Galli et al. determined the basal concentrations of plasma α- and γ-CEHC in nine healthy subjects at 12.6 ± 7.5 nmol/L and 160.7 ± 44.9 nmol/L, respectively (68). Radosavac et al. assessed the serum levels of α- and γ-CEHC in 21 healthy subjects after a single dose supplementation with 306 mg of RRR-α-tocopherol and 1.77 mg of γ-tocopherol, and found the peak serum levels of α-tocopherol and α-CEHC is reached at 12 hours after ingestion, and return to baselines at 72 hours (69). On the other hand, the γ-tocopherol concentration gradually decreased from baseline without peaking, but there was an increase in γ-CEHC concentration. The authors concluded that there appears to be a parallel increase in α-CEHC metabolite concentration compared to its parent compound after supplementation. In contrast, γ-tocopherol is more effectively metabolized to γ-CEHC compared to α-tocopherol.

This study attempts to measure and compare α-tocopherol and γ-tocopherol to α-CEHC and γ-CEHC concentrations in pairs of maternal vs. cord blood samples to determine if the placenta possibly metabolizes vitamin E via the cytochrome P450 enzyme system. This may have an impact on clinical practice of supplementing pregnant

mothers for the purpose of increasing fetal stores. If the placenta actively metabolizes vitamin E, supplementation during pregnancy to increase fetal stores may be ineffective.

CHAPTER 3

METHODS

General Design

This was an observational, prospective cohort sub-study of the ongoing "In-Utero Smoke: Vitamin C and Newborn Lung Function" study, conducted by Dr. Cynthia McEvoy at Oregon Health & Science University (OHSU). The vitamin C study is a double-blind randomized trial, which investigates whether supplementation of vitamin C given to smoking pregnant women has a positive effect on newborn's lung function. In the sub-study, the non-smoking reference group was used to determine normal vitamin E and metabolite concentrations of pregnant women and their newborns. The OHSU Institutional Research Board (IRB) approved this as a part of the "vitmain C" study. Participation in the study was voluntary and written informed consent was obtained from each subject.

Sample Recruitment and Selection

A total of 17 healthy, non-smoking pregnant mothers were recruited for this sub-study from the OHSU obstetrical clinics and followed throughout routine prenatal hospital visits, and 15 pairs were used for analysis. The inclusion and exclusion criteria for the subjects followed that of "In-Utero Smoke: Vitamin C and Newborn Lung Function" protocol, listed in Table 1. Exclusion criteria was established to exclude individuals whose medications, personal characteristics, obstetric conditions, or diseases

Table 1.

Inclusion and exclusion criteria for reference group of non smokers.

Inclusion criteria	Exclusion criteria
1. Maternal age of at least 15 years old.	1. Multiple gestation.
2. Pregnant with consent and enrollment/randomization prior to 22 weeks of gestation by last menstrual period (LMP) and confirmed by ultrasound when available.	2. Documented major fetal congenital anomalies.
3. Gives history of never smoking with confirmatory urine cotinine.	3. Current maternal use of heroin, cocaine, crack, LSD, or methamphetamines.
4. Singleton gestation.	4. Recent history of alcohol abuse: $\geq$ 3 drinks on $\geq$ 5 days / week since LMP, hospitalization for alcohol abuse.
5. Informed consent signed.	5. Continuous use of daily high dose vitamin C and / or vitamin E supplements since LMP or refusal to abstain from using vitamins other that those provided through study. PNV (85 mg of vitamin C) will be provided to all mothers in the 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 diabetic.
	10. Initial ascorbic acid level > 100 μmol/L when available.
	11. Urine cotinine of >0.50 ng/ml to exclude a closet smoker.

could have had significantly affected newborn lung function, vitamin C or its metabolism, or poor compliance.

Study Design

Subject recruitment took place between June and September of 2008, at the OHSU obstetrical clinic. After the primary care provider had given the patient initial study information and the patient agreed to speak to a study investigator, the patient was approached about the study. At that initial encounter, the subject was consented and two follow-up meetings with a study investigator were coordinated with routine prenatal visits around 22 and 28 weeks of gestation. At subsequent scheduled appointments, a fasting blood draw a minimum of one-half cc was collected into Vacutainer tubes containing 1 mg/ml EDTA, and the previous day's 24 hour diet recall history was obtained and entered into a computer database system. At admission to the hospital for delivery, one of the nurses paged a study investigator informing them of the mother's arrival to the hospital. After delivery, the placenta was transported to a utility room; the study investigator collected a minimum of one-half cc of mixed cord blood sample into the EDTA tube. If available before hospital discharge, a maternal blood sample was also collected. Whole blood was centrifuged and plasma was stored at -80ºC until analysis. Maternal and infant hospital records were reviewed for standard maternal data and morbidities. The design of the study is summarized in Table 2.

Table 2.

Summary of study design.

	~ 22 weeks GA	**~ 28 weeks GA**	**Delivery**
Fasting blood draw	X	X	X
24-hr diet history	X	X	X
Umbilical cord blood			X

Data Collection Methods

Demographic, Anthropometric and Delivery Indices

Demographic and anthropometric indices were obtained from OHSU's electronic charting system, Epic, to determine the participants' age, ethnicity, profession, marital status, number of previous pregnancies, pre-pregnancy BMI, newborn's birth weight, type of delivery, gestational age at delivery, and time between admission to Labor and Delivery and the actual delivery of the baby.

Dietary Assessment

Nutrient intake data obtained from 24 hour dietary recalls was calculated using Nutrition Data System for Research (NDSR, 2006 and 2007, University of Minnesota). Dietary intake of total vitamin E, α-tocopherol, γ-tocopherol, total fat, total cholesterol, and energy was calculated and recorded for each available visit.

Follow-up Questionnaire

Due to a high variability in metabolite concentrations for both maternal and cord samples, a follow-up phone call questionnaire was administered asking the participating mothers about the type of prenatal vitamin they took during pregnancy, the time of day and how many times a week they took it, and the time between the last meal they consumed and delivery of their baby. These variables were thought to possibly influence the metabolite concentrations in plasma. Only 12 out of 15 mothers responded to the questionnaire. The questionnaire is presented in Appendix A.

Blood Sample Analysis

Plasma was obtained by centrifugation of blood samples at 2500 x g, 4°C for five minutes, which was then aliquoted into appropriate storage tubes and stored at -80°C until analysis. Samples for vitamin E analysis were transported to and run at Dr. Traber's laboratory at the Linus Pauling Institute (Corvallis, OR). Samples for lipid analysis were measured at OHSU's Lipid Clinic.

Tocopherol analysis

Plasma α- and γ-tocopherol concentrations were measured using high-performance liquid chromatography (HPLC) with amperometric detection (Appendix B). Plasma vitamin E was extracted using a liquid-liquid extraction protocol. Briefly, samples were placed into a 70ºC water bath for 30 minutes after the addition of 2 ml ethanol/1% ascorbic acid, 900 µl Milli-Q water and 0.3 ml saturated potassium hydroxide. Following saponification, 25 µl butylated hydroxytoluene (1 mg/ml EtOH), 1ml Milli-Q water/1% ascorbic acid, and 2 ml of hexane were added. Samples were mixed and an aliquot of the hexane layer was evaporated under nitrogen, re-suspended in 50% methanol mixture, and briefly vortexed. Twenty µl of the sample was injected onto the column, and concentrations were calculated using external standard curves.

CEHC analysis

α- and γ-CEHC were separated by reverse-phase HPLC and quantified by single quadrupole mass spectrometric detection, as previously determined (70). Sensitive methodology was originated to attain accurate measurements for plasma concentrations in low-nanomole-per-liter ranges. The samples were incubated at 37ºC for 30 minutes

with β-glucuronidase enzyme solution, 1% ascorbic acid, and internal standard (trolox). The samples were then acidified with 10 µL 12 M HCl and extracted with diethyl ether. An aliquot of the ether layer was dried under nitrogen, re-suspended in 100 µl of 50% methanol solution, and injected onto the column. Using single-ion-recording, analyte retention times were: α-CEHC 15.57 minutes, γ-CEHC 14.70 minutes, and trolox 14.60 minutes using an electrospray probe set to negative mode . Standard curves were generated using external standards and trolox as the internal standard. Metabolite concentrations were calculated by adjusting the unknown areas to that of the internal standard and plotted on the corresponding curve.

Total cholesterol and triglycerides analysis

Lipid panels including total cholesterol and triglycerides were measured by standard autoanalyzer in the OHSU lipid lab. In order to calculate lipid standardized vitamin E concentration, tocopherol was divided by total lipid (calculated as cholesterol plus triglycerides) to obtain a ratio.

Statistical Analysis

Sample Size Determination

Maternal and cord plasma concentrations of tocopherols were previously determined in maternal and cord blood samples at 20.65 ± 4.0 µmol/L and 7.21 ± 1.9 µmol/L with an approximate 13 unit change, and α-tocopherol/lipid ratios at 3.41 ± 0.8 µmol/mmol and 2.95 ± 0.7 µmol/mmol with an approximate 0.46 unit difference between maternal and fetal concentrations (10). The estimated difference between sample means for this study design was based on the smaller unit difference between maternal and cord

blood plasma α-tocopherol/lipid ratio. To determine the sample size for a one-tailed paired-sample t-test, with a significance level of $\alpha = 0.05$ and power of $1-\beta = 0.90$, a non-centrality parameter formula for a t-distribution was used (71). The estimated sample size required to detect a 0.5 unit difference in α-tocopherol/lipid ratio with an estimated standard deviation of 0.5 was 19 pairs. In the initial proposal, we anticipated collecting a sample size of 20 maternal cord blood pairs, which provided a power of 93% to detect a difference in α-tocopherol/lipid ratio of 0.5 μmol/mmol. The final sample size for the study that was actually collected equaled 15 pairs. This provided a power of 85% to detect the expected change in α-tocopherol/lipid ratio.

Data Management

STATA (version 10.1) was used to perform statistical analyses. The means and the standard deviations were calculated for all outcome variables. One-tailed paired-sample t-tests were used to calculate differences between maternal plasma and cord blood in α- and γ-tocopherol concentrations, α- and γ-CEHC concentrations, and α- and γ-tocopherol/total lipid ratios, based on the directional hypothesis that were originally made. Pearson's correlation analysis was used to assess a possible relationship between maternal and cord blood in biochemical data and to determine a possible association in plasma and dietary α- and γ-tocopherol. A statistical *p*-value of <0.05 was considered significant for all performed tests.

Administrative Responsibilities during Data Collection

Cynthia McEvoy, M.D., was the Prinicapal Investigator of "In-Utero Smoke: Vitamin C and Newborn Lung Function" study and assembled the research protocol for IRB approval. Participants were recruited and consented by study investigators, Cynthia McEvoy, M.D., Nakia Clay, M.A., and myself, Svetlana Zubkova, B.S., of the Graduate Program of Clinical Nutrition at OHSU. A study investigator or one of the OHSU obstetric clinic's nurses drew maternal blood samples, which were frozen and stored at Dr. Gillingham's laboratory. Frozen samples were transported to the Linus Pauling Institute (Corvallis, OR) Dr. Traber's, laboratory for vitamin E and metabolite analysis, and to the OHSU's lipid clinic for total lipid analysis by medical lab technologist, Carol Marsh. α- and γ-Tocopherol concentrations were measured and calculated by Katie Lebold, and α- and γ-CEHC concentration were measured and calculated by Scott Leonard, senior research assistant. I assisted the study investigators in collecting subject's diet-histories, performing dietary analysis, and collecting cord blood samples.

Limitations

Limitations of the current study included a small sample size of maternal/cord blood pairs. Though this was a pilot study in determining average vitamin E metabolite concentrations in cord blood in comparison to maternal blood, which hasn't been previously investigated, a larger sample size is warranted in future studies to represent a more heterogeneous population. Most of study participants were affiliated with the hospital as nurses and other staff, and were primarily Caucasian. Though this reduced variability between subjects, the study wasn't able to capture differences in population

groups, ethnically or in socioeconomic status. Also, not all anticipated samples were collected for every participant. The unpredictable nature of delivery time and inclement weather during the study period, made it difficult to obtain some cord samples at the time of delivery.

Another limitation of the study was the reliance on 24-hour dietary recalls to collect dietary intake information. This method is typically preferred for its readily available format, open-ended type questions, the subject is less likely to alter eating behavior, and there is a minimal burden on the respondent. On the other hand, the method relies on the subject's memory estimating portions sizes, and the single day intake may not represent a typical nutrient intake of the individual. The goal of the 24 hour recall in this study was to assess a possible relationship between dietary intake of vitamin E and blood concentrations, and to control for any confounding variables.

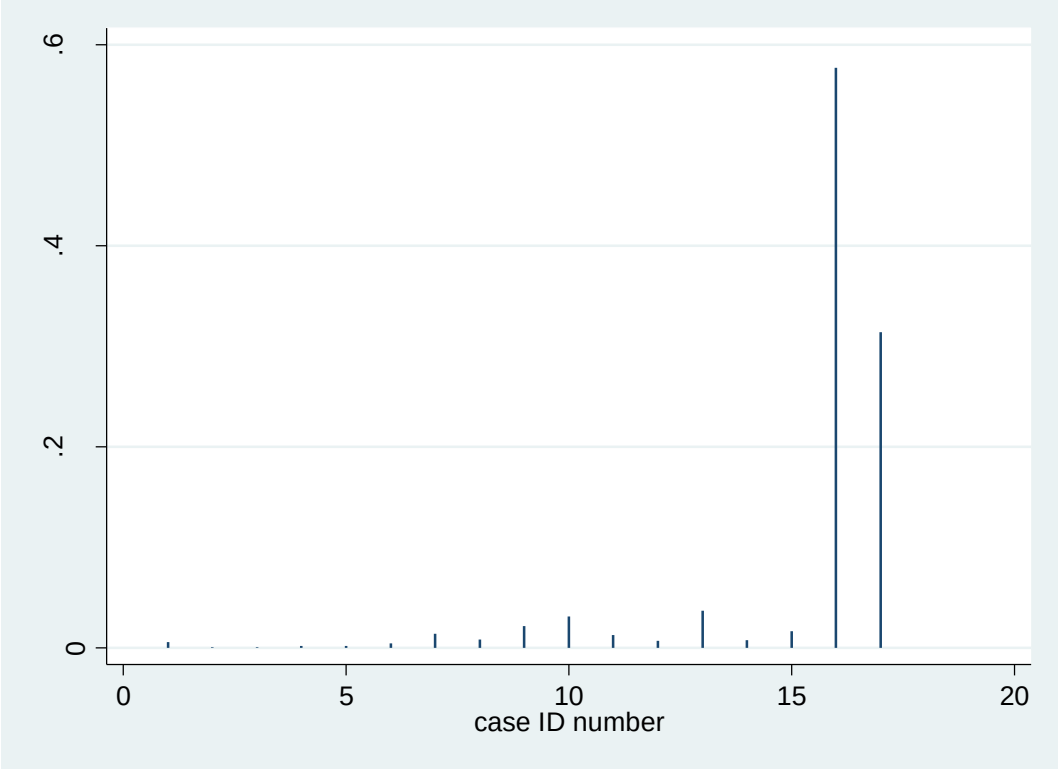
CHAPTER 4

RESULTS

Sample Characteristics

α-/γ-Tocopherol and α-/γ-CEHC concentrations were measured in normal pregnancy, in both maternal and cord blood pairs. A total of 17 pairs were collected during the course of the sub-study. Two of the last obtained cord samples had α-tocopherol values unreasonably high for a newborn, 29.8 and 24.4 μmol/L, considering that previously determined average of term cord blood α-tocopherol concentration was at 6 ± 2 μmol/L (72). Statistical analysis showed that when examined together, those values had a significant influence on the sample mean (Cook's distance plot: Figure 5) and could potentially be outliers. Thus, 15 pairs were used for statistical analysis of α/γ tocopherol and α/γ CEHC concentrations. Furthermore, one cord sample had inadequate amount of plasma for total lipid analysis, therefore 14 pairs were used for α/γ tocopherol:total lipid ratio analysis .

Figure 5. Cook's distance of each subject's α-tocopherol value.



The demographic information of participating mothers is described in Table 3. The average age was 30 ± 3 yr, ranging from 24 to 36 years. Thirteen participants were Caucasian, one was Hispanic and one was of Asian ethnicity. Forty percent worked in a health related profession and were affiliated with the OHSU hospital. The majority had no previous children (67%), and 12 out of 15 were married. The average pre-pregnancy body mass index was 25 ± 5 kg/m², and based on the World Health Organization's adult weight classifications (73), 10 of the 15 participants were normal weight (BMI range 18.50 – 24.99 kg/m²), three were overweight (BMI range 25.00 – 29.99 kg/m²), and two were obese (BMI range ≥ 30.00 kg/m²).

Delivery outcome information is presented in Table 4. All deliveries were uncomplicated, full-term (born at 37 weeks or more), and took place at the OHSU hospital. The average time between admission to the hospital and actual delivery was 12.7 ± 8.8 hours, ranging from 0.72 hours to 28.62 hours. Thirteen of the fifteen participants delivered vaginally. The average birth weight was 3494 ± 503 g (7.7 ± 1.1 lb), one newborn weighed less than 2500 g, and two weighted over 4000 g.

Table 3.

Demographic indices of participating mothers.

Characteristic	**Total mothers (n = 15)**
Age (years):	24 – 36
Ethnicity (n):	
Caucasian	13
Hispanic/Latino	1
Asian	1
Health profession (%):	40
Marital status (n):	
Single	3
Married	12
Previous children (n):	
0	10
1	5
Pre-pregnancy BMI (mean ± SD):	25 ± 5 kg/m²
Pre-pregnancy BMI* (n):	
Underweight	0
Normal	10
Overweight	3
Obese	2

*BMI: Body Mass Index classification from World Health Organization 1995.

Table 4.

Delivery outcomes.

Delivery outcome	**Total newborns (n = 15)**
Type of delivery (n):	
Cesarean	2
Vaginal	13
GA at delivery (wk):	37 – 41
Birth weight (g)	3494 ±503*
Birth weight (n):	
1500 – 2499 g	1
2500 – 3999 g	12
4000 – 4500 g	2
Time between admission and delivery (hr):	12.7 ± 8.8*

* mean ±SD

Plasma Vitamin E and Metabolite Concentrations

A summary of tocopherol and CEHC concentrations in maternal and cord blood pairs is presented in Table 5.

Both α- and γ- tocopherol concentrations were significantly lower in cord blood compared to maternal blood (α-tocopherol 6.8 ± 1.7 μmol/L vs. 34.2 ± 6.6 μmol/L, $p < 0.001$; and γ- tocopherol 0.4 ± 0.2 μmol/L vs. 1.9 ± 0.8 μmol/L, $p < 0.001$, respectively). Tocopherol/total lipids ratio, determined by adding plasma triglycerides and total cholesterol concentrations, remained significantly lower in cord blood (α-tocopherol:total lipids $p < 0.002$, and γ-tocopherol:total lipids $p < 0.05$).

The average α-CEHC concentration in maternal blood was 56.0 ± 53.0 nmol/L and 33.4 ± 29.4 nmol/L in cord blood; the average γ-CEHC concentration was 157.4 ± 69.3 nmol/L in maternal blood and 115.8 ± 55.8 nmol/L in cord blood. Absolute α- and γ-CEHC concentrations were not significantly different between maternal and cord blood values ($p = 0.07$ and $p = 0.08$, respectively), but there was a trend for lower concentrations in cord blood. On the other hand, the metabolite to tocopherol ratio was significantly different, both α-CEHC/α-tocopherol and γ-CEHC/γ-tocopherol was higher in the cord blood.

Table 5.

Maternal and cord plasma total lipids (n = 14), tocopherol (n = 15), tocopherol/total lipids ratio (n = 14), and CEHC concentrations (n = 15), with paired t-test statistics.

	Maternal (mean ± SD)	Cord (mean ± SD)	p[1]
total lipids[2] (mmol/L)	8 ± 1	2 ± 0.5	< 0.001*
α-tocopherol (μmol/L)	34.2 ± 6.6	6.8 ± 1.7	< 0.001*
α-tocopherol : total lipids (μmol/mmol)	4.4 ± 0.5	3.1 ± 0.7	< 0.001*
γ-tocopherol (μmol)	1.9 ± 0.8	0.4 ± 0.2	< 0.001*
γ-tocopherol : total lipids (μmol/mmol)	0.24 ± 0.09	0.19 ± 0.08	< 0.05*
α-CEHC (nmol/L)	56.0 ± 53.0	33.4 ± 29.4	0.07
γ-CEHC (nmol/L)	157.4 ± 69.3	115.8 ± 55.8	0.08
α-CEHC/α-tocopherol	1.7 ± 1.9	5.1 ± 4.5	< 0.01*
γ-CEHC/γ-tocopherol	96.1 ± 62.9	371.0 ± 248.4	< 0.001*

[1] Analysis performed using one-sided paired t-test.
[2] Total lipids determined by adding triglyceride and cholesterol.
* Significant at α = 0.05.

Dietary Parameters

The dietary intake analysis was based on 14 maternal diet histories; one participant did not have a diet recall for analysis. The averages of dietary parameters are presented in Table 6. The mean energy intake was 1977 ± 494 kcals, and total fat and cholesterol intake was 74 ± 25 g and 283 ± 261 mg. The total dietary vitamin E intake from food was 14 ± 10 IU, α- tocopherol was 9.1 ± 5.2 mg and γ-tocopherol was 13.6 ± 8.4 mg.

Follow-up phone call questionnaire results are presented in Table 7. Only 12 out of 15 subjects responded and completed the questionnaire. Because mothers took different types of prenatal vitamin during the course of pregnancy, the supplemental amount of vitamin E greatly varied, from 15 to 100 IU. Most mothers took their prenatal vitamin daily (82%) and at night time (55%). The time between last meal and delivery ranged from 7 to 34 hours. The total vitamin E was calculated as the vitamin E intake from food (measured by NDSR) combined with vitamin E from prenatal vitamins, and was based on 11 subjects, ranging from 19 to 144 IU with a mean of 48 ± 37 IU.

Table 6.

Diet history summary.

	Minimum	Maximum	Mean ± SD
Energy (kcal)	1573	2761	1977 ± 404
Fat (g)	47	130	74 ± 25
Total cholesterol (mg)	121	1023	283 ± 261
Vitamin E[1] (IU)	4	44	14 ± 10
α-Tocopherol (mg)	2.7	22.7	9.1 ± 5.2
γ-tocopherol (mg)	3.1	34.9	13.6 ± 8.4
Prenatal vitamin E (IU)	15	100	32 ± 26
Total vitamin E[2] (IU)	19	144	48 ± 37

[1] Vitamin E from food.
[2] Vitamin E from food + prenatal vitamins.

Table 7.

Follow-up questionnaire summary.

	Total mothers (n = 12)
Prenatal vitamin E content (IU*)	
Mean ± SD	15 – 100
(Range)	(32 ± 25)
Time of day (n)	
Morning	3
Night	6
Afternoon	1
Twice a day	1
Times taken per week (n)	
Daily	9
5x	1
4x	1
3x	1
Time between last meal and delivery (hr)	
Mean ± SD	17 ± 9
(Range)	(7 – 34)

*IU: international units

Influence of Demographics and Delivery Characteristics

No significant associations were found between demographic indices and maternal tocopherol and CEHC concentrations (data not presented).

A negative correlation was found between cord α-tocopherol concentration and maternal pre-pregnancy BMI ($r = -.54$; $p < 0.05$). Maternal obesity was associated with signigicantly lower cord α-tocopherol concentrations (4.6 ± 0.9 μmol/L) compared to nomal weight mothers (7.3 ± 1.7 μmol/L). Cord α-tocopherol concentration from mothers with no previous children was significantly higher than from mothers with one previous child (7.4 ± 1.6 μmol/L vs. 5.6 ± 1.0 μmol/L, respectively; $p < 0.05$). Once standardized for total lipids, these associations became insignificant. The time between admission to the hospital and the actual delivery was negatively correlated only with cord blood α-tocopherol concentrations ($r = -0.54$, $p < 0.05$), but the time between last meal and delivery was not associated with any of the blood parameters ($r = -0.44$, $p = 0.15$). No significant associations were found between the newborn's birth weight or the type of delivery and cord plasma variables. Neither γ-tocopherol nor CEHC concentrations in the cord blood were found to be significantly associated with any of the demographic or delivery indices.

Relationship between Maternal and Cord Biochemical Parameters

Maternal

Maternal plasma γ-tocopherol and γ-tocopherol/total lipids ratio was negatively correlated with α-CEHC ($r = -0.57$, $p < 0.05$; and $r = -0.54$, $p < 0.05$). There was a substantial correlation between maternal α-CEHC and γ-CEHC concentrations ($r = 0.80$, $p < 0.001$), with γ-CEHC concentration significantly higher than the α-CEHC (157.4 ± 69.3 nmol/L vs. 56.0 ± 53.0 nmol/L, respectively; $p < 0.0001$). No other correlations were found between maternal biochemical parameters.

Cord

Both γ-tocopherol and γ-tocopherol/total lipids ratio were negatively correlated with α-CEHC ($r = -0.53$, $p < 0.05$ and $r = -0.55$, $p < 0.05$). No other correlations were found between cord biochemical parameters.

Maternal vs. Cord

Correlations between maternal and cord plasma concentrations are presented in Table 8. No significant correlation was found between maternal and cord plasma α-tocopherol concentrations ($r = 0.26$, $p = 0.34$), but after adjustment for total lipids, the association became significant ($r = 0.56$, $p < 0.05$) (Figure 6). Neither cord γ-tocopherol concentrations nor γ-tocopherol/total lipids ratio was significantly correlated with maternal concentrations ($r = 0.51$, $p = 0.051$ and $r = 0.42$, $p = 0.32$, respectively), but cord γ-tocopherol was significantly correlated with maternal α-CEHC concentration ($r = -0.52$, $p < 0.05$). Cord α-CEHC was correlated with maternal α-tocopherol/total lipids ratio (r =

0.78, p <0.001) (Figure 7), maternal α-CHEC concentration (r = 0.53, p <0.05), and maternal γ-CEHC concentration (r = 0.52, p <0.05). No other associations between maternal and cord plasma reached significance.

Table 8.

Pearson's correlations between maternal and cord tocopherol and CEHC concentrations.

	Cord					
Maternal	**α-tocopherol**	**α-tocopherol: total lipids**	**γ-tocopherol**	**γ-tocopherol: total lipids**	**α-CEHC**	**γ-CEHC**
α-tocopherol	No correlation	r = 0.54 (p < 0.05)	No correlation	No correlation	No correlation	No correlation
α-tocopherol: total lipids	No correlation	r = 0.56 (p < 0.05)	No correlation	No correlation	r = 0.78 (p < 0.001)	No correlation
γ-tocopherol	No correlation	No correlation	No correlation	No correlation	No correlation	No correlation
γ-tocopherol: total lipids	No correlation	No correlation	No correlation	No correlation	No correlation	No correlation
α-CEHC	No correlation	No correlation	r = -0.52 (p < 0.05)	No correlation	r = 0.53 (p < 0.05)	No correlation
γ-CEHC	No correlation	No correlation	No correlation	No correlation	r = 0.52 (p < 0.05)	No correlation

Figure 6. Corrrelation between maternal and cord α-tocopherol/total lipids ratio ($r = 0.56$, $p < 0.05$).

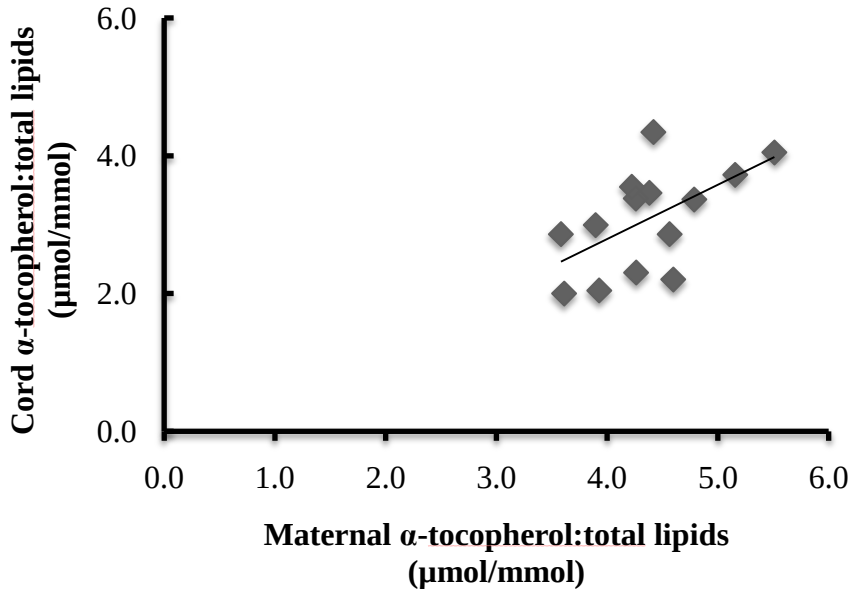
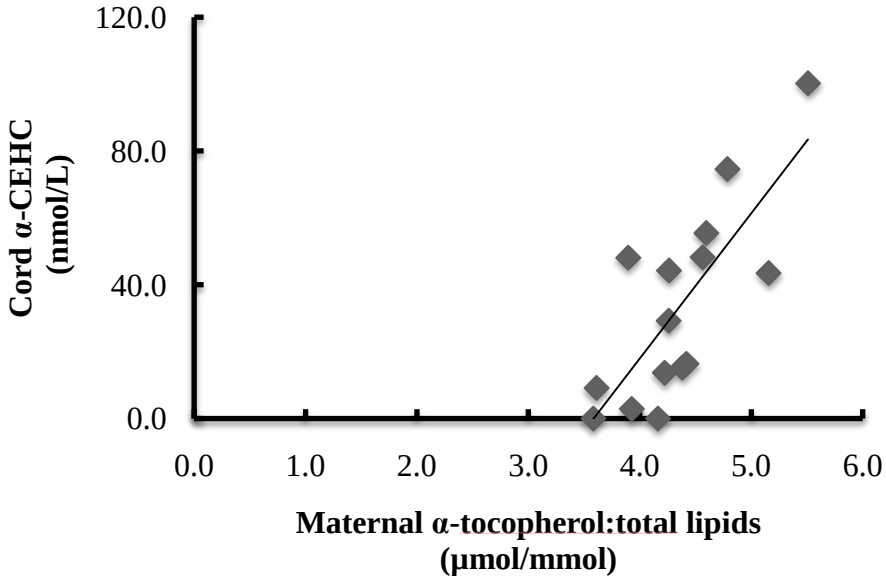


Figure 7. Correlation between maternal α-tocopherol/total lipids ratio and cord α-CEHC ($r = 0.78$, $p < 0.001$).



Relationship between Dietary and Biochemical Parameters

Maternal and cord associations between dietary and plasma parameters are presented in Tables 9 and 10.

Maternal

No significant correlations were found between either calories or cholesterol and any of the maternal plasma variables. Only the maternal plasma γ-tocopherol/total lipids ratio was significantly correlated with dietary fat ($r = -0.54$, $p < 0.05$). Both plasma α-tocopherol and α-tocopherol/total lipids ratio were significantly correlated with dietary vitamin E from food ($r = 0.71$, $p < 0.05$; and $r = 0.62$, $p < 0.05$), dietary α-tocopherol ($r = 0.64$, $p < 0.05$; and $r = 0.59$, $p < 0.05$), and vitamin E from food plus prenatal supplements ($r = 0.72$, $p < 0.05$; and $r = 0.65$, $p < 0.05$) (Figure 8). Dietary γ-tocopherol was correlated with maternal γ-CEHC concentrations ($r = 0.83$, $p < 0.001$), but maternal α-CEHC concentrations were not correlated with any of the dietary parameters.

Cord

No correlations were found between dietary calories, fat or cholesterol and cord plasma biochemical parameters. α-CEHC was significantly correlated with dietary vitamin E from food ($r = 0.65$, $p < 0.05$), dietary α-tocopherol ($r = 0.61$, $p < 0.05$), and total vitamin E from food plus prenatal supplements ($r = 0.66$, $p < 0.05$) (Talbe 9). γ-CEHC was only correlated with total vitamin E from food plus prenatal supplements ($r = 0.63$, $p < 0.05$). Neither cord plasma α-tocopherol nor γ-tocopherol concentrations were correlated with any of the dietary parameters. No associations were found among

prenatal intake per week, time of day the prenatal was taken, or the time between last meal and delivery and biochemical parameters.

The plasma metabolite to tocopherol ratio was also analyzed for possible associations with dietray parameters. Neither the maternal nor the fetal ratios were significantly correlated with dietary vitamin E intake, but there seemed to be a trend for a positive correlation between both cord α-CEHC/α-tocopherol and cord λ-CEHC/λ-tocopherol ratios and total dietary vitamin E intake ($p = 0.09$ and $p = 0.05$, respectively) (Table 11).

Table 9.

Maternal Pearson's correlations between dietary and biochemical parameters.

	α-tocopherol	**α-tocopherol: total lipids**	**γ-tocopherol**	**γ-tocopherol: total lipids**	**α-CEHC**	**γ-CEHC**
Energy	No correlation	No correlation	No correlation	No correlation	No correlation	No correlation
Fat	No correlation	No correlation	No correlation	$r = -0.54$ ($p < 0.05$)	No correlation	No correlation
Cholesterol	No correlation	No correlation	No correlation	No correlation	No correlation	No correlation
Vitamin E[1]	$r = 0.71$ ($p < 0.01$)	$r = 0.62$ ($p < 0.05$)	No correlation	No correlation	No correlation	No correlation
α-tocopherol	$r = 0.64$ ($p < 0.05$)	$r = 0.59$ ($p < 0.05$)	No correlation	No correlation	No correlation	No correlation
γ-tocopherol	No correlation	No correlation	No correlation	No correlation	No correlation	$r = 0.83$ ($p < 0.001$)
Prenatal vitamin E	No correlation	No correlation	No correlation	No correlation	No correlation	No correlation
Total vitamin E[2]	$r = 0.72$ ($p < 0.05$)	$r = 0.65$ ($p < 0.05$)	No correlation	No correlation	No correlation	No correlation

[1] Vitamin E from food.
[2] Vitamin E from food and prenatal vitamins.

Table 10.

Cord Pearson's correlations between dietary and biochemical parameters.

	α-tocopherol	**α-tocopherol: total lipids**	**γ-tocopherol**	**γ-tocopherol: total lipids**	**α-CEHC**	**γ-CEHC**
Vitamin E[1]	No correlation	No correlation	No correlation	No correlation	r = 0.65 ($p < 0.05$)	No correlation
α-tocopherol	No correlation	No correlation	No correlation	No correlation	r = 0.61 ($p < 0.05$)	No correlation
γ-tocopherol	No correlation	No correlation	No correlation	No correlation	No correlation	No correlation
Prenatal vitamin E	No correlation	No correlation	No correlation	No correlation	r = 0.66 ($p < 0.05$)	No correlation
Total vitamin E[2]	No correlation	No correlation	No correlation	No correlation	r = 0.72 ($p < 0.05$)	r = 0.63 ($p < 0.05$)

[1] Vitamin E from food.
[2] Vitamin E from food and prenatal vitamins.

Table 11.

Pearson's correlations between CEHC/tocopherol ratios and total dietary vitamin E intake (n = 11).

Cord plasma	**Dietary total vitamin E[1]**	***p*[2]**
α-CEHC/α-tocopherol	r = 0.54	0.09
λ-CEHC/λ-tocopherol	r = 0.60	0.05

[1] Vitamin E from food and prenatal vitamins.
[2] $p < 0.05$ concidered significant.

Figure 8. Correlation between maternal total vitamin E intake and maternal α-tocopherol/total lipids ratio (r = 0.65, p < 0.05).

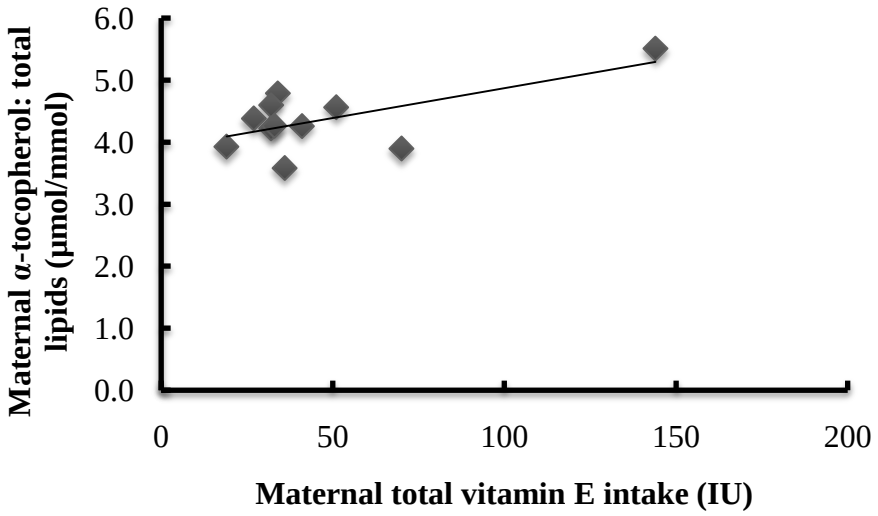
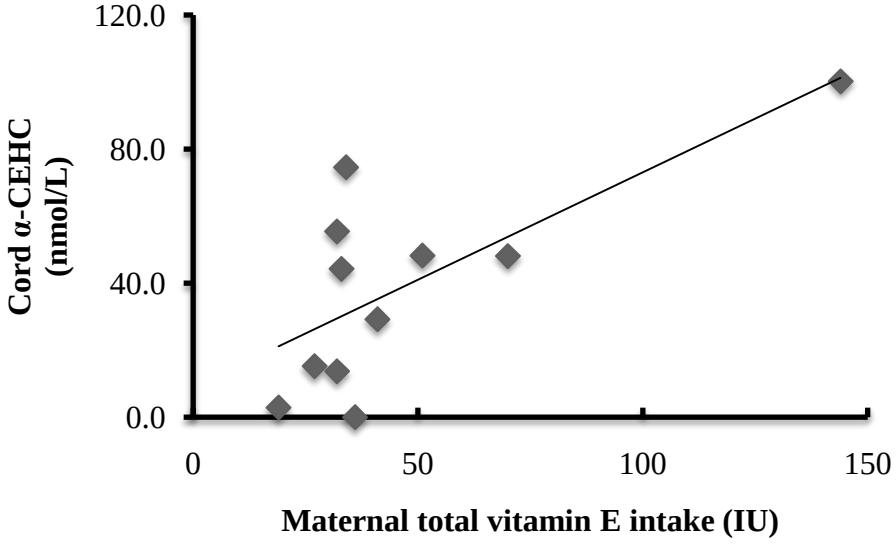


Figure 9. Correlation between maternal total vitamin E intake and cord α-CEHC (r = 0.72, p < 0.05).



CHAPTER 5

DISCUSSION

Summary

This observational, prospective cohort sub-study was conducted in a normal, healthy pregnancy sample to compare vitamin E to its metabolite concentration in maternal cord blood pairs. Fifteen pairs of maternal fasting plasma and cord plasma samples were collected and analyzed for α-tocopherol, γ-tocopherol, α-CEHC, and γ-CEHC concentrations. Diet histories were obtained from participating mothers for the day previous to blood draw. Analysis was performed to investigate possible associations between biochemical, dietary, and demographic parameters.

Vitamin E status has been extensively investigated in humans, including during pregnancy. Normal plasma α-tocopherol concentrations in humans are considered to be from 11.6 to 46.4 μmol/L, while γ-tocopherol concentrations are typically about one tenth those of α-tocopherol. Adult deficiency levels are reached at levels < 11.6 μmol/L, or < 1.6 μmol α-tocopherol/mmol total lipids. During pregnancy, maternal plasma α-tocopherol concentrations increase with gestation (65, 74), and this rise is typically associated with gestational secondary hyperlipidemia; but the adjustment of tocopherol for total lipids reduces the variability among trimesters. In the present study, fasting maternal samples were taken at different time points during pregnancy, and α-tocopherol concentration was found to significantly increase with gestational age, but after standardizing for total lipids, the variability in concentrations was not associated with gestational age, consistent with previous studies.

Although γ-tocopherol is the most abundant form of vitamin E in the US diet, it has not been thoroughly investigated due to its considerably lower bioavailability and bioactivity than that of α-tocopherol. Its plasma concentration has a higher disappearance rate and an increased metabolite production rate compared to that of α-tocopherol (18), but recent findings have suggested that the γ isoform might have a number of distinctive biochemical functions in the human body, such as inhibition of cyclooxygenase 2 (COX2), which reduces inflammation, and greater quenching ability of reactive nitrogen species compared to other isoforms of tocopherol (75). The γ-tocopherol concentration in human plasma is around 1.9 μmol/L (43). It has been shown to increase from first to third trimester during pregnancy (74), but after adjustments for total lipids in the blood, no difference is observed (65). In the present study, the average plasma γ-tocopherol concentration was about 20 times lower than that of α-tocopherol and was not associated with the gestational age of when the maternal sample was drawn. It was interesting to note that the highest γ-tocopherol concentration in the present study was for the one Asian participant. The γ-tocopherol concentration for this sample was twice as high as the overall mean, though the dietary γ-tocopherol level was not different. Scholl et al., had found a significantly lower concentrations of both α- and γ-tocopherol for the African American ethnic group compared to White, Hispanic, and Asian/other group ($p < 0.0001$), but no difference was observed for the Asian/other group. No inferences can be made based on a single person's results, but dietary patterns among ethnic groups might present differences in tocopherol concentrations. Furthermore, it was found that genetic factors possibly regulate plasma concentrations of vitamin E (76).

Placental transfer of vitamin E appears to be inefficient (56), and is only slightly or not at all influenced by maternal dietary vitamin E variation and supplement intake (9, 77). Previously determined averages of α-tocopherol in cord blood vary between 3.63 and 7.21 μmol/l, and for γ-tocopherol between 0.19 to 0.50 μmol/L (10, 65, 74, 78). Cord values are considerably lower than maternal concentrations, and remain significantly lower after adjusting for total lipids in the blood. The present study observed a similar phenomenon; both α- and γ-tocopherol concentrations were approximately five times lower than those in maternal plasma, and remained significantly lower for the tocopherol/total lipids ratio. Possibly due to the relatively hypoxic environment in utero, the fetus does not require substantial amounts of vitamin E, which could explain the lower concentrations observed in the cord plasma. On the other hand it has been shown that unlike vitamin E, the concentrations of vitamin C are significantly higher in cord blood compared to maternal concentrations (92.11 μmol/L vs. 36.90 μmol/L; $p < 0.001$) (77), possibly to protect newborn's cells against oxidative insult and to promote collagen synthesis. No present studies have looked at the relationship between the two vitamins in fetal circulation of humans. It is possible that due to vitamin C's recycling properties of vitamin E, the increased concentration in one compensates for the lower levels in the other. Bruno et al. has demonstrated that faster plasma vitamin E disappearance in smokers is normalized by vitamin C supplementation (79). Therefore, it would be interesting to investigate whether the higher plasma vitamin C concentrations are related to the cord plasma vitamin E status.

No significant correlations were found in the present study between maternal and cord plasma in total α- and γ-tocopherol concentrations. However, after adjustment for

total lipids, the cord α-tocopherol/total lipids ratio correlated with maternal α-tocopherol/total lipids ratio. While most studies find no significant correlation in α-tocopherol concentrations (10, 63, 74, 78, 80), there is some disparity in the relationship of α-tocopherol after adjustment for lipids. The γ-tocopherol concentrations in maternal and cord plasma have been analyzed by only a few studies, but have demonstrated a positive correlation. Kiely et al. measured vitamin E concentrations in non-fasting maternal samples between 10-20 weeks gestation and umbilical cord blood pairs ($n = 40$) and found a significant correlation in γ-tocopherol ($r = 0.45$, $p < 0.005$), but α-tocopherol concentrations were not correlated (10). The authors did not discuss the measured tocopherol/total lipids ratio associations. Yeum et al. assessed relationship of tocopherols in healthy pregnant women at delivery and in cord blood pairs ($n = 10$) (78). The authors found a positive correlation in γ-tocopherol concentrations between maternal and cord plasma ($r = 0.808$, $p = 0.0047$), but none for α-tocopherol concentrations or α-tocopherol adjusted for triglyceride concentrations. Oostenburg et al. found no correlation either between α-tocopherol concentrations or between γ concentrations (74), though their measurement of γ-tocopherol was in combination with β-tocopherol, which could have affected the relationship. Jain et al. found no correlation between α-tocopherol in maternal circulation and paired cord plasma ($p = 0.64$), but after normalization for total lipids in the blood, the relationship became significant ($r = 0.54$, $p = 0.007$) (63). Sanchez-Vera et al. obtained maternal blood at the time of delivery and paired cord blood samples ($n = 50$) and found a significant correlation between α-tocopherol/cholesterol concentrations ($r = 0.506$, $p < 0.001$) (80). This present study did not reach a significant correlation in γ-tocopherol concentrations between maternal and cord samples ($r = 0.49$, p

> 0.05), probably due to the small sample size of the study. Furthermore, it is well documented that plasma and tissue γ-tocopherol are suppressed by α-tocopherol supplementation, and since the participants took prenatal vitamins that contained varied amounts of either RRR- or all-rac-α-tocopherol forms, this might explain the lack of an association between maternal and cord γ-tocopherol concentrations. The α-tocopherol intake levels could have suppressed γ-tocopherol concentrations to varying degrees. The studies mentioned above did not discuss participants' prenatal vitamin use.

CEHC is a fairly new metabolite; it was first identified in human urine and recently quantified in human serum. Measurement of CEHC has been recognized as the reference strategy to assess vitamin E metabolism and has been applied in both animal and human studies. One of the first studies to quantify vitamin E metabolites in human serum used high-performance liquid chromatography (HPLC) to determine baseline levels in four healthy unsupplemented subjects. The averages observed were 7.1 ± 3.0 nmol/L for α-CEHC and 66.4 ± 15.5 nmol/L for γ-CEHC (66). A later studied by Galli et al. assessed vitamin E metabolite concentrations in eight healthy human subjects using gas chromatography-mass spectrometry and obtained slightly higher values, 12.56 ± 7.54 nmol/L for α-CEHC and 160.74 ± 44.88 nmol/L for γ-CEHC (68). The authors concluded that the difference observed between the two studies was likely due to the lack of an internal standard in the HPLC analyses; however, the ratio between α- and γ-CEHC concentrations of 1 to 10 was comparable between the two studies. In the present study, the mean maternal α-CEHC concentration was 56.0 ± 53.0 nmol/L and γ-CEHC was 157.4 ± 69.3 nmol/L. The mean ratio between the two metabolites, 1 to 3, was much lower than in the studies mentioned above. The higher concentration of α-CEHC

obtained in the current study was likely due to the prenatal supplement use by the subjects. Though the correlation between total vitamin E intake and α-CEHC concentration did not reach significance, studies have shown a direct relationship between metabolite concentration and its parent compound. Radosavac et al. (69) looked at the actual change in metabolite concentrations after a single dose supplement of vitamin E from a natural source (306 mg of RRR-α-tocopherol and 1.77 mg of γ-tocopherol), and arrived at several conclusions. The level of α-CEHC in serum followed the time course of α-tocopherol, increasing from 15.1 ± 7.0 nmol/L at baseline to a maximum of 42.4 ± 18.3 nmol/L at 12 hours after ingestion. The authors concluded that the α-CEHC levels increase after administration of a single dose of vitamin E in humans, and the appearance of the metabolite in blood parallels that of the α-tocopherol. On the other hand the γ-tocopherol concentrations decreased, while the γ-CEHC concentrations increased and reached a maximum of 151.3 ± 64.4 nmol/L at 24 hours. The authors also concluded that γ-tocopherol appears to be metabolized more efficiently than α-tocopherol. In a different study, after a single dose supplementation with 500 IU RRR-α-tocopherol/day for 4-7 weeks, the subject's serum α-CEHC concentrations increased up to 198.8 nmol/L (66). In the present study, the three participants whose α-CEHC concentration was above 100 nmol/L and γ-CEHC concentration was above 200 nmol/L, was reflected by a high dietary vitamin E intake of above 50 IU. Another possible explanation for the variations in metabolite concentrations might be the difference in gestational age at which the maternal sample was drawn. No previous studies have investigated the change in metabolite concentration throughout pregnancy, and it is possible the concentrations vary with gestation. The present study did not find an

association between gestational age when the sample was taken and the maternal metabolite concentrations, but this could be due to the small sample size of the study.

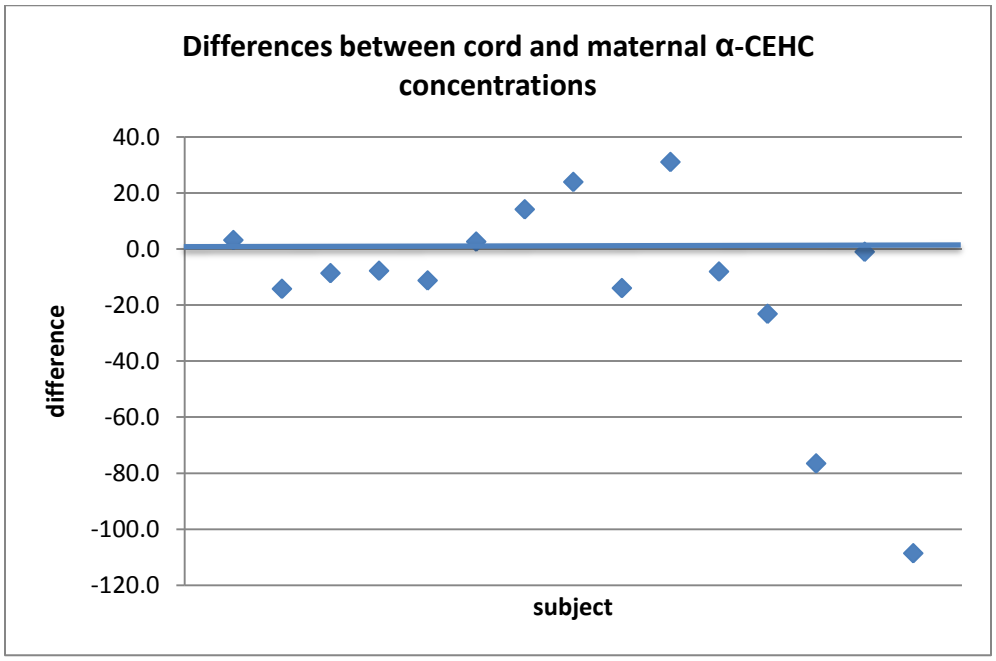
A negative association was found between maternal concentrations of α-CEHC and γ-tocopherol, and with γ-tocopherol/total lipids ratio, but a highly positive association was found with γ-CEHC. This most likely relates to the prenatal vitamin intake of the subjects. It has been shown that with increased α-tocopherol intake, γ-tocopherol concentration is decreased while the metabolite concentration of γ-tocopherol is increased. Furthermore, studies have demonstrated that high intakes of α-tocopherol can induce expression of cytochrome p450 enzymes that are involved in vitamin E metabolism, and thus high doses could stimulate the nutrient's own metabolism. Since both of the isoforms are metabolized by the same enzyme system, an increase in the enzyme could cause a similar increase in both metabolites. We in fact observed a positive correlation between α- and γ-CEHC in this study.

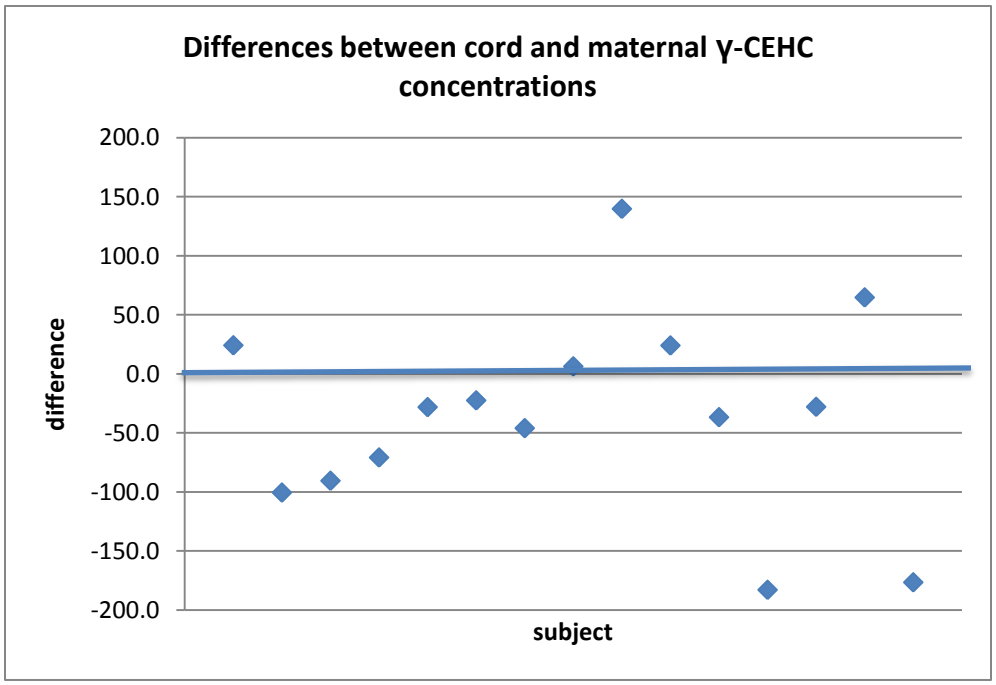
The primary aim of the study was to determine α- and γ-CEHC concentrations in cord blood and how it compares to maternal concentrations. No previous studies have looked at CEHC metabolite concentrations in cord blood. The present study determined cord α-CEHC concentration at 33 ± 29.4 nmol/L, which is slightly higher than the previously determined healthy adult average in the study by Galli et al. (68), and cord γ-CEHC concentration at 115.8 ± 55.8 nmol/L, which is slightly lower than the previously determined adult average. The data analysis showed that as within the maternal circulation, cord α-CEHC concentration was significantly lower than γ-CEHC concentration, but the correlation between the two metabolites did not reach significance. The lack of an association could be due to the small sample size of the

study. Also, as with the maternal values, cord α-CEHC concentration was negatively correlated with cord γ-tocopherol and γ-tocopherol/total lipids ratio. It is possible that the cord circulation reflects the tocopherols and metabolite concentrations found in maternal blood.

Another aim of the study was to indirectly estimate whether the placenta metabolizes vitamin E. During pregnancy, the body becomes a complex unit of mother, placenta, and the fetus. Umbilical cord contains metabolites that are transferred from maternal circulation across the placenta to the baby, and represents the fetal circulation. In the present study, tocopherol and its metabolite concentrations were measured in maternal and cord blood pairs, and the hypothesis was that a higher metabolite concentration in fetal blood could possibly indicate that the placenta metabolized the tocopherol from the maternal circulation, and thus explain the lower tocopherol concentrations that have been observed in cord blood. The results obtained show no significant difference between maternal and cord α-CEHC. In fact, there was a strong correlation between the two concentrations. Figure 10 demonstrates the difference between maternal and cord plasma metabolite concentrations. Though the difference was not statistically significant, there seems to be a trend for the lower α- and γ-CEHC concentrations in cord blood. It is plausible that the water-soluble α-CEHC passes the placental barrier to the fetus, equilibrating with the maternal circulation. At phase II metabolism of xenobiotics in the liver, a bulky endogenous compound is conjugated to the site of the oxidation, such as glucuronate and sulfate, before hepatic excretion. This makes the metabolite highly polar and water soluble, thus requiring special proteins to pass the cell membrane. Multidrug resistance-associated proteins (MRPs) are thought to

Figure 10. Differences in CEHC concentrations between maternal and cord plasma.





be one group of enzymes that regulate the efflux of conjugated metabolites from the liver, and have also been located in the placental syncytiotrophoblast membrane. Whether the metabolite passes straight from the maternal circulation or the placenta contributes to vitamin E metabolism, α-CEHC could be transported into the fetal circulation using these transporters, though the exact reason for that is not clear. It is possible that the metabolites possess some biological role and might be necessary for fetal development. On the other hand, it is also possible that the metabolite produced by the placenta is transported back into the maternal circulation for excretion, which could explain the observed higher α-CEHC concentration in pregnant women compared to the previously determined non pregnant normal adult values.

Cord γ-CEHC concentrations, were not correlated between maternal and cord plasma, though still present at significant concentrations in cord blood. This could be due to α-TTP located in the placenta, which selectively transfers α-tocopherol, while discriminating against other isoforms, including γ-tocopherol. This probably causes an enhanced metabolism of the γ isoform, resulting in a higher γ-CEHC concentration on the maternal side. Interestingly enough, cord α-CEHC was significantly correlated with maternal intake of vitamin E from prenatal vitamins, but vitamin E intake did not have a significant effect on the cord plasma tocopherol concentrations. Therefore it seems that higher intake of supplemented vitamin E during pregnancy results in elevated metabolite levels in fetal blood, without increasing its vitamin E concentration, though further studies are warranted.

Maternal supplementation with vitamin E and vitamin C has been considerably explored due to its plausible role in prevention of preeclampsia. Several clinical trials

supplemented pregnant women with these antioxidants to investigate its effect on pregnancy outcomes, particularly preeclampsia and birth weight. A large randomized placebo-controlled trial (n = 2410) failed to show a protective effect of vitamin supplementation (400 IU of RRR-α-tocopherol and 1000 mg of vitamin C), but found a significant increase in the incidence of low birth weight babies (< 2500 gm; 19% in control group vs. 28% in supplemented group; RR = 1.15, 95% CI: 1.02-1.30) (81). Two smaller studies in high-risk women used vitamin C and E in the same doses, and found a slight, non-significant decrease in birth weight in the supplemented group (82, 83). On the other hand, in an Australian clinical trial by Rumbold et al. (n = 1877), using a similar dose in vitamins, found no effect on either birth weight or preeclampsia (4). Furthermore, a WHO multicenter trial evaluating effects of vitamin C and E supplementation in pregnant women of low nutritional status from developing countries, also found no significant difference in low birth weight rate between supplemented and control groups (33.2% vs. 36.4%, RR: 0.9; 95% CI: 0.8-1.1) (84). It could possibly be that the two vitamins in combination have some affect on the growth and function of the placenta. However, in a prospective study of pregnant women exposed to high doses of just the vitamin E (≥ 400 IU/day) during the first trimester of pregnancy, found that newborns of the supplemented mothers had a significantly lower birth weight compared to the matched control group (3173 ± 467 gm vs. 3417 ± 565 gm; p = 0.0015) (85). The study concluded that this outcome was due to behavioral differences; the mothers who took extra supplements were probably more health conscious and more likely to exercise and thus deliver lighter babies. On the other hand, in an epidemiologic study by Scholl et al., a positive association was found between plasma concentrations of α-tocopherol and

increased fetal growth (birth weight for gestation) in a vulnerable population of low socioeconomic status (86). The researchers discussed vitamin E's role in enhanced release of prostacyclin, leading to a vasodilatory effect, thus increasing blood flow between the placenta and the fetus and increasing the fetus's nutrient supply. Saker et al. (87) determined the antioxidant status in Algerian mothers and their newborns according to birth weight groups, small for gestational age (SGA: < 2500 gm or < 10^{th} percentile), appropriate for gestational age (AGA), and large for gestational age (LGA: > 4000 gm or > 90^{th} percentile), and found significantly lower plasma vitamin E concentrations for both mothers and newborns in the SGA group ($p < 0.01$). The maternal blood samples were taken 48 hours port partum, and the newborn levels were measured from cord blood. Interestingly enough, the maternal vitamin E concentrations of LGA group were not significantly different from the AGA group (32.68 ± 3.13 μmol/L vs. 29 ± 2.03 μmol/L), but their newborn vitamin E concentrations were significantly lower (6.29 ± 1.08 μmol/L vs. 12.26 ± 1.04 μmol/L, $p <0.01$), closer to the concentrations of the SGA babies (4.68 ± 0.65 μmol/L). The study group concluded that oxidative stress is present in both SGA and LGA newborns, supported by low plasma total antioxidant activity and high plasma hydroperoxide and carbonyl protein levels, and supplementary therapy with antioxidants might help to shift this oxidant and antioxidant balance. To investigate possible detrimental effects of high vitamin C and E levels on the placental function, Aris et al. (88) exposed isolated placental cells in vitro to high concentrations of these antioxidants and measured the secretion of human chorionic gonadotropin (hCG) and the production of tumor necrosis factor-alpha (TNF-α). They found that exposure of human cytotrophoblasts to above physiological concentrations of vitamin C and E, separately or

combined, decreased secretion of hCG. This hormone is involved in many regulatory functions and is critical to the maintenance of gestation and successful pregnancy. Additionally, high levels of vitamin C and E increased cytotrophoblasts' production of TNF-α. This major proinflammatory cytokine is related to adverse pregnancy outcomes and placental immunity. The authors hypothesized that due to the nutrients' antioxidant capacities, a significant depletion of oxidative metabolites by high doses of vitamin C and E may cause an imbalance in the oxidative status, which is necessary for normal hCG secretion. These changes have been known to result in endothelial dysfunction and adverse pregnancy outcomes, such as fetal growth restriction. It seems that nature would have a mechanism in place to reduce the negative effects of high vitamin E concentrations on the function of the placenta and the growth of the baby. Though pregnancies of low nutritional status seem to benefit from increased vitamin E concentrations to establish normal levels for their fetus' environment, for those groups that are already consuming an adequate diet, additional vitamin E seems to negatively influence baby's growth through oxidative imbalance and other unknown mechanisms. It is probable that in order to protect the developing fetus from high doses of this antioxidant, the placenta assists in metabolizing above normal plasma vitamin E concentrations. None of this study's participants took prenatal vitamins with levels higher than the ones mentioned in above studies that resulted in lower birth weight. It is possible that we didn't see a significant difference in the metabolite concentrations between maternal and cord plasma due to insufficient vitamin E levels for the placenta to notably metabolize the vitamin.

Conclusions

In conclusion, α- and γ-tocopherol concentrations were significantly lower in cord blood compared to maternal blood, even after adjustment for total lipids. Of the tocopherols, only α-tocopherol/total lipids ratio was significantly correlated between maternal and cord concentrations. The absolute vitamin E metabolite concentrations, α- and γ-CEHC, were not significantly different between maternal and fetal circulations, but the metabolite to tocopherol ratios were signficantly higher in cord blood. The higher α-tocopherol/total lipids ratio in maternal blood was reflected by an increased α-tocopherol/total lipids ratio in fetal blood, and an even more prominent increase in the fetal metabolite concentration of α-CEHC. Furthermore, an increased vitamin E level of prenatal vitamins was associated with higher α-tocopherol concentration in the maternal circulation, but higher α-CEHC concentration in the fetal circulation. This might imply that the higher levels of supplemented vitamin E intake do not produce an increase in fetal vitamin E concentrations, but result in an increased metabolism of the vitamin. It is possible that the placenta contributes to the metabolism of the higher vitamin E concentrations, which is supported by the significant correlation between supplement intake and cord blood α-CEHC concentration. If the goal of supplemental vitamin E in pregnancy is to increase fetal store, it appears that maternal supplements do not increase the fetal tocopherol concentrations, but may increase their CEHCs concentrations. Since it is possible that high dose supplementation during pregnancy may have negative effects, further studies are needed to examine the relationship of supplemented levels of vitamin E and its affect on the fetus.

References

1. Brigelius-Flohe R. Vitamin E and drug metabolism. Biochem Biophys Res Commun 2003;305:737-40.

2. National Center for Health Statistics. final natality data. Version current 2009. Internet: www.marchofdimes.com/peristats (accessed 11/02 2009).

3. Brion LP, Bell EF, Raghuveer TS. Vitamin E supplementation for prevention of morbidity and mortality in preterm infants. Cochrane Database Syst Rev 2003;(4):CD003665.

4. Rumbold AR, Crowther CA, Haslam RR, Dekker GA, Robinson JS, ACTS Study Group. Vitamins C and E and the risks of preeclampsia and perinatal complications N Engl J Med 2006;354:1796-806.

5. Hakkola J, Pelkonen O, Pasanen M, Raunio H. Xenobiotic-metabolizing cytochrome P450 enzymes in the human feto-placental unit: role in intrauterine toxicity Crit Rev Toxicol 1998;28:35-72.

6. Yu SM, Keppel KG, Singh GK, Kessel W. Preconceptional and prenatal multivitamin-mineral supplement use in the 1988 National Maternal and Infant Health Survey Am J Public Health 1996;86:240-2.

7. Evans HM, Bishop KS. On the Existence of a Hitherto Unrecognized Dietary Factor Essential for Reproduction. Science 1922;56:650-1.

8. Debier C. Vitamin E during pre- and postnatal periods. Vitam Horm 2007;76:357-73.

9. Leger CL, Dumontier C, Fouret G, Boulot P, Descomps B. 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 1998;68:293-9.

10. Kiely M, Cogan PF, Kearney PJ, Morrissey PA. Concentrations of tocopherols and carotenoids in maternal and cord blood plasma. Eur J Clin Nutr 1999;53:711-5.

11. Mustacich DJ, Bruno RS, Traber MG. Vitamin E. Vitam Horm 2007;76:1-21.

12. Cohn W. Bioavailability of vitamin E. Eur J Clin Nutr 1997;51 Suppl 1:S80-5.

13. Bieri JG, Evarts RP. Gamma tocopherol: metabolism, biological activity and significance in human vitamin E nutrition. Am J Clin Nutr 1974;27:980-6.

14. Bieri JG, Evarts RP. Vitamin E activity of gamma-tocopherol in the rat, chick and hamster. J Nutr 1974;104:850-7.

15. Burton GW, Traber MG, Acuff RV, et al. Human plasma and tissue alpha-tocopherol concentrations in response to supplementation with deuterated natural and synthetic vitamin E. Am J Clin Nutr 1998;67:669-84.

16. Kiyose C, Muramatsu R, Kameyama Y, Ueda T, Igarashi O. Biodiscrimination of alpha-tocopherol stereoisomers in humans after oral administration. Am J Clin Nutr 1997;65:785-9.

17. Traber MG, Elsner A, Brigelius-Flohe R. Synthetic as compared with natural vitamin E is preferentially excreted as alpha-CEHC in human urine: studies using deuterated alpha-tocopheryl acetates. FEBS Lett 1998;437:145-8.

18. Leonard SW, Paterson E, Atkinson JK, Ramakrishnan R, Cross CE, Traber MG. Studies in humans using deuterium-labeled alpha- and gamma-tocopherols demonstrate faster plasma gamma-tocopherol disappearance and greater gamma-metabolite production. Free Radic Biol Med 2005;38:857-66.

19. Traber MG, Ramakrishnan R, Kayden HJ. Human plasma vitamin E kinetics demonstrate rapid recycling of plasma RRR-alpha-tocopherol. Proc Natl Acad Sci U S A 1994;91:10005-8.

20. Bjorneboe A, Bjorneboe GE, Drevon CA. Absorption, transport and distribution of vitamin E. J Nutr 1990;120:233-42.

21. Traber MG, Sokol RJ, Burton GW, et al. Impaired ability of patients with familial isolated vitamin E deficiency to incorporate alpha-tocopherol into lipoproteins secreted by the liver. J Clin Invest 1990;85:397-407.

22. Hosomi A, Arita M, Sato Y, et al. Affinity for alpha-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. FEBS Lett 1997;409:105-8.

23. Copp RP, Wisniewski T, Hentati F, Larnaout A, Ben Hamida M, Kayden HJ. Localization of alpha-tocopherol transfer protein in the brains of patients with ataxia with vitamin E deficiency and other oxidative stress related neurodegenerative disorders. Brain Res 1999;822:80-7.

24. Kaempf-Rotzoll DE, Horiguchi M, Hashiguchi K, et al. Human placental trophoblast cells express alpha-tocopherol transfer protein. Placenta 2003;24:439-44.

25. Traber MG, Burton GW, Hamilton RL. Vitamin E trafficking. Ann N Y Acad Sci 2004;1031:1-12.

26. Schultz M, Leist M, Petrzika M, Gassmann B, Brigelius-Flohe R. Novel urinary metabolite of alpha-tocopherol, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman, as an indicator of an adequate vitamin E supply? Am J Clin Nutr 1995;62:1527S-34S.

27. Chiku S, Hamamura K, Nakamura T. Novel urinary metabolite of d-delta-tocopherol in rats. J Lipid Res 1984;25:40-8.

28. Galli F, Cristina Polidori M, Stahl W, Mecocci P, Kelly FJ. Vitamin E biotransformation in humans. Vitam Horm 2007;76:263-80.

29. Syme MR, Paxton JW, Keelan JA. Drug transfer and metabolism by the human placenta. Clin Pharmacokinet 2004;43:487-514.

30. Birringer M, Drogan D, Brigelius-Flohe R. Tocopherols are metabolized in HepG2 cells by side chain omega-oxidation and consecutive beta-oxidation. Free Radic Biol Med 2001;31:226-32.

31. Ikeda S, Tohyama T, Yamashita K. Dietary sesame seed and its lignans inhibit 2,7,8-trimethyl- 2(2'-carboxyethyl)-6-hydroxychroman excretion into urine of rats fed gamma-tocopherol. J Nutr 2002;132:961-6.

32. Parker RS, Sontag TJ, Swanson JE. Cytochrome P4503A-dependent metabolism of tocopherols and inhibition by sesamin. Biochem Biophys Res Commun 2000;277:531-4.

33. Sontag TJ, Parker RS. Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status. J Biol Chem 2002;277:25290-6.

34. Mustacich DJ, Leonard SW, Devereaux MW, Sokol RJ, Traber MG. Alpha-tocopherol regulation of hepatic cytochrome P450s and ABC transporters in rats. Free Radic Biol Med 2006;41:1069-78.

35. Brigelius-Flohe R, Kelly FJ, Salonen JT, Neuzil J, Zingg JM, Azzi A. The European perspective on vitamin E: current knowledge and future research. Am J Clin Nutr 2002;76:703-16.

36. Niki E, Noguchi N, Tsuchihashi H, Gotoh N. Interaction among vitamin C, vitamin E, and beta-carotene. Am J Clin Nutr 1995;62:1322S-6S.

37. Wang X, Falcone T, Attaran M, Goldberg JM, Agarwal A, Sharma RK. Vitamin C and vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. Fertil Steril 2002;78:1272-7.

38. Kaempf-Rotzoll DE, Igarashi K, Aoki J, et al. Alpha-tocopherol transfer protein is specifically localized at the implantation site of pregnant mouse uterus. Biol Reprod 2002;67:599-604.

39. Jishage K, Tachibe T, Ito T, et al. Vitamin E is essential for mouse placentation but not for embryonic development itself. Biol Reprod 2005;73:983-7.

40. Jishage K, Arita M, Igarashi K, et al. Alpha-tocopherol transfer protein is important for the normal development of placental labyrinthine trophoblasts in mice. J Biol Chem 2001;276:1669-72.

41. Food and Nutrition Board, Institute of Medicine. Dietary reference intake for vitamin C, vitamin E, selenium, and carotenoids: a report of the Panel on Dietary Antioxidants and Related Compounds. Washington, D.C.: National Academy Press, c2000.

42. Blatt DH, Leonard SW, Traber MG. Vitamin E kinetics and the function of tocopherol regulatory proteins. Nutrition 2001;17:799-805.

43. Hensley K, Benaksas EJ, Bolli R, et al. New perspectives on vitamin E: gamma-tocopherol and carboxyelthylhydroxychroman metabolites in biology and medicine. Free Radic Biol Med 2004;36:1-15.

44. Huang HY, Appel LJ. Supplementation of diets with alpha-tocopherol reduces serum concentrations of gamma- and delta-tocopherol in humans. J Nutr 2003;133:3137-40.

45. Handelman GJ, Epstein WL, Peerson J, Spiegelman D, Machlin LJ, Dratz EA. Human adipose alpha-tocopherol and gamma-tocopherol kinetics during and after 1 y of alpha-tocopherol supplementation. Am J Clin Nutr 1994;59:1025-32.

46. Traber MG, Siddens LK, Leonard SW, et al. Alpha-tocopherol modulates Cyp3a expression, increases gamma-CEHC production, and limits tissue gamma-tocopherol accumulation in mice fed high gamma-tocopherol diets. Free Radic Biol Med 2005;38:773-85.

47. Cooney RV, Franke AA, Harwood PJ, Hatch-Pigott V, Custer LJ, Mordan LJ. Gamma-tocopherol detoxification of nitrogen dioxide: superiority to alpha-tocopherol. Proc Natl Acad Sci U S A 1993;90:1771-5.

48. Traber MG. Vitamin E regulatory mechanisms. Annu Rev Nutr 2007;27:347-62.

49. Robles R, Palomino N, Robles A. Oxidative stress in the neonate. Early Hum Dev 2001;65 Suppl:S75-81.

50. Kelly FJ, Rodgers W, Handel J, Smith S, Hall MA. Time course of vitamin E repletion in the premature infant. Br J Nutr 1990;63:631-8.

51. Saladin KS. Human Development. In: Anonymous Anatomy & Physiology: the unity of form and function. New York, NY: McGrw-Hill Companies, Inc., 2004:1089-1101.

52. Smith CH, Moe AJ, Ganapathy V. Nutrient transport pathways across the epithelium of the placenta. Annu Rev Nutr 1992;12:183-206.

53. Hakkola J, Pasanen M, Hukkanen J, et al. Expression of xenobiotic-metabolizing cytochrome P450 forms in human full-term placenta. Biochem Pharmacol 1996;51:403-11.

54. Pasanen M. The expression and regulation of drug metabolism in human placenta. Adv Drug Deliv Rev 1999;38:81-97.

55. Pavek P, Dvorak Z. Xenobiotic-induced transcriptional regulation of xenobiotic metabolizing enzymes of the cytochrome P450 superfamily in human extrahepatic tissues. Curr Drug Metab 2008;9:129-43.

56. Schenker S, Yang Y, Perez A, et al. Antioxidant transport by the human placenta. Clin Nutr 1998;17:159-67.

57. Godel JC. Vitamin E status of northern Canadian newborns: relation of vitamin E to blood lipids. Am J Clin Nutr 1989;50:375-80.

58. Rotzoll DE, Scherling R, Etzl R, Stepan H, Horn LC, Poschl JM. Immunohistochemical localization of alpha-tocopherol transfer protein and lipoperoxidation products in human first-trimester and term placenta. Eur J Obstet Gynecol Reprod Biol 2008;140:183-91.

59. Muller-Schmehl K, Beninde J, Finckh B, et al. Localization of alpha-tocopherol transfer protein in trophoblast, fetal capillaries' endothelium and amnion epithelium of human term placenta. Free Radic Res 2004;38:413-20.

60. Acuff RV, Dunworth RG, Webb LW, Lane JR. Transport of deuterium-labeled tocopherols during pregnancy. Am J Clin Nutr 1998;67:459-64.

61. Gonzalez-Corbella MJ, Lopez-Sabater MC, Castellote-Bargallo AI, Campoy-Folgoso C, Rivero-Urgell M. Influence of caesarean delivery and maternal factors on fat-soluble vitamins in blood from cord and neonates. Early Hum Dev 1998;53 Suppl:S121-34.

62. Baydas G, Karatas F, Gursu MF, et al. Antioxidant vitamin levels in term and preterm infants and their relation to maternal vitamin status. Arch Med Res 2002;33:276-80.

63. Jain SK, Wise R, Bocchini JJ,Jr. Vitamin E and vitamin E-quinone levels in red blood cells and plasma of newborn infants and their mothers. J Am Coll Nutr 1996;15:44-8.

64. Dison PJ, Lockitch G, Halstead AC, Pendray MR, Macnab A, Wittmann BK. Influence of maternal factors on cord and neonatal plasma micronutrient levels. Am J Perinatol 1993;10:30-5.

65. Herrera E, Ortega H, Alvino G, Giovannini N, Amusquivar E, Cetin I. Relationship between plasma fatty acid profile and antioxidant vitamins during normal pregnancy. Eur J Clin Nutr 2004;58:1231-8.

66. Stahl W, Graf P, Brigelius-Flohe R, Wechter W, Sies H. Quantification of the alpha- and gamma-tocopherol metabolites 2,5,7, 8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman and 2,7, 8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman in human serum. Anal Biochem 1999;275:254-9.

67. Wechter WJ, Kantoci D, Murray ED,Jr, D'Amico DC, Jung ME, Wang WH. A new endogenous natriuretic factor: LLU-alpha. Proc Natl Acad Sci U S A 1996;93:6002-7.

68. Galli F, Lee R, Dunster C, Kelly FJ. Gas chromatography mass spectrometry analysis of carboxyethyl-hydroxychroman metabolites of alpha- and gamma-tocopherol in human plasma. Free Radic Biol Med 2002;32:333-40.

69. Radosavac D, Graf P, Polidori MC, Sies H, Stahl W. Tocopherol metabolites 2, 5, 7, 8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman (alpha-CEHC) and 2, 7, 8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman (gamma-CEHC) in human serum after a single dose of natural vitamin E. Eur J Nutr 2002;41:119-24.

70. Leonard SW, Traber MG. Measurement of the Vitamin E Metabolites, Carboxyethyl Hydroxychromans (CEHCs), in Biological Samples. Current Protocols in Toxicology 2006;7.8.1-7.8.12:Copyright by John Wiley & Sons, Inc.

71. BAM Software Download Site--STPLAN software. Internet: http://biostatistics.mdanderson.org/SoftwareDownload/SingleSoftware.aspx?Software_Id=41 (accessed 6/26/2009 2009).

72. Galinier A, Periquet B, Lambert W, et al. Reference range for micronutrients and nutritional marker proteins in cord blood of neonates appropriated for gestational ages Early Hum Dev 2005;81:583-93.

73. Report of a WHO Expert Committee. Physical status: the use and interpretation of anthropometry. World Health Organ Tech Rep Ser 1995;854:1-452.

74. Oostenbrug GS, Mensink RP, Al MD, van Houwelingen AC, Hornstra G. Maternal and neonatal plasma antioxidant levels in normal pregnancy, and the relationship with fatty acid unsaturation Br J Nutr 1998;80:67-73.

75. Jiang Q, Christen S, Shigenaga MK, Ames BN. gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention Am J Clin Nutr 2001;74:714-22.

76. Borel P, Moussa M, Reboul E, et al. Human plasma levels of vitamin E and carotenoids are associated with genetic polymorphisms in genes involved in lipid metabolism. J Nutr 2007;137:2653-9.

77. Scaife AR, McNeill G, Campbell DM, Martindale S, Devereux G, Seaton A. Maternal intake of antioxidant vitamins in pregnancy in relation to maternal and fetal plasma levels at delivery Br J Nutr 2006;95:771-8.

78. Yeum KJ, Ferland G, Patry J, Russell RM. Relationship of plasma carotenoids, retinol and tocopherols in mothers and newborn infants J Am Coll Nutr 1998;17:442-7.

79. Bruno RS, Leonard SW, Atkinson J, et al. Faster plasma vitamin E disappearance in smokers is normalized by vitamin C supplementation. Free Radic Biol Med 2006;40:689-97.

80. Sanchez-Vera I, Bonet B, Viana M, Sanz C. Relationship between alpha-tocopherol content in the different lipoprotein fractions in term pregnant women and in umbilical cord blood. Ann Nutr Metab 2004;48:146-50.

81. Poston L, Briley AL, Seed PT, Kelly FJ, Shennan AH, Vitamins in Pre-eclampsia (VIP) Trial Consortium. Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial Lancet 2006;367:1145-54.

82. Beazley D, Ahokas R, Livingston J, Griggs M, Sibai BM. Vitamin C and E supplementation in women at high risk for preeclampsia: a double-blind, placebo-controlled trial Am J Obstet Gynecol 2005;192:520-1.

83. Chappell LC, Seed PT, Briley AL, et al. Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomised trial Lancet 1999;354:810-6.

84. Villar J, Purwar M, Merialdi M, et al. World Health Organisation multicentre randomised trial of supplementation with vitamins C and E among pregnant women at high risk for pre-eclampsia in populations of low nutritional status from developing countries BJOG 2009;116:780-8.

85. Boskovic R, Gargaun L, Oren D, Djulus J, Koren G. Pregnancy outcome following high doses of Vitamin E supplementation Reprod Toxicol 2005;20:85-8.

86. Scholl TO, Chen X, Sims M, Stein TP. Vitamin E: maternal concentrations are associated with fetal growth Am J Clin Nutr 2006;84:1442-8.

87. Saker M, Soulimane Mokhtari N, Merzouk SA, Merzouk H, Belarbi B, Narce M. Oxidant and antioxidant status in mothers and their newborns according to birthweight Eur J Obstet Gynecol Reprod Biol 2008;141:95-9.

88. Aris A, Leblanc S, Ouellet A, Moutquin JM. Detrimental effects of high levels of antioxidant vitamins C and E on placental function: considerations for the vitamins in preeclampsia (VIP) trial J Obstet Gynaecol Res 2008;34:504-11.

Appendix A

<u>Questionnaire Regarding Prenatal Vitamin Intake and Study Visits</u>

1) What prenatal vitamins were you taking during your pregnancy?
    a. The ones supplied by the study (Prenatal Rx 1) or other brands?

        _____

    b. Did you take it in the morning or at night? _____
2) What is the vitamin E and vitamin C content of that prenatal?
3) Were you taking them on a regular basis?
    a. Daily
    b. 5 times a week
    c. 3 times a week
    d. Once a week
    e. Not consistently
4) Do you remember the last time you ate before you delivered? _____

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 a 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/100 mL)
EtOH/MeOH 1:1 mixture

Column: C18, 4.6 x 100mm, 3μM, 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 min.

Sample Prep:
1. Add 100 μL plasma into a 10 mL screwcap glass tube containing: 2 mL EtOH/1% AA, and 900 μL Milli-Q H_2O. Then add 0.3 mL saturated KOH and vortex briefly.
2. Place the sample rack into 70°C water bath for 30 min.
3. Cool on ice then add 25 μL BHT (1 mg/mL EtOH) and 1 mL Milli-Q H2O/1% AA. Then add 2 mL hexane.
4. Mix by hand inversion for ~1 min. Allow to separate to partition, or centrifuge on low speed for 3 min.
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 an 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 ~20 μM. 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. The α-Tocopherol stock is 1500 μM (pmol/μL), so diluting 50 μL to 5000 μL and injecting 5 μL = 75 pmol inj. Injecting 10 μL = 150 pmol inj, and so on.
2. Take out 50 μL from the stock α-tocopherol std 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 μM. 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.

HPLC Directions:

1. Turn on the computer, 2 pumps, auto sampler, and controller. Purge the system with the freshly sonicated vitamin E mobile phase by opening the "Purge" valves and pushing the purge button on both pumps. The system will stop purging after 2 minutes. Close the purge valves and hit "Activate" on the controller. If the wrong method is open you will need to open method 6 on the controller (press menu key, then 1, then function key, then F1, then 6, then F2) for the correct flow. For the above column the flow should be 1.5 mL/min (0.75 mL/min/pump), with a back pressure of ~150 bar.
2. Once you have flow, turn on the ECD (power on, then cell on). Settings: Range 0.2 μA, Potential 0.5 V, Output ~0.005.
3. Set up the sequence on the controller to match where the vials have been placed in the autosampler. End with low flow program (vial 60, 10 μL inj, and F1 for run time/file). The system has a 50 μL loop, do not inject more than 50 μL.
4. Open the Shimadzu Class*VP* software icon on the computer and set up a new batch (under File pull down menu). Find the correct Method path by clicking on the blue folder on the right. Use the current date for the Data path (ex. c:\your name\date, ex. 112602). Sample ID does not matter, just make sure to put something. Put in the correct Method by clicking on the folder on the right. File name, ex. 112602.(001). Then enter the total number of runs. Click OK. Change the run type to calibration for STDSs. Fill in Sample ID descriptors, and 1-5 for STDS under Level. Save the batch (under File pull down menu) as the same date in the current date folder under your name on the c:/ drive.
5. Click on the green "Batch" arrow icon when your samples are loaded and you are ready to begin analyzing. You should see a "waiting for trigger" prompt in the bottom left hand corner of the screen. The trigger is the controller.
6. Check to make sure you have the correct column, plenty of room in the waste container, enough mobile phase as well as rinse solution, and that your samples and standards are in the correct positions. Hit "Run" on the controller to start the batch. Watch the first 2 standards come off to make sure everything is working properly.

Chromatogram Analysis:

1. If your batch was the last one on the screen, open a Data file (under File pull down menu) for one of your standards. If not, open your Method, and your Batch first. Then, click the "Analyze" icon. Open Peak Table (under Method pull down menu). Clear the Peak Table (under the Edit pull down menu). Define Peaks (under the Method pull down menu, under Graphic Events Programming). Click on chromatogram anywhere before the peaks of interest, and then anywhere after the peaks.
2. Fill in the new Peak Table. First, name the peaks (ex. g-T & a-T). Fit Type = Linear, DO NOT FORCE ZERO, put in your standard levels (pmol inj). Save the Method (under the File pull down menu).
3. Batch Summary (under Batch pull down menu). Click on: area, and ESTD conc under Peak Parameters, and channel B. Save to folder with current date.
4. Batch Reprocess (under Batch pull down menu). Click start and scroll down to analyze the files using the down arrow. If any of the chromatograms need to be changed, you will need to reprocess again after finishing (screwy software bug).

Sample Calculations:

Open the batch summary in Excel. When the Delimited data screen opens up click Finish.

ESTD conc. (pmol inj)/starting volume*hexane used/extracted*amount resuspended in/amount injected

Ex. pmol inj/100 μL*2 mL/1.6 mL*100 μL/20 μL = μM

Reference:Simultaneous determinationof tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. 1996. Podda M, Weber C, Traber MG, and L Packer. J Lipid Res. 37(4):893-901.