Nutrition and Health
Series Editors: Adrianne Bendich · Connie W. Bales

Peter Weber
Marc Birringer
Jeffrey B. Blumberg
Manfred Eggersdorfer
Jan Frank *Editors*

Vitamin E in Human Health

Humana Press

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The Nutrition and Health series has an overriding mission in providing health professionals with texts that are considered essential since each is edited by the leading researchers in their respective fields. Each volume includes: 1) a synthesis of the state of the science, 2) timely, in-depth reviews, 3) extensive, up-to-date fully annotated reference lists, 4) a detailed index, 5) relevant tables and figures, 6) identification of paradigm shifts and consequences, 7) virtually no overlap of information between chapters, but targeted, inter-chapter referrals, 8) suggestions of areas for future research and 9) balanced, data driven answers to patient/health professionals questions which are based upon the totality of evidence rather than the findings of a single study.
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Nutrition and Health

ISBN 978-3-030-05314-7 ISBN 978-3-030-05315-4 (eBook)
https://doi.org/10.1007/978-3-030-05315-4

Library of Congress Control Number: 2019934811

This Humana Press imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

We dedicate this book to the diverse community of biochemical, molecular, clinical, and public health scientists exploring the many roles of vitamin E in human health. We especially appreciate the commitment shown by the many devoted scientists who have contributed directly to this book. Last but not the least, we are most grateful to the unending support of our families for the work we do in nutrition science to promote health and treat disease.

Peter Weber, MD, PhD
Marc Birringer, PhD
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Foreword

Progress in nutrition science is continuing at an ever more rapid pace. New data from experimental and preclinical studies are emerging at the same time as advanced designs in prospective cohorts, metabolic studies, and clinical trials are providing evidence that needs to be evaluated and translated into dietary guidelines for health promotion and disease prevention. This is no small challenge when nutrition research is criticized as based on confounded or inaccurate methods and scorned for changing recommendations to follow or avoid specific nutrients, foods, and dietary patterns. In fact, when properly evaluated, there is a great deal of concordance between conclusions drawn from multiple research approaches, while changes in the science and recommendation largely follow these advances. When there is a discordance between these results, for example, between observational studies and clinical trials, then we need to examine them more closely. By doing so, we often gain valuable insights and learn to ask new and more targeted questions. A book like this one provides an opportunity to bring together the disparate information collected from basic research and human studies to be considered jointly in a broad context of applications from food science to public policy.

The early reductionist approaches in nutrition research focused on the discovery of individual nutrients and their mechanisms in the prevention of corresponding deficiency syndromes. However, much like more recent holistic approaches to tackling complex biological pathways and to addressing sociocultural factors that underlie health outcomes, parallels exist for individual nutrients like vitamin E. Most micronutrients have pleiotropic effects on multiple physiological systems, including the microbiome, and interact broadly with other dietary components as well as drugs and environmental toxins. We need to understand better how the various congeners of vitamin E and their metabolites affect the molecular and cellular functions and structures of the body that ultimately impact human health. These issues are urgent because the consumption of vitamin E from foods falls below recommended intakes in about 90 percent of populations around the globe. Together with other under-consumed micronutrients and healthy foods, we continue to be faced with the problem of "hidden hunger" where the effect of these chronic shortfalls may not be immediately apparent but whose consequences can be long

term and profound. After being studied for close to a century, we are far from knowing all that is relevant about vitamin E and other micronutrients. This volume brings together the history and the science of vitamin E and looks to its future in human health.

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Series Editor Page

The great success of the Nutrition and Health Series is the result of the consistent overriding mission of providing health professionals with texts that are essential because each includes (1) a synthesis of the state of the science; (2) timely, in-depth reviews by the leading researchers and clinicians in their respective fields; (3) extensive, up-to-date, fully annotated reference lists; (4) a detailed index; (5) relevant tables and figures; (6) identification of paradigm shifts and the consequences; (7) virtually no overlap of information between chapters but targeted, interchapter referrals; (8) suggestions of areas for future research; and (9) balanced, data-driven answers to patient as well as health professional questions which are based upon the totality of evidence rather than the findings of any single study.

The series volumes are not the outcome of a symposium. Rather, each editor has the potential to examine a chosen area with a broad perspective, both in subject matter and in the choice of chapter authors. The international perspective, especially with regard to public health initiatives, is emphasized where appropriate. The editors, whose trainings are both research- and practice-oriented, have the opportunity to develop a primary objective for their book, define the scope and focus, and then invite the leading authorities from around the world to be part of their initiative. The authors are encouraged to provide an overview of the field, discuss their own research, and relate the research findings to potential human health consequences. Because each book is developed de novo, the chapters are coordinated so that the resulting volume imparts greater knowledge than the sum of the information contained in the individual chapters.

Vitamin E in Human Health edited by **Peter Weber, MD, PhD; Marc Birringer, PhD; Jeffrey B. Blumberg, PhD; Manfred Eggersdorfer, PhD, and Jan Frank, PhD,** is a very welcome and timely addition to the Nutrition and Health Series and fully exemplifies the series' goals. There has been a continuous stream of basic as well as clinical research over the last decade on the associations between oxidative stress and increased risk of cellular damage that provide increasing evidence of the critical antioxidant role of vitamin E. Moreover, the recent findings of non-antioxidant functions of the vitamin, including biological activities of metabolites as well as activation of enzymes and, of great significance, vitamin E-gene interactions, are reviewed in depth so that the reader is brought up-to-date on the many new findings at the cellular and intracellular levels. With regard to vitamin E, the major lipid-soluble antioxidant capable of reducing the adverse effects of oxidative stress, significant clinical research has been published on the importance of vitamin

E in reducing the damage to the vessels and organs of the cardiovascular and cerebrovascular systems in aging populations as well as in the very youngest newborns. Of equal importance are the detailed studies of the critical role of vitamin E in tissues of the nervous and immune systems. Contemporary data on the vitamin E status in at-risk populations, especially in children and women of childbearing potential, are also of great importance; these studies alone warrant the development of this 31-chapter tome.

Vitamin E, as an essential fat-soluble nutrient for humans and animal species, plays key roles in the retina and related brain tissues, lymphocytes, macrophages and other immune cells, red cells, sperm and ova, and basically every cell membrane in the body. As a former vitamin E researcher, and Roche colleague of Dr. Peter Weber, I am personally very pleased to note the thoughtful organization of this new volume. There are chapters that review data on the biology and biochemistry of the vitamin, its metabolism and metabolomics, and effects on gene regulation and the genetic defect that results in severe neurological and muscular defects associated with ataxia in isolated vitamin E deficiency; intakes and status in healthy individuals are included as well as an extensive section that is devoted to clinically relevant discussions of safety in healthy individuals as well as in patient populations. The volume is designed as an important resource for nutritionists and dietitians, research and public health scientists, diabetes specialists, ophthalmologists, nephrologists, cardiologists, gastroenterologists, and related physicians and healthcare professionals who interact with clients, patients, and/or family members. The volume provides objective, relevant information for professors and lecturers, advanced undergraduates and graduates, researchers, and clinical investigators who require extensive, up-to-date literature reviews, instructive tables and figures, and excellent references on all aspects of the importance of vitamin E in human health and disease.

The editors of this volume are experts in their respective fields. Dr. Peter Weber, MD, PhD, is Adjunct Professor of Nutrition at the Institute of Nutritional Sciences at the University of Hohenheim, Germany. Dr. Weber received his PhD in Nutritional Sciences from the University of Bonn, Germany, and his MD degree from the University of Mainz, Germany. He then worked at the Research Institute of Child Nutrition in Dortmund, Germany, and then trained in Internal Medicine with a subspecialty in endocrinology at the University of Mainz, Germany. Dr. Weber practiced medicine and clinical research for 10 years before joining the vitamins R&D area at Hoffmann-La Roche in New Jersey, USA, and following the purchase of the Vitamins Division by DSM, he continued to lead the research and development team human nutrition in various functions until his recent retirement. Dr. Weber has more than 70 peer-reviewed publications in the fields of iodine deficiency and goiter, thyroid diseases, metabolic syndrome, postprandial lipid metabolism, vitamin K, vitamin status of populations, and the role of vitamins and polyunsaturated fatty acids in human health. He is a Coeditor of several books on micronutrients and health. His scientific interests include the role of micronutrients in the prevention of chronic diseases, nutritional status in risk groups such as the elderly, and the emerging topic of nutrition security.

Dr. Marc Birringer received his PhD in Chemistry from the University of Siegen (Germany). His postdoctoral research included investigations of the

anticancer activity of seleno-amino acids at the University of Albany and examination of human vitamin E metabolism and anticancer properties of newly synthesized tocopheryl derivatives at the German Institute of Human Nutrition. Dr. Birringer co-founded a biopharmaceutical company where he was Head of R&D and developed a high-throughput synthesizer for peptide libraries and the development of tissue-specific drug-peptide conjugates. He also researched diet-induced mitochondrial activation and aging. Dr. Birringer was appointed Full Professor for Applied Nutritional and Environmental Biochemistry in the Department of Nutritional, Food and Consumer Sciences at Fulda University of Applied Sciences. His current research is focused on the metabolism of dietary lipophilic micronutrients. Dr. Birringer is President of the Gesellschaft für Angewandte Vitaminforschung eV and Member of the Editorial Board of *NFS Journal* and has authored more than 80 scientific articles and reviews.

Dr. Jeffrey B. Blumberg, PhD, is a Full Professor in the Friedman School of Nutrition Science and Policy and also serves as the Senior Scientist in the Antioxidants Research Laboratory at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University (USA). His research is focused on the biochemical basis for the role of antioxidant nutrients and their dietary requirements in promoting health and preventing disease during the aging process via changes in the status of oxidative stress, glucoregulation, and inflammation. He has published more than 400 scientific articles and serves on the Editorial Boards of several scientific journals. Dr. Blumberg was included in the 2015 Thomson Reuters' top 1% of cited researchers in his field. He has been the Recipient of the ASN Mary Swartz Rose Senior Investigator Award for outstanding research on the safety and efficacy of bioactive compounds for human health. Dr. Blumberg participates in activities relevant to the incorporation of sound nutrition science into public health policy and has served as a Member of the Workshop on Health Promotion and Aging in the Office of the US Surgeon General, Sports Medicine Committee of the US Olympic Committee, Consultation on Preparation and Use of Food-Based Dietary Guidelines for the WHO/FAO, Food Advisory Committee of the FDA, and other committees.

Dr. Manfred Eggersdorfer is Professor for Healthy Ageing at the University Medical Center in Groningen, Netherlands. He received his PhD in Organic Chemistry at the Technical University of Munich, Germany, and undertook postdoctoral work at Stanford University, California, working with Dr. Carl Djerassi on the isolation and characterization of sterols from marine origin. Dr. Manfred Eggersdorfer worked for BASF and was Head of R&D for the Fine Chemicals Division and then joined Roche as Head of Research Vitamins and DSM where he was responsible for Nutrition Science & Advocacy. Dr. Eggersdorfer is a Member of the Advisory Board of Johns Hopkins Bloomberg School of Public Health, a Board Member of the Gesellschaft für Angewandte Vitaminforschung eV, and Member of the Tufts Nutrition Council. He is an Honorary Member of the Oxygen Club of California and Author of numerous publications, reviews, and book chapters in the field of vitamins, carotenoids, and innovation in nutritional ingredients. He is a Reviewer for several scientific journals as well as Associate Editor of the *International Journal of Vitamin and Nutrition Research*.

Professor Frank graduated with a Diploma in Nutrition from Bonn University and obtained his PhD in Food Science at the Swedish University of Agricultural Sciences. He received postdoctoral training at the Universities of Kiel and Hohenheim and was a Visiting Scientist at the Linus Pauling Institute (USA), the University of Reading (UK), and the University of Surrey (UK). Dr. Frank served as Professor of Human Metabolomics at the Institute of Nutrition and Food Science at the University of Bonn and is now Full Professor and Head of the Division of Food Biofunctionality at the Institute of Nutritional Sciences at the University of Hohenheim (Germany). He is the Founding President of the Society of Nutrition and Food Science (www.snfs.org), Member of the Board of Directors of the Society for Applied Vitamin Research (Gesellschaft für Angewandte Vitaminforschung eV), and Editor-in-Chief of *NFS Journal*, Associate Editor of *Nutrition*, and Member of the Editorial Boards of *The Journal of Nutritional Biochemistry*, *BioFactors*, and *Plant Foods for Human Nutrition*. His primary research interests lie in factors that determine the absorption, metabolism, and elimination of phytochemicals and different vitamin E congeners, especially long-chain vitamin E metabolites.

The 31 chapters in this comprehensive volume are organized in five parts: historic perspective and basic biochemical review; intake and status data; safety, drug-nutrient interactions, and nutrient-nutrient interactions; cardiovascular, neurological, and immune functions; and public health implications.

Part I: Biochemistry, Metabolism, and Molecular Effects of Vitamin E

The first of the four introductory chapters of the volume, Chap. 1, written by the editors, provides readers with the rationale for this timely review of vitamin E. Chapter 2, *the first chapter in Part One*, examines the first 100 years of vitamin E research using a historical perspective of the discovery of the vitamin, its importance in the maintenance of growth and animal reproduction, and the gradual appreciation of its essentiality for human life. We learn that vitamin E has eight forms that provide the biological activities associated with the broad term. The vitamin is ubiquitously distributed throughout the body as it is a critical component of all membranes. Chapter 3 describes in detail the physical structures and biological activities of the eight forms of vitamin E and concentrates on their antioxidant activities in both foods and living organisms. α-Tocopherol's role in cell membranes is to protect unsaturated fatty acids from oxidation and maintain membrane integrity. Even though the concentration of α-tocopherol in membranes is small, ranging 0.1–1 mol% relative to phospholipids, it efficiently prevents the oxidation of these fatty acids. The extensively referenced chapter describes the process of scavenging free radicals and the detailed movement of vitamin E within cell membranes in animal tissues as well as the protective role in food production involving oils and other lipids. Chapter 4 reviews the absorption, metabolism, and excretion of vitamin E. We learn that vitamin E has a high bioavailability of about 50–80% and follows the general absorptive route of dietary fats; the

liver preferentially incorporates α-tocopherol into lipoproteins that are released into the blood stream for the distribution of lipids to peripheral tissues. Metabolism is described in detail and outstanding research questions are included.

Chapters 5 and 6 examine the bioactivities of some of the vitamin E derivatives and metabolites. Chapter 5, containing 11 detailed figures and over 100 relevant references, reviews the new data concerning the biosynthetic steps leading to a chromanol ring system found only in photosynthetic organisms, such as plants, algae, and cyanobacteria as well as fungi, corals, sponges, and tunicates. The chapter focuses on the structural diversity and bioactivity of minor vitamin E derivatives, meroditerpenes (135 in number that have so far been described) that belong to the class of chromanols and chromenols. This unique chapter provides details concerning the biosynthesis of these compounds and lists of plants that contain these compounds and their functions. Chapter 6 expands our knowledge of new research concerning the bioactivities of vitamin E metabolites by explaining that during hepatic metabolism of vitamin E, the long-chain metabolites, 13-hydroxychromanol and 13-carboxychromanol, are formed by oxidative modification of the vitamin E side chain. These metabolites have been detected in human serum, indicating a physiological relevance for the human body. Given that only 1% of the total body tocopherol is transported in blood, the determination of the long-chain metabolites in extrahepatic tissues is important for assessing the total value of vitamin E for human health. Methodologies are described in detail, and preliminary in vitro studies show activities with regard to anti-inflammatory functions, lipid metabolism, chemoprevention, and xenobiotic metabolism.

New research into the gene-regulatory functions of vitamin E is reviewed in Chap. 7. The chapter examines the non-antioxidant properties of vitamin E including α-tocopherol's inhibition of protein kinase C and 5-lipoxygenase and activation of protein phosphatase 2A and diacylglycerol kinase. Vitamin E can modulate the immune response and activities of many of the immune cells resulting in reduced inflammation and enhanced responses to certain pathogens (discussed in more detail in Chaps. 25 and 26). There is a discussion of the long-term effects of vitamin E deficiency on hepatic gene expression by regulating specific hepatic factors and messenger RNA responses (as reviewed in Chap. 20). The authors' animal studies indicate that dietary vitamin E induces changes in steroidogenesis by affecting cholesterol homeostasis in the testes and adrenal glands. The chapter includes informative figures, an extensive and detailed table, and 120 key references. Another relatively new research area, looked at in Chap. 8, is the study entitled "Metabolomics." Metabolomics is a technology that examines all of the metabolites in a given sample and the changes in these metabolites when challenged with a substance such as vitamin E. The chapter reviews the technologies used to examine the metabolome (all of the metabolites in a sample). The chapter describes the studies in humans that have demonstrated changes to the urinary and the plasma metabolome with vitamin E supplementation and the studies in animals that have found changes to the metabolome with models of vitamin E deficiency. As examples, in humans, vitamin E supplementation influences phospholipid metabolism and amino acid metabolism. Metabolomic studies in animal models, including studies with zebra fish, showed changes to anti-

oxidant status and lipid peroxidation with vitamin E deficiency; rodent models of vitamin E deficiency showed influences on central metabolism.

Chapter 9 provides an in-depth review of the structure and function of the specific α-tocopherol transfer protein (TTP) which serves as the major determinant for the distribution of newly absorbed dietary tocopherol throughout the body. The authors present a model for the directional transport of tocopherol from endosomal membranes to the plasma membrane of hepatocytes and review recent data showing that regulation of TTP serves to maintain vitamin E homeostasis in cells and tissues. Expression of TTP is highest in the liver and is also expressed in the brain and placenta and that expression in these tissues is of high physiological importance. Mutations in the TTP gene are the only known cause for the autosomal recessive disorder ataxia with isolated vitamin E deficiency (AVED). AVED patients present with progressive neurodegeneration and low (or undetectable) serum levels of α-tocopherol.

As discussed in earlier chapters, there is growing interest in the biological activities of a number of vitamin E isoforms. New data on the in vitro studies of the effects of γ-tocotrienol on cancer cell types in cell culture are described in Chap. 10. The chapter reviews the importance of lipid rafts, which are specialized micro-domains within the plasma membrane that are required for receptor tyrosine kinase dimerization, activation, and signal transduction. Recent studies demonstrate that the antiproliferative effects of γ-tocotrienol are associated with its accumulation in the lipid raft micro-domain where it appears to interfere with the tyrosine kinase dimerization and activation in human breast cancer cells. Additional experiments showed that γ-tocotrienol added to cell cultures directly disrupted lipid raft integrity by directly interfering with HER receptor dimerization that is linked to breast cancer cell viability. This early evidence of the bioactivity of γ-tocotrienol is critical to moving to the next step of laboratory animal testing in breast cancer models.

The final chapter in this Part, Chap. 11, describes, in detail, the interactions between vitamin E and polyunsaturated fatty acids (PUFA) and reviews the mechanisms of uptake, transport, distribution, metabolism, interaction, and regulatory roles of both these classes of essential nutrients in signal transduction, gene expression, and maintenance of normal cell membrane physiology. Vitamin E protects PUFA in cellular membranes from oxidative damage, and the key tissues and organs most affected by either nutrient deficiency are described. Emphasis is placed on the review of research in brain function and the roles of active lipids and vitamin E and how each has independent effects and interdependent effects.

Part II: A Global View on Vitamin E Intake and Status

The five chapters in this part examine the processes behind setting healthful intake levels of vitamin E and review the current intake levels in populations based upon age, sex, and the types of foods that are consumed. Chapter 12 describes the challenges facing regulators, academicians, and clinicians in setting recommended intakes for vitamin E. The chapter summarizes the current approaches used by national organizations around the world to set vitamin E intake recommendations and compares the most recent

recommendations. The authors posit that new research on the importance of increased omega-3 long-chain (LC) PUFA intakes should also result in further increases in vitamin E intake to protect the PUFA from oxidation. Specifically, even though the current intake of omega-3 LC PUFA is low and below the recommended dietary intakes of most Western nations, vitamin E intakes are even lower than needed to protect PUFA. In fact, a worldwide review of nutritional surveys showed that only 45 of the 266 countries achieved the recommended PUFA intake level. Similarly, the current vitamin E intakes are below recommended levels in more than 90% of North American as well as in some European countries. Even so, national recommendations have increased for LC PUFA. Thus, the authors suggest that the ratio of vitamin E to PUFA is even more critical and requires a deeper examination by regulatory authorities. Chapter 13 reviews the current data from over 120 relevant references on vitamin E intakes globally and clearly indicates that, virtually, all populations examined had low intakes relative to whatever their country recommended. Likewise, serum vitamin E concentrations reflected the low intakes, and given that the standard for adequacy is also relatively low, based upon PUFA intakes, these even lower serum levels attest to the validity of the intake data across nations. Of importance are the data that show that plasma vitamin E concentrations less than 8 μmol/L are associated with neurologic disturbances and diseases including peripheral neuropathy, spinocerebellar ataxia, and skeletal myopathy. Concentrations lower than 12 μmol/L have been seen in women with miscarriages and in patients with increased erythrocyte fragility. Preliminary data suggest that low plasma α-tocopherol concentrations are associated with increased risk of developing mild cognitive impairment and Alzheimer's disease. The studies that have correlated higher serum vitamin E with health benefits are reviewed. Chapter 14 presents the current reference values for vitamin E intake and compares these values to the published data on vitamin E intake as well as on vitamin E status for toddlers, elementary school-age children, and adolescents. The chapter includes tables and text that contain the published dietary intakes in these age groups, including infants (preterm and term) from the USA, Mexico, and Brazil and several European countries and a discussion of the rationale used to determine these values. The studies that determined serum/plasma vitamin E in children are reviewed, and the data, like that of intake, are inconsistent.

Chapter 15 provides new research concerning the determination of vitamin E status and its correlation with other biological factors. Vitamin E, as a lipid-soluble molecule, has a high affinity for circulating lipids, resulting in a strong correlation with total cholesterol and fasting triacylglycerols. Many studies correct this by analyzing vitamin E serum/plasma concentrations as a ratio of vitamin E concentrations divided by total cholesterol plus fasting triglycerides. The authors are concerned that there is also a positive correlation between total cholesterol and fasting triglycerides, the summation of total cholesterol and fasting triglycerides to calculate total lipids, and correction for this by dividing vitamin E concentrations may result in an unintended double correction for variance shared by total cholesterol and fasting triglycerides and unwanted weakening, disappearance, or even inversion of existing associations with vitamin E. The chapter, using data from the LifeLines Cohort Study, calculates linear regressions of vitamin E serum concentrations

with numerous other clinical factors and found the association between circulating α-tocopherol and γ-tocopherol to be positive, while most of the associations of γ-tocopherol with biological variables, if present, were opposite to those that were observed for α-tocopherol. The associations are described in detail in the text and included tables.

Chapter 16 reviews the major sources of vitamin E in the diet with emphasis on natural sources. The effects of food processing are also included and examined from the point of harvest until the product is consumed. Wheat germ oil contains about 149 mg of α-tocopherol per 100 g, making it the edible oil that has the highest amount of total vitamin E. Other edible oils that are excellent sources of α-tocopherol include sunflower, safflower, and olive oils. In addition to oils, the comprehensive chapter examines numerous foods and types of preparation that are further summarized in relevant tables and figures.

Part III: Safety of Vitamin E and Interactions with Other Nutrients and Drugs

This clinically important part of the volume provides the reader with three chapters that examine the safety of supplementation with vitamin E, its potential interaction with commonly used pharmaceuticals, as well as the reported interactions with vitamin K. Chapter 17 reviews the analyses of data associating vitamin E supplementation with potential increased risk of death. The author performed a meta-analysis with 68 published studies. The total numbers of study participants were 124,836 in the vitamin E group and 124,925 in the control group. Dosages of vitamin E supplementation ranged from 16.5 to 5000 IU/d. Vitamin E supplementation, regardless of the length of use or dose, age, or sex of participant, did not affect mortality risk. The comprehensive chapter provides all citations, methods of the multiple types of analyses, and findings. Additionally, vitamin E intake is considered to be safe up to an established upper tolerable intake (UL) level of 300–1,000 mg/day (depending on the regulatory authority and the age and sex of the individual) and in any amount that is naturally occurring in foods or in multivitamin supplements. These values are agreed upon by expert groups of the US-IOM, EFSA, and the UK's Expert Group on Vitamins and Minerals (EVM).

Chapter 18 looks at the potential for vitamin E supplementation to affect the drugs commonly used to treat medical conditions often associated with reported beneficial vitamin E effects. The chapter reviews the basics of nutrient-drug interactions: interactions affecting the pharmacokinetics (the metabolism of a drug) or pharmacodynamics (the effect of a drug) of drugs. There is also a review of the evidence in the scientific literature on the potential for any of the eight vitamin E congeners to interact with drugs. Hepatic metabolism is reviewed in detail, and it appears that vitamin E does not affect these enzyme systems at dosage levels known to be consumed, including high doses of supplements. No evidence for vitamin E-drug interactions at vitamin E intakes achievable by diet was found. High-dose (≥ 300 mg/d) supplementation of vitamin E, especially of α-tocopherol, however, may lead

to interactions with aspirin, warfarin, tamoxifen, and cyclosporine A. For the majority of drugs, interactions with vitamin E, even at high doses, have not been observed and are considered by the authors to be unlikely. Chapter 19 looks at the nutrient-nutrient interactions with emphasis on the interactions between vitamin E and another fat-soluble vitamin, vitamin K. Vitamin K is required for normal blood clotting, and vitamin E supplementation can affect platelet membranes, and thus there is the potential for vitamin E to affect the clotting function of vitamin K. However, the extensive review of in vitro as well as clinical studies with and without the anticlotting drug, warfarin, indicates that vitamin E supplementation did not affect clotting time or other indices of clotting.

Part IV: Benefits of Vitamin E on Human Health and Disease

The next four chapters examine the clinical data associating vitamin E status and/or supplementation with beneficial effects on the cardiovascular system and related data in individuals with diabetes or the metabolic syndrome. The final chapter looks at nonalcoholic fatty liver disease (NAFLD). Chapter 20 focuses on the clinical trials evaluating vitamin E supplementation and various cardiovascular outcomes. The chapter examines the major primary prevention peer-reviewed studies published starting in 1998 and ends with the study published in 2015. Secondary prevention studies are then reviewed including studies looking at vitamin E as well as those testing vitamins E and C, β-carotene, and certain pharmacological agents. All data are tabulated; the authors posit that there may be specific patient populations that will benefit from vitamin E supplementation, however, to date; the published studies do not support vitamin E supplementation for either primary or secondary prevention of cardiovascular disease.

One possible reason that the intervention studies have not shown statistically positive effects of vitamin E may be that the populations are genetically heterogeneous and vitamin E may benefit some and harm other patients, resulting in an overall null effect. Chapter 21 reviews the important finding that a polymorphism in the haptoglobin (Hp) gene strongly defines individuals with diabetes who are at greater risk of vascular disease. The chapter describes the three polymorphisms and their effects on the removal of free hemoglobin from the bloodstream. The Hp 2-2 genotype is an independent risk factor for incident atherosclerotic cardiovascular disease (CVD) in type 1 and type 2 diabetes patients. Vitamin E supplementation benefits diabetics with the Hp 2-2 genotype. The data from clinical trials as well as the pharmacogenomic consequences of the polymorphism are examined in detail.

Chapter 22 examines the factors associated with the metabolic syndrome (MetS) which is considered an early risk factor for diabetes, cardiovascular, as well as nonalcoholic liver diseases. MetS is defined by the presence of a cluster of conditions that includes at least three of the five risk factors: hypertension, hyperglycemia, central obesity, hypertriglyceridemia, or depressed high-density lipoprotein (HDL). Compared with healthy individuals,

individuals with MetS often have increased oxidative stress that is evidenced by circulating biomarkers of lipid peroxidation including F_2-isoprostanes and oxidized LDL and low antioxidant capacity. Similarly, inflammatory responses, including circulating pro-inflammatory interleukins, tumor necrosis factor-α, C-reactive protein, myeloperoxidase, and other inflammatory mediator concentrations, are increased. The chapter explains the consequences of the metabolic aberrations seen in the syndrome and focuses on obese patients with MetS as a clinical model and emphasizes the dysregulation of α-tocopherol trafficking and catabolism and related health consequences. Individuals with MetS have poor α-tocopherol intakes and compromised α-tocopherol status. The inability to efficiently achieve adequate α-tocopherol serum concentrations can create a vicious cycle of increasing inflammatory damage that further provokes the depletion of α-tocopherol.

Chapter 23 provides a clinical perspective on the hepatic disease, nonalcoholic fatty liver disease (NAFLD), which is one of the leading causes of chronic liver disease worldwide, especially in Western countries where obesity and metabolic syndrome have been on the rise. NAFLD is broadly subdivided into nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NASH, characterized by histological hallmarks of steatosis, lobular inflammation, and hepatocellular ballooning, can progress to fibrosis and liver cancer. Intracellular evidence points to oxidative damage to the liver mitochondria and other organelles in NAFLD. At the same time, antioxidant capacity of the liver is reduced, resulting in further damage. Laboratory animal studies of NASH and human clinical trials with vitamin E are reviewed and tabulated

Chapter 24 examines the rationale for looking at vitamin E status and supplementation in the prevention and/or slowing of progression of Alzheimer's disease (AD) or mild cognitive impairment (MCI). Vitamin E, as an essential antioxidant micronutrient, is a significant factor in protecting cells from oxidative damage, such as the harmful effects of free radicals observed in brain aging and AD. The chapter reviews the animal studies that showed sufficient evidence to consider treatment with vitamin E for patients suffering from AD. However, the results of clinical studies with AD patients are conflicting. To date, there are no convincing data from meta-analyses of randomized, placebo-controlled trials that vitamin E alone or in combination with other antioxidants prevents the progression or improves cognitive function in people with MCI or AD.

Two major functions of the human immune system are to protect from pathogens and also to recognize our own cells, tissues, and organs. If the immune system is not working properly, there is a greater risk of infections, and, as important, there is also a greater risk of allergies and asthma and other auto-immune diseases. Certainly, genetics plays a critical role, but environmental factors, including nutrients, like vitamin E, can affect these risks. Chapter 25 looks at the development of allergies and asthma and identifies the opposite responses to two isoforms of vitamin E, α-tocopherol and γ-tocopherol. The complex responses seen during allergy and asthma episodes are described as are the specific components of the immune system that respond to these challenges. We learn that in addition to antioxidant functions of tocopherols, the non-antioxidant functions of tocopherol regulate allergic inflammation. Important for interpretation of tocopherol regulatory effects is

that although γ-tocopherol is at tenfold lower concentrations in vivo, γ-tocopherol is very potent and, at these tenfold lower concentrations, can block the benefit of α-tocopherol during allergic inflammation and asthma. Data, from the over 200 important references cited, are presented from laboratory animal studies, epidemiological data globally, and from clinical studies including maternal to child transmission of vitamin E, increased use of soybean oil rich in γ-tocopherol in infant formula and other relevant factors that help to explain the potential benefits and risks of examining vitamin E in allergy and asthma without paying attention to the particular isoforms. Chapter 26 reviews the data showing the immunoenhancing capabilities of vitamin E that can beneficially affect responses to infections. The authors present their data as well as that of others that increased vitamin E intake (α-tocopherol) above recommended levels enhances T-cell function, particularly in aged animals and humans. The mechanisms for this effect of vitamin E involve both a direct effect of enhancing T-cell activation and effector function, and a suppressing effect on the production of prostaglandin E2, a T-cell-suppressing lipid mediator known to increase with aging. Vitamin E's enhancement of immune functions has significant clinical implications as supplementation is associated with increased resistance to respiratory infections in both aged mice and older adults. The chapter reviews the mechanism of action of the immune system and the detailed analysis of the role of vitamin E in assuring that responses to pathogens, especially respiratory infections, are optimal. The authors indicate that the intake level of vitamin E needed to assure robust immune responses in seniors is about 200 mg/d as compared to the US recommended level of 15 mg/d.

Two other important topics are included in this part of the impressive volume: vitamin E and air pollution, and pregnancy. The chapter on vitamin E's role in reproductive function is especially apt as the first establishment of the international units of activity (IU) of vitamin E was based upon the prevention of fetal reabsorption in a rat model. Chapter 27, containing over 200 relevant references, reviews the sources of air pollution, their adverse health effects, and ways to mitigate these effects with primary decreases in the sources as well as secondary measures to help protect the lungs and overall health. Air pollution is mainly caused by vehicle engine combustion, fossil fuel combustion, power plants, and other industrial sources. Compounds found in air pollution discussed include particulate matter from various sources, carbon monoxide, lead, nitrogen dioxide, sulfur dioxide, and ozone. Children, the elderly, and individuals with health conditions such as asthma, chronic obstructive pulmonary disease (COPD), hypertension, ischemic heart disease, obesity, and diabetes are more susceptible than other populations to the toxicity of air pollution. Both laboratory and clinical studies, where vitamin E, often with vitamin C, is given to at-risk populations, are reviewed. We learn that exposure to pollutants increases the risk of many common diseases, including asthma, COPD, and cardiovascular, metabolic, gastrointestinal, skin, and CNS diseases. While the specific mechanisms leading to the development and progression of each of these diseases vary, all have a common underlying pathology of inflammation and oxidative stress.

Chapter 28 describes our current understanding of vitamin E's role in pregnancy. The vitamin E status of the pregnant woman and its impact on the developing fetus are reviewed. Complications of pregnancy and the neonatal

period, including, but not limited to, infection, chronic lung disease, necrotizing enterocolitis, and intraventricular hemorrhage, are characterized by inflammation. Also, pregnancy outcomes such as length of gestation, mode of delivery, and associations with preeclampsia, pregnancy-induced hypertension, and gestational diabetes are discussed. The authors acknowledge that considerable scientific work remains to fully understand the role of vitamin E in fertility, pregnancy, and its ability to optimize maternal and neonatal outcomes.

Part V: Public Health Implications of Vitamin E

The last part of this comprehensive volume explores unique questions concerning important public health issues. The first chapter examines the health economics of vitamin E supplementation followed by an investigation of consumer choices including vitamin E use and, finally, a critical examination of the current knowledge gaps which are always linked to research priorities and funding sources. Chapter 29 describes the applications of health economic theories and models to an example of clinical use of vitamin E to obtain a better understanding of the possible impact of this essential micronutrient on healthcare costs. The chapter includes a general introduction into health economics and the main concept of the cost per quality-adjusted life year (QALY) gained. This calculation is used to determine the value of vitamin E supplementation using the clinical data discussed in Chap. 20, where there was a reduction in the risk of cardiovascular complications in type 2 diabetes mellitus patients with the haptoglobin Hp 2-2 genotype. The author calculated that, irrespective of Hp genotype, there was an incremental cost-effectiveness ratio (ICER) of £ 684 per QALY gained compared with patients who did not take vitamin E supplementation. Given that in the UK, the value of one QALY is about £40,000, the data indicate that vitamin E supplementation is a very cost-effective intervention for this indication. Different scenarios are provided, and the factors that are used to determine the health economics of an intervention in different healthcare environments are discussed.

Chapter 30 takes a pragmatic perspective on consumer nutrition knowledge and extrapolates to the degree of consumer awareness of vitamin E and its functions as these are critical issues in making the decision to use vitamin supplements. We learn that most consumers have learned that there is a link between the food consumed and their health. Using consumer feedback, it is also clear that not everyone is highly motivated to eat healthily, because food serves other purposes than maintaining a healthy body. The chapter discusses the concept of nutrition knowledge, presents insights on how and from which sources consumers obtain their nutrition knowledge and how this nutrition knowledge affects their food choices and other decisions relevant to their dietary intake, and, finally, reviews possible options for behavioral change regarding the intake of micronutrients such as vitamin E. The author notes that the instruments used to ask consumers about their nutrition knowledge rarely include questions about any vitamins, but when vitamins and minerals were included in questions, consumers rated their importance as high. Recent data indicate that the Internet is a significant resource for nutrition informa-

tion for consumers. The complexity of affecting consumer intake of any food, let alone a single nutrient such as vitamin E, appears from this analysis to be daunting. The conclusion is that the role of vitamin E in consumer decision-making is probably very small and that attempts to change vitamin E intake will be difficult.

The final chapter, Chap. 31, describes the difficulties in comparing study findings between healthy individuals and those with clinical disease; moreover, there is not a clear definition of the term "healthy individual." Thus, building a totality of the evidence of a consistent biological effect of vitamin E supplementation on any outcome remains quite difficult. Even when the finding is accepted, as with the haptoglobin data in Chap. 20, the finding is so specific, it affects individuals with diabetes and thus not healthy individuals, and the benefits are quite difficult to explain to the general consumer. The chapter includes several examples of basic knowledge gaps including the fact that in the case of vitamin E, the Institute of Medicine (IOM) acknowledges that persons consuming a diet high in polyunsaturated fatty acids (PUFA) require a higher intake of vitamin E to meet the estimated average requirement (EAR) levels for adults in the USA. However, since studies used to support the current EAR levels for vitamin E were conducted in men only, the IOM lacked the scientific data to deliberate a potential EAR for women or any other subpopulations based on age, as an example. The chapter presents insights into the process used for the funding of nutrition research from the National Institutes of Health and other national sources of funding. As with consumer interest, funding research studies are based upon many disparate factors, and priorities are constantly in flux, resulting in the difficulty in comparing results over time. Of note, there are many new initiatives that are being implemented to broaden the scope of research funding, including collaboration across national borders and collaboration with private companies.

Conclusions

The above description of the volume's 31 chapters attests to the depth of information provided by the 50 highly respected chapter authors and volume editors. Each chapter includes complete definitions of terms with the abbreviations fully defined and consistent use of terms between chapters. Key features of the comprehensive volume include over 100 detailed tables and informative figures; an extensive, detailed index; and more than 2600 up-to-date references that provide the reader with excellent sources of worthwhile practice-oriented information that will be of great value to health providers as well as graduate and medical students.

In conclusion, ***Vitamin E in Human Health***, edited by **Peter Weber, Marc Birringer, Jeffrey B. Blumberg, Manfred Eggersdorfer, and Jan Frank**, provides health professionals in many areas of research and practice with the most current and well-referenced volume on the importance of vitamin E in the maintenance of the overall health of the individual as well as reducing the risk of adverse effects in patients with chronic and/or infectious diseases that increase the risk of oxidative stress. The volume serves the reader as the benchmark for integrating the complex interrelationships

between vitamin E intake and the consumption of dietary and supplemental sources of lipids, especially omega-3 long-chain PUFA. Moreover, the physiological, genetic, and environmental interactions between vitamin E, its eight isoforms, metabolites, and cofactors are clearly delineated so that students as well as practitioners can better understand the complexities of these interactions. Practice-oriented chapters examine the clinical importance of a specific vitamin E transfer protein, metabolomics effects of vitamin E, its use as an antioxidant in foods, the clinical value of vitamin E in asthma and allergy, respiratory disease prevention, exposure to air pollution, and human reproduction. The final chapters of this valuable volume provide unique and relevant data on the positive health economic value of vitamin E supplementation in certain patient populations and the importance of understanding the drivers of consumer use of specific supplements, such as vitamin E, and a final chapter provides valuable insights into the processes involved in determining the funding for health research in the USA and how vitamin E research funding fits into these objective processes. The broad scope as well as in-depth reviews of each chapter's topic makes this excellent volume a very welcome addition to the Nutrition and Health Series.

Adrianne Bendich, PhD, FACN, FASN
Series Editor

About the Series Editor

Adrianne Bendich PhD, FASN, FACN, has served as the *"Nutrition and Health" Series Editor* for more than 20 years and has provided leadership and guidance to more than 200 editors that have developed the 80+ well-respected and highly recommended volumes in the series.

In addition to ***Vitamin E in Human Health*** **edited by Peter Weber, Marc Birringer, Jeffrey B. Blumberg, Manfred Eggersdorfer, and Jan Frank,** major new editions published in 2012–2019 include:

1. ***Handbook of Nutrition and Pregnancy*****, Second Edition,** edited by Carol J. Lammi-Keefe, Sarah C. Couch, and John P. Kirwan, 2019
2. ***Dietary Patterns and Whole Plant Foods in Aging and Disease***, edited as well as written by Mark L. Dreher, PhD, 2018
3. ***Dietary Fiber in Health and Disease*****,** edited as well as written by Mark L. Dreher, PhD, 2017
4. ***Clinical Aspects of Natural and Added Phosphorus in Foods***, edited by Orlando M. Gutierrez, Kamyar Kalantar-Zadeh, and Rajnish Mehrotra, 2017
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6. ***Nutrition and Diet in Maternal Diabetes***, edited by Rajendram Rajkumar, Victor R. Preedy, and Vinood B. Patel, 2017
7. ***Nitrite and Nitrate in Human Health and Disease*****, Second Edition**, edited by Nathan S. Bryan and Joseph Loscalzo, 2017
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Earlier books included ***The Vitamin D Solution*****, Second Edition,** edited by Dr. Michael Holick; ***Dietary Components and Immune Function*** edited by Dr. Ronald Ross Watson, Dr. Sherma Zibadi, and Dr. Victor R. Preedy; ***Bioactive Compounds and Cancer*** edited by Dr. John A. Milner and Dr. Donato F. Romagnolo; ***Modern Dietary Fat Intakes in Disease Promotion*** edited by Dr. Fabien De Meester, Dr. Sherma Zibadi, and Dr. Ronald Ross Watson; ***Iron Deficiency and Overload: From Basic Biology to Clinical Medicine*** edited by Dr. Shlomo Yehuda and Dr. David Mostofsky; ***Nutrition Guide for Physicians*** edited by Dr. Edward Wilson, Dr. George A. Bray, Dr. Norman Temple, and Dr. Mary Struble; ***Nutrition and Metabolism*** edited by Dr. Christos Mantzoros; and ***Fluid and Electrolytes in Pediatrics: A Comprehensive Handbook*** edited by Leonard Feld and Dr. Frederick Kaskel. Recent volumes include ***Handbook of Drug-Nutrient Interactions*** edited by Dr. Joseph Boullata and Dr. Vincent Armenti; ***Probiotics in Pediatric Medicine*** edited by Dr. Sonia Michail and Dr. Philip Sherman; ***Handbook of Nutrition and Pregnancy*** edited by Dr. Carol Lammi-Keefe, Dr. Sarah Couch, and Dr. Elliot Philipson; ***Nutrition and Rheumatic Disease*** edited by Dr. Laura Coleman; ***Nutrition and Kidney Disease*** edited by Dr. Laura Byham-Grey, Dr. Jerrilynn Burrowes, and Dr. Glenn Chertow; ***Nutrition and Health in Developing Countries*** edited by Dr. Richard Semba and Dr. Martin Bloem; ***Calcium in Human Health*** edited by Dr. Robert Heaney and Dr. Connie Weaver; and ***Nutrition and Bone Health*** edited by Dr. Michael Holick and Dr. Bess Dawson-Hughes.

Dr. Bendich is President of Consultants in Consumer Healthcare LLC and is the Editor of ten books including ***Preventive Nutrition: The Comprehensive Guide for Health Professionals*****, Fifth Edition**, coedited with Dr. Richard Deckelbaum (www.springer.com/series/7659). Dr. Bendich serves on the Editorial Boards of the *Journal of Nutrition in Gerontology and Geriatrics* and *Antioxidants* and has served as Associate Editor for *Nutrition*, the International Journal, served on the Editorial Board of the *Journal of Women's*

Health & Gender-Based Medicine, and served on the Board of Directors of the American College of Nutrition.

Dr. Bendich was Director of Medical Affairs at GlaxoSmithKline (GSK) Consumer Healthcare and provided medical leadership for many well-known brands including TUMS and Os-Cal. Dr. Bendich had primary responsibility for GSK's support for the Women's Health Initiative (WHI) intervention study. Prior to joining GSK, Dr. Bendich was at Roche Vitamins Inc. and was involved with the groundbreaking clinical studies showing that folic acid-containing multivitamins significantly reduced major classes of birth defects. Dr. Bendich has coauthored over 100 major clinical research studies in the area of preventive nutrition. She is recognized as a leading authority on antioxidants, nutrition and immunity, and pregnancy outcomes, vitamin safety, and the cost-effectiveness of vitamin/mineral supplementation.

Dr. Bendich received the Roche Research Award, is a *Tribute to Women and Industry* Awardee, and was a recipient of the Burroughs Wellcome Visiting Professorship in Basic Medical Sciences. Dr. Bendich was given the Council for Responsible Nutrition (CRN) Apple Award in recognition of her many contributions to the scientific understanding of dietary supplements. In 2012, she was recognized for her contributions to the field of clinical nutrition by the American Society for Nutrition and was elected a Fellow of ASN (FASN). Dr. Bendich served as an Adjunct Professor at Rutgers University. She is listed in Who's Who in American Women.

Connie W. Bales is a Professor of Medicine in the Division of Geriatrics, Department of Medicine, at the Duke University School of Medicine and Senior Fellow in the Center for the Study of Aging and Human Development at Duke University Medical Center. She is also Associate Director for Education/Evaluation of the Geriatrics Research, Education, and Clinical Center at the Durham VA Medical Center. Dr. Bales is a well-recognized expert in the field of nutrition, chronic disease, function, and aging. Over the past two decades, her laboratory at Duke has explored many different aspects of diet and activity as determinants of health during the latter half of the adult life course. Her current research focuses primarily on enhanced protein as a means of benefiting muscle quality, function, and other health indicators during geriatric obesity reduction and for improving perioperative outcomes in older patients. Dr. Bales has served on NIH and USDA grant review panels and is Past-Chair of the Medical Nutrition Council of the American Society for Nutrition. She has edited three editions of the *Handbook of Clinical Nutrition and Aging*, is Editor-in-Chief of the *Journal of Nutrition in Gerontology and Geriatrics*, and is a Deputy Editor of *Current Developments in Nutrition.*

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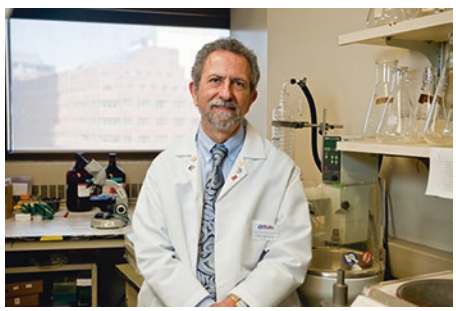
Peter Weber, MD, PhD Professor of Nutrition, University of Stuttgart-Hohenheim, Stuttgart, Germany

Peter Weber received his PhD in Nutritional Sciences from the University of Bonn, Germany, and his MD from the University of Münster, Germany. After working for 2 years at the "Research Institute of Child Nutrition," Dortmund, Germany, he trained in Internal Medicine with a subspecialty in endocrinology at the University of Mainz, Germany, obtaining 10 years of experience in patient care and clinical research. He is a Professor of Nutrition at the University of Hohenheim, Stuttgart, Germany, and gives lectures in Human Nutrition and Health. He has more than 70 peer-reviewed publications in the field of iodine deficiency and goiter, thyroid diseases, metabolic syndrome, postprandial lipid metabolism, vitamin K, vitamin status of populations, and the role of vitamins and polyunsaturated fatty acids in human health, and he is a Coeditor of several books on micronutrients and health. His scientific interests include the role of micronutrients in the prevention of chronic diseases, nutritional status in risk groups such as elderly, and the emerging topic of nutrition security. After a successful career in various R&D functions at Hoffmann-La Roche in New Jersey, USA, and as Corporate Scientist for Human Nutrition & Health in DSM Nutritional Products in Kaiseraugst, Switzerland, he retired from his industrial appointment in August 2017.

Marc Birringer, PhD Professor for Applied Nutritional and Environmental Biochemistry at the Department of Nutritional, Food and Consumer Sciences of Fulda University of Applied Sciences, Germany

Dr. Marc Birringer started his academic career at the University of Siegen (Germany), where he received his PhD in Chemistry. In 1998, he studied the anticancer activity of seleno-amino acids with Eric Block at the University of Albany (NY/USA). In 1999, he joined the lab of Regina Brigelius-Flohé at the German Institute of Human Nutrition where he investigated human vitamin E metabolism and anticancer properties of newly synthesized tocopheryl derivatives. In 2002, Dr. Birringer co-founded *peptides&elephants* GmbH, where he was Head of the Research and Development Unit and responsible for the conception of a high-throughput synthesizer for peptide libraries and the development of tissue-specific drug-peptide conjugates. From 2005 to 2010, he worked in the laboratory of Michael Ristow at the University of Jena on diet-induced mitochondrial activation and aging. During that time, he received his *habilitation* in human nutrition.

In 2011, Marc Birringer was appointed Full Professor for Applied Nutritional and Environmental Biochemistry in the Department of Nutritional, Food and Consumer Sciences of Fulda University of Applied Sciences. His current research is focused on the metabolism of dietary lipophilic micronutrients, such as vitamin E and coenzyme Q10. Dr. Marc Birringer is President of the Gesellschaft für Angewandte Vitaminforschung eV, member of the Editorial Board of *NFS Journal*, and author and coauthor of more than 80 scientific articles and reviews.



Jeffrey B. Blumberg, PhD, is a Professor in the Friedman School of Nutrition Science and Policy and also serves as the Senior Scientist in the Antioxidants Research Laboratory at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University. His research is focused on the biochemical basis for the role of antioxidant nutrients and their dietary requirements in promoting health and preventing disease during the aging process via changes in the status of oxidative stress, glucoregulation, and inflammation. He has published more than 400 scientific articles and serves on the editorial boards of several scientific journals. Dr. Blumberg was included in the 2015 Thomson Reuters' top 1% of cited researchers in his field. He has been the recipient of the ASN Mary Swartz Rose Senior Investigator Award for outstanding research

on the safety and efficacy of bioactive compounds for human health. Dr. Blumberg also participates in activities relevant to the incorporation of sound nutrition science into public health policy and has served as a member of the Workshop on Health Promotion and Aging in the Office of the US Surgeon General, Sports Medicine Committee of the US Olympic Committee, Consultation on Preparation and Use of Food-Based Dietary Guidelines for the WHO/FAO, Food Advisory Committee of the FDA, and other committees.

Manfred Eggersdorfer, PhD Professor for Healthy Ageing, University Medical Center, Groningen, Netherlands

Dr. Manfred Eggersdorfer studied chemistry at the Technical University of Munich, Germany, and did his PhD in Organic Chemistry on the synthesis and characterization of unusual amino acids. He undertook postdoctoral work at Stanford University, California, working with Carl Djerassi on the isolation and characterization of sterols from marine origin.

Dr. Manfred Eggersdorfer worked for BASF in various positions, including Head of R&D Fine Chemicals, joined Roche as Head of Research Vitamins, and continued in this position after the acquisition by DSM. In the last years, he was responsible for Nutrition Science & Advocacy.

Dr. Manfred Eggersdorfer is member of the Advisory Board of Johns Hopkins Bloomberg School of Public Health, Baltimore, a board member of the Gesellschaft für Angewandte Vitaminforschung eV, and member of the Tufts Nutrition Council, Boston. He is an Honorary Member of the Oxygen Club of California and author of numerous publications, reviews, and book chapters in the field of vitamins, carotenoids, and innovation in nutritional ingredients. He is a reviewer for several scientific journals as well as Associate Editor of the *International Journal for Vitamin and Nutrition Research*.

Jan Frank, PhD Professor of Food Biofunctionality, Institute of Nutritional Sciences, University of Hohenheim, Stuttgart, Germany

Professor Frank graduated with a Diploma in Nutrition from Bonn University (2000), obtained a PhD in Food Science at the Swedish University of Agricultural Sciences (2004), and received postdoctoral training at the Universities of Kiel and Hohenheim. He was a Visiting Scientist at the Linus Pauling Institute (USA), the University of Reading (UK), and the University of Surrey (UK).

Dr. Frank was appointed Professor of Human Metabolomics at the Institute of Nutrition and Food Science at the University of Bonn in 2012 and, in 2013, Full Professor and Head of the Division of Food Biofunctionality at the Institute of Nutritional Sciences at the University of Hohenheim.

He is the Founding President of the Society of Nutrition and Food Science (www.snfs.org), member of the board of directors of the Society for Applied Vitamin Research (Gesellschaft für Angewandte Vitaminforschung eV), and Editor-in-Chief of *NFS Journal*, Associate Editor of *Nutrition*, and member of the editorial boards of *The Journal of Nutritional Biochemistry*, *BioFactors*, and *Plant Foods for Human Nutrition*.

His research interests lie in factors that determine the absorption, metabolism, and elimination of phytochemicals and different vitamin E congeners and, in particular, long-chain vitamin E metabolites. Dr. Frank and his team examine novel strategies to overcome the low intrinsic oral bioavailability of phytochemicals and investigate their biological activities.

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Chapter 1
Introduction: Why (Another) Book on Vitamin E?

Peter Weber, Marc Birringer, Jeffrey B. Blumberg, Manfred Eggersdorfer, and Jan Frank

Vitamin E is a well-described, fat-soluble essential micronutrient and, as such, must be provided to the human body on a regular basis to prevent deficiency symptoms and maintain a healthy status. As vitamin E was discovered close to a century ago, there is a wealth of data about this vitamin in the public domain, reported in original research articles and reviews as well as in many books addressing its mechanisms of action and preclinical investigations as well as its benefits and safety profiles in human studies. So, one may ask: Why another book on vitamin E?

Despite the huge body of evidence about vitamin E accumulated over time, many issues remain unsettled, and inconsistent study results always pose new questions. However, the scientific progress we have experienced over the last decade has provided new insights into the mode(s) of action of vitamin E and the biological roles of individual tocopherol and tocotrienol congeners that shed new light on the roles of vitamin E in human health. Nonetheless, as with other areas of nutrition research, there are wide-ranging impressions by the media that often contrast with our scientific knowledge. This situation has generated a misleading perception about vitamin E that questions its importance for human health. This is concerning for an essential nutrient which the human body requires for proper function and therefore needs to be ingested regularly in adequate amounts. In addition, the recent gain in our knowledge from the emerging evidence of putative clinical benefits, in health promotion and disease prevention, especially in the context of personalized nutrition, suggests an importance of vitamin E that extends beyond its function as an essential antioxidant micronutrient. So, there are at least

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_1

two good reasons to publish a new book on vitamin E: first, we are excited about the recent scientific "Renaissance" about this vitamin; and, second, in a world where malnutrition and "hidden hunger" remain a critical challenge to public health, continuing attention to the intake and status of micronutrients, including vitamin E, is required not only within the scientific community but also among healthcare providers and policymakers.

Given the broad application and relevance of vitamin E to human health, this book should interest the nutrition and medical community, healthcare professionals, public health decisionmakers, as well as students and the educated lay person. We have organized this book into five sections: (i) biochemistry, metabolism, and molecular actions of vitamin E, (ii) a global perspective on vitamin E intake and status, (iii) safety of vitamin E and its interactions with other nutrients and drugs, (iv) benefits of vitamin E on human health and disease, and (v) public health implications of vitamin E.

The section on "Biochemistry, metabolism, and molecular effects" of vitamin E summarizes pertinent information on the established biological properties of vitamin E and also includes new insights from the fields of transcriptomics, metabolomics, and proteomics, including emerging data on bioactivities of minor vitamin E derivatives. Vitamin E is the key lipid chain-breaking antioxidant in the human body and plays a fundamental but complex role in protecting polyunsaturated fatty acids (PUFA) from oxidation. Especially in light of recent recommendations to increase PUFA intake, the many interactions between vitamin E and PUFA are reviewed. Indeed, the chapter "A global view on vitamin E intakes" discusses the current dietary recommendations from the Institute of Medicine (IOM) [1], European Food Safety Agency (EFSA) [2], and the Nordic Nutrition Recommendations (NNR) [3]; all stress the importance of vitamin E in the context of PUFA intake. However, it is noteworthy that the evidence these organizations use to set Recommended Dietary Allowances (RDA) for vitamin E are based on other considerations. We therefore revisit the evidence that intake levels higher than the current RDA of vitamin E are required to protect PUFA from oxidation at usual and recommended intakes of PUFA. The NNR, EFSA, and IOM report an RDA for vitamin E in adults ranging from 8 to 15 mg per day. Nonetheless, a global overview on vitamin E intakes and serum concentrations reveals that in many countries, including affluent and industrialized nations, vitamin E status and intakes are still below current recommendations. Paying more attention to the stability of vitamin E in food processing as well as during storage may help improve this situation as there are significant losses of vitamin E in food items even before reaching the consumer. In the section on "Safety of vitamin E," we present an updated meta-analysis including 68 studies comprising more than 120,000 participants which clearly reveals no evidence of an increased all-cause mortality as suggested by earlier publications. As the number of adults over 60 years of age is increasing in most countries to levels never before experienced, so too is the regular consumption of dietary supplements and drugs. Hence, two chapters are included which review the known interactions between vitamin E and drugs as well as between vitamin E and other nutrients.

Vitamin E has been implicated in the prevention of many noncommunicable diseases (NCD), as discussed in the section "Benefits of vitamin E on human health and disease." As a lipid-soluble antioxidant, vitamin E was first suggested to reduce the risk of cardiovascular diseases (CVD), which was indeed observed in epidemiological studies. These results, however, could not be confirmed in most of the ensuing randomized clinical trials. Various reasons were proffered to explain those discrepant findings, as it appeared at the time. However, the epidemiological findings and the clinical data remained controversial until researchers began to examine variables such as genotype and risk factors like diabetes mellitus and other conditions associated with metabolic oxidative stress. For example, in one randomized clinical trial in diabetics carrying the haptoglobin (Hp) 2–2 genotype (a mutant of Hp 1–1), a significant reduction of composite CVD outcomes was reported [4], and those results were strengthened by retrospective analyses of clinical studies reporting a null effect when not considering the Hp 2–2 variable [5, 6]. Haptoglobin acts as an antioxidant, as it binds hemoglobin, and polymorphisms in the Hp gene correlate strongly with a greater risk for CVD in diabetics. Further, vitamin E

was shown to be critical for an adequate immune responses among older adults; a randomized clinical trial demonstrated a significant reduction of upper respiratory infections in those supplemented with vitamin E as compared to the placebo group. In addition, emerging evidence suggests that vitamin E may be beneficial in patients with asthma and in people living in regions with high air pollution. Nonalcoholic fatty liver disease (NAFLD) is a health issue of rising importance associated with the epidemic of overweight and obesity. There is currently no drug therapy approved for NAFLD. However, randomized clinical trials showing that vitamin E improves histological parameters in patients with nonalcoholic steatohepatitis (NASH), an advanced form of NAFLD with an inflammatory component, have led some medical associations in the USA [7] and in Europe [8], to establish guidelines suggesting vitamin E be considered as a treatment option. The results from clinical intervention studies suggest a role of vitamin E in clinical practice at doses between 200 and 800 mg per day which is a multiple of the current RDA and which is considered safe.

Vitamin E has established clinical benefits in combination with other antioxidants for age-related macular degeneration (AMD). The supplement formulation employed in the Age-Related Eye Disease 2 trial that contained vitamin E (400 IU/d), vitamin C (500 mg/d), lutein (10 mg/d), zeaxanthin (2 mg/d), zinc oxide (80 mg/d), and cupric oxide (2 mg/d) is now recommended by the American Academy of Ophthalmology (AAO) [9] for patients at particular stages of AMD. This formula was investigated in a large study with more than 4000 participants at risk to develop advanced stages of AMD. Compared to placebo, the active group had a 25% risk reduction to develop AMD. While there is no chapter on the role of vitamin E in diseases of the eye, we encourage readers interested in this topic to explore elsewhere. Other clinical fields, for which there is encouraging evidence emerging that vitamin E could be of benefit, include cognitive function, pregnancy, and cancer, topics which are discussed in detail within this book.

The last section on "Public health implications of vitamin E" presents important information and challenging questions. Applying established models for healthcare costs, the available clinical data on vitamin E treatment in diabetics suffering from CVD and carrying the Hp 2–2 genotype are examined and the treatment options considered. The progress in understanding the interplay of the genotype and nutrition will not only provide opportunities in the field of precision medicine but may also contribute to a better explanation of many individual variations in response to vitamin E intake or supplementation [10] which may be one reason for inconsistencies in data in this field. As the knowledge in this emerging field of research will increase, it may pave the way for a realistic concept of personalized nutrition. Consumer knowledge about vitamin E is also reviewed. Given the wealth of emerging science and the potential benefits for public health that have been put forward for vitamin E and other micronutrients, it is of concern that availability of resources for this research field has declined [11]. The final chapter is devoted to considerations about the future funding of research that will be required to explore the health benefits of vitamin E further and to apply the biological power of nutrition for the improvement of public health.

We hope that this book reemphasizes and updates the role of vitamin E as an essential micronutrient for human health, the risk of inadequate intakes of vitamin E contributing to the pathogenesis of a variety of NCD, and the opportunity for dietary and supplemental vitamin E to positively impact clinical conditions in a cost-effective fashion. We feel this is the time to put forward the case for vitamin E into an up-to-date, science-based manner that is applicable to today's world and offers pragmatic solutions for its safe and effective use to benefit the public as well as specific patients. The evidence in this book supports a dose-dependent *dual role* of vitamin E in its:

- *Essentiality* for human health
- *Clinical benefits* for people at risk of selected noncommunicable diseases

We also hope this book will contribute to facilitating a forward-looking discussion about the role of vitamin E and stimulate further resources to support new investigations to its benefits to human health.

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Part I
Biochemistry, Metabolism and Molecular Effects of Vitamin E

Chapter 2
History of Vitamin E Research

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Keywords Vitamin E · Discovery · History · Research

Discovery of Vitamin E as Reproductive Factor in Rats (1920s)

Vitamin E research made significant progress, and a wealth of data is published about this essential micronutrient; however, there remains much to be learned about its fundamental metabolic functions, optimal daily intakes, and overall impact across the life cycle on public health. In their search of dietary factors important to the healthy growth of animals, Katherine J. Scott Bishop and Herbert M. Evans observed in 1922 that besides the already known vitamins A, B, C, and D, a new factor X appeared responsible for the successful reproduction of rats (Fig. 2.1) [1]. They found factor X in butterfat, lettuce, and different plant-based oils such as wheat germ and cottonseed oil. They further observed that the quotient of factor X and the amount of lard within the diet was critical for the production of littermates. Based on that observation, it became clear that the grade of rancidity of lard corresponded to the destruction of the fertility factor. In the same year, Charles Moureu and Charles Dufraisse found that phenolic compounds could prevent the oxidation of acrolein and developed the concept of auto-oxidation and the function of antioxidants [2]. Shortly after the initial investigations by Evans and Bishop, it became clear that factor X was an antioxidant preventing the (auto-)oxidation

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_2

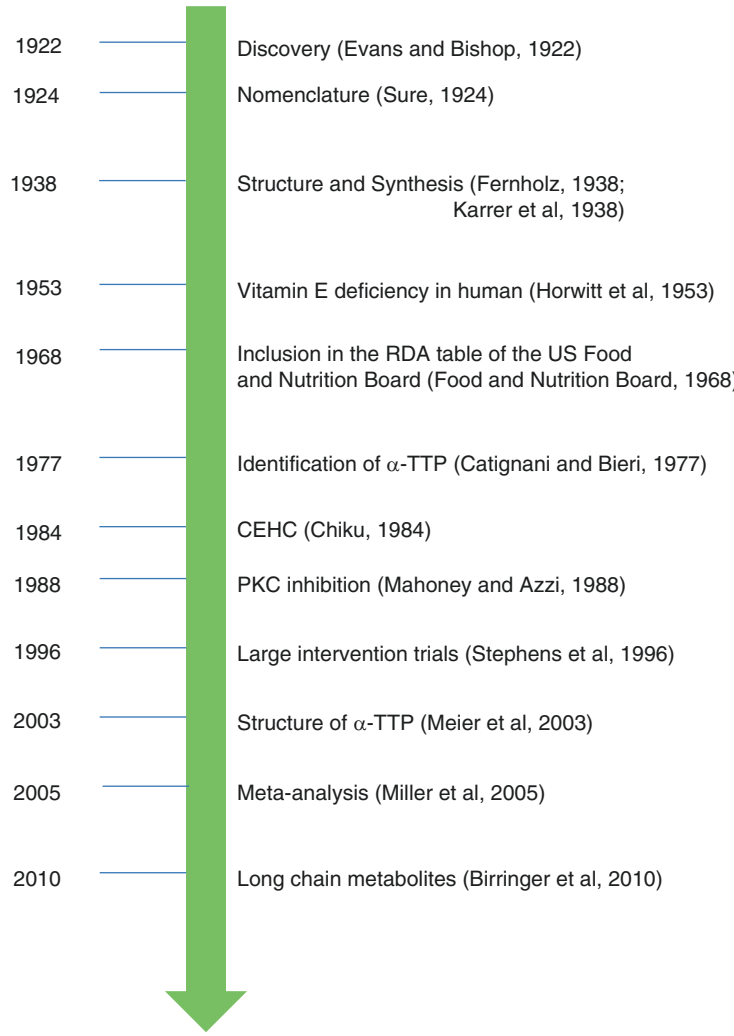


Fig. 2.1 Milestones of vitamin E research. *RDA* Recommended Dietary Allowance, *α-TTP* α-tocopherol transfer protein, *PKC* protein kinase C, *CEHC* carboxyethylhydroxychromane

of lard [3, 4]. However, rancidity was not responsible for the resorption of the fetus since the acetylation product of factor X (vitamin E acetate) showed full bioactivity in the presence of a rancid diet [5–7]. Conversely, the chemical oxidation of vitamin E or methylation of the phenolic hydroxyl group leads to inactivity in fertility assays. Even today, important questions about vitamin E and auto-oxidation are still an issue for debate. Whether the only task of vitamin E is the protection of other molecules against oxidation or if it should be protected against oxidation to fulfill its vitamin activity (i.e., anti sterility properties) is unanswered yet.[1] The early days of vitamin E research have been concisely summarized by George Wolf [8].

Evans et al. described the chemical and biological activities of factor X and named the active compound α-tocopherol composed from the Greek words “tokos” (childbirth) and “phero” (to bear) [9]. The name was suggested by George M. Calhoun from the University of California. The yellow-brownish oil showed an absorption maximum at 294 nm and was able to stop chain reactions among

[1] Olcott and Mattill suggested the following interpretation of the experimental data: “…has thus far prevented an understanding of the function of vitamin E whether it is itself an antioxidant or whether its presence in food is dependent upon the presence of antioxygenic substances which protect it against oxidation” [4].

lipids induced by free radicals. α-Tocopherol thus became the most important lipid "chain-breaking" antioxidant used in chemical, biochemical, and industrial applications.

Shortly after the structural elucidation of α-tocopherol as a 6-hydroxychromane in 1938 by Erhard Fernholz, the Nobel laureate Paul Karrer and Otto Isler presented the first chemical synthesis of *d*,*l* α-tocopherol realized by the condensation of phytol and the corresponding 1,4-hydroquinone [10, 11].[2] In addition to α-tocopherol, β- and γ-tocopherol were described as structurally related compounds albeit with reduced antioxidant vitamin E activity [12].

Vitamin E Deficiency Symptoms in Animals (1930–1950)

In 1934, HS Olcott and Henry A. Mattill stated that "…experiments with vitamin E without adequate biological assays are valueless" [5]. Besides infertility, several avitaminoses were observed in different animal models. Depending on the vitamin E content of the diet, muscular dystrophy was most prominently found in vitamin E-deficient rats, guinea pigs, and rabbits [13]. From a biochemical point of view, most animal models revealed decreased muscle creatine, creatinuria, reduced ATP, and diminished cholinesterase activity. Interestingly, the oxygen consumption of muscle tissue was increased in animals fed with a vitamin E-deficient diet. Today, and with hindsight of recent publications by Maret Traber and others on vitamin E-deficient zebrafish embryos (*Danio rerio*), these early findings can be explained by the fact that vitamin E-deficient animals can suffer from a glucose deficit [14, 15]. This unexpected condition is caused by a glucose shunt to produce NADPH via pentose phosphate pathway. Instead of vitamin E, NADPH is oxidized due to increased oxidation of unsaturated fatty acids, most notably the n-3 docosahexaenoic acid. As a consequence, the limited glucose supply causes starving via reduced ATP production, most critically of neurons in the brain. A switch of energy metabolism toward the production of ketone bodies via deamination of amino acids or beta-oxidation of fatty acids also leads to an increased oxygen consumption.

In 1949, the *Annals of the New York Academy of Science* dedicated an entire issue to vitamin E [16]. Neurological and muscular lesions were described in vitamin E-deficient animals, and the first measurements of plasma tocopherol were presented together with clinical implications such as preeclampsia and elevated blood pressure [17–21]. The search for a role of tocopherols and their esters (tocopherol phosphate) as cofactors or enzyme inhibitors then became of central interest [22, 23]. These important topics and their implications for the promotion of health and reduction in the risk of chronic diseases remain active areas of vitamin E research today.

Vitamin E Deficiency in Humans (1950–1960)

Vitamin E deficiency in animals was well described by the early 1950s, but this condition had not yet been observed in humans. The Elgin Project, co-funded by the National Research Council, aimed to investigate the effect of vitamin E deprivation in man. Between 1953 and 1967, Max K. Horwitt and his coworkers composed basal diets providing ~2 mg tocopherol daily. The effect of a limited tocopherol intake on erythrocyte hemolysis over a period of more than 6 years was examined. Surprisingly, clinical and physiological parameters were found to remain within normal ranges. However, low tocopherol plasma concentrations were associated with an elevated peroxide-induced erythrocyte hemolysis and a reduced half-life of erythrocytes [24]. Nonetheless, no sign of anemia was observed in the study subjects. These results led to two major conclusions. First, dietary deprivation of vitamin E to achieve a true deficiency status in adults is difficult, likely because liver and adipose tissues

[2] Isler synthesized *d*,*l* α-tocopherol in the laboratories of Hoffmann-La Roche and showed its biological activity.

serve as a tocopherol depot which maintains a minimum of plasma tocopherol over a long period. Second, the recycling and trafficking of tocopherol (by the tocopherol transfer protein, see Chap. 9) is quite effective in conserving vitamin E in humans. It is also worth noting that during this period creating a tocopherol-free diet was a difficult challenge. In conclusion, the Elgin Project estimated an intake level of 12 mg α-tocopherol per day to prevent cells from hydroperoxide-induced hemolysis and to maintain α-tocopherol serum levels at 12 μmol/L. This experiment contributed greatly to the definition of the Estimated Average Requirements (EAR) and calculation of the Recommended Daily Allowance (RDA) for vitamin E in the United States. The US Institute of Medicine (IOM) defined a status serum α-tocopherol concentration < 12 μmol/L as deficient (for more details see Chap. 11) [25]. In the 1960s, Binder and Spiro described several malabsorption conditions associated with steatorrhea that resulted in vitamin E deficiency in man, including patients with sprue, cystic fibrosis, chronic pancreatitis, intestinal lymphangiectasia, and abetalipoproteinemia [26, 27].

Interplay of Vitamins E and C and Radical Scavenging Chemistry (1970–1980)

The work of L. Michaelis and SH Wollman in the 1940s served to characterize the semiquinone radical of tocopherol and the capability of tocopherols to scavenge free radicals in vitro [28]. However the knowledge of the complex and dynamic interplay of pro- and antioxidants in living systems was in its infancy during this period. By the 1960s, Al L. Tappel and his colleagues were investigating the antioxidative activity of vitamin E in membranes of mitochondria, microsomes, and artificial liposomes [29]. They found increased amounts of oxidized lipids (thiobarbituric acid-reactive compounds or TBARs) in liver cells from vitamin E-deficient rats [30]. They were the first to suggest the coupling of vitamin E recycling to other biological redox systems [31]. Lester Packer and his coworkers investigated the interplay of vitamin E and C after pulse radiolysis using 4 MeV electrons from a linear accelerator and demonstrated a rapid, first-order transfer of the tocopheryl radical to ascorbate in an aerated aquatic solution [32]. Later, the ascorbyl radical was found to be reduced back to vitamin C by NADH-dependent semidehydroascorbate reductase or NADPH-dependent thioredoxin reductase [33, 34]. Nonetheless, the biological relevance of this in vitro model was questioned since vitamin C is water-soluble and resides in the cytosol, whereas vitamin E remains in the lipid-soluble membranes. Then, in the 1980s, GW Burton and KU Ingold showed that vitamins E and C worked as a redox pair when multilamellar phospholipid liposomes were tested in a model of biomembranes [35, 36]. The free radical scavenging chemistry of vitamin E in membranes was further developed by Etsuo Niki and his colleagues by investigating the rate constants of the tocopheryl radical with different reductants and their biological abundance and carefully considering the compartmentalization of the tocopherol molecule within biological membranes [37]. In 1982, Burton et al. showed that vitamin E was the only lipid-soluble antioxidant in blood plasma capable of scavenging generated radical ex vivo (for more details see Chap. 3) [38]. Stimulated by these provocative biochemical results plus observational studies suggesting an inverse association of vitamin E intake/status and some chronic diseases, randomized clinical trials using vitamin E supplements were planed and conducted during the 1990s.

Discovery of α-Tocopherol Transfer Protein and AVED (1980s)

In 1977, John Bieri and George L. Catignani discovered a rat liver protein that selectively bound α-tocopherol, determined its molecular mass at 32 kDa, and proved its selectivity by binding assays with several structural-related molecules [39]. The proteins were eventually named α-tocopherol

transfer protein (α-TTP) after its active transfer of α-tocopherol from phosphatidylcholine liposomes to liver microsomes and mitochondria, respectively [40]. α-TTP was determined in human liver, and mutations of the protein were connected to a rare autosomal recessive neurodegenerative disease with clinical phenotypes that resemble Friedreich's ataxia [41, 42]. This disease was associated with very low plasma vitamin E and characterized by Ben Hamida et al. as ataxia with isolated vitamin E deficiency (AVED) [43].

Mainly expressed in the liver, α-TTP facilitates the active incorporation of α-tocopherol into very low density lipoprotein (VLDL) and subsequent transport to peripheral tissues. Receptor-mediated uptake of lipoprotein-bound α-tocopherol into the liver restarts the recycling process and helps to maintain stable α-tocopherol plasma concentrations [44]. α-TTP also maintains the transport of α-tocopherol from endosomal membranes to the plasma membrane of hepatocytes to help regulate vitamin E homeostasis (see Chap. 9).

Eventually, several other tocopherol-binding proteins were discovered although only limited information is available about them today. A 15 kDa protein with tocopherol-binding activity and a 75 kDa phospholipid transfer protein that facilitates the exchange of tocopherol between HDL and LDL have been described [45, 46]. In the 1990s, Zimmer et al. identified a 46 kDa tocopherol-associated protein (TAP) that appears to be involved in the intracellular transport of tocopherols between membranes [47, 48]. More recently, the expression of TAP has been associated with clinical outcomes in breast cancer patients [49].

Vitamin E Metabolism/Excretion and Biomarkers (~1984)

Vitamin E homeostasis is controlled by biliary transport and via enterohepatic circulation of tocopherols as well as by degradation of tocopherols (and tocotrienols) followed by urinary excretion. Activation of the side chain of tocopherols leads to carboxy-derivatives that undergo β-oxidation, similar to fatty acids (see Chap. 4). In the 1980s, Chiku et al. investigated the fate of deuterium-labeled δ-tocopherol in rats and found a water-soluble short-chain metabolite, δ-carboxy-ethyl-hydroxychromanol (δ-CEHC), excreted in the urine [50]. Later, α- and γ-CEHCs were found in rat and human urine. CEHCs may be indicators of an adequate vitamin E status, only being excreted at a certain threshold level of hepatic tocopherols [51, 52]. Interestingly, γ-CEHC has been found to be a natriuretic factor [53].

Besides short-chain metabolites, long-chain metabolites (LCMs) of tocopherols exhibit high biological activity and have been discussed as active metabolites of vitamin E (see Chap. 6) [54].

Non-antioxidative Actions of Vitamin E (~1988)

Even the early investigators stressed the question of whether the antioxidant properties of vitamin E would fully explain its functions in living organisms as well as its essentiality. The search for non-antioxidative functions of vitamin E such as actions as a cofactor or a prosthetic group for enzymes or a putative role as hormone or a transcription factor intensified in the late 1980s. The first evidence of a non-antioxidative function of α-tocopherol was carried out by Angelo Azzi and his colleagues in 1988 [55]. They investigated the inhibitory activity of α- and β-tocopherols toward protein kinase C activity and found a more pronounced inhibition by α-tocopherol despite the fact that β-tocopherol has the same or even greater antioxidant activity. Based on this observation, numerous specific non-antioxidative effects of *RRR*-α-tocopherol have been described, including the inhibition of platelet adhesion and aggregation, inhibition of 5-lipoxygenase, activation of protein phosphatase 2 (PP2 or PP2A) and diacylglycerol kinase, and modulation of α-TTP expression [56].

Randomized Clinical Trials (1990–2005)

Many age-associated diseases are accompanied by an increase in oxidative stress status. Whether this stress is the cause or the result of one or more of these disorders remains elusive. Several observational studies have correlated the incidence of cardiovascular diseases, cancer, and type 2 diabetes mellitus with reduced plasma vitamin E status. Since vitamin E is the most prominent lipid-soluble antioxidant and is able to reduce inflammatory markers in vitro, large-scale, during the 1990s and early 2000s, randomized intervention trials with vitamin E supplements were planned and conducted. However, these trials have often yielded equivocal or contradictory results on the efficacy of vitamin E supplementation. Mentioned here briefly are some of the more noteworthy clinical trials on vitamin E and risk for cardiovascular disease or cancer. The Cambridge Heart Antioxidant Study (CHAOS) with 2002 patients given 400 IU/d or 800 IU/d significantly reduced the risk of nonfatal myocardial infarction [57]. Later, the Gruppo Italiano per la Sperimentazione della Streptochinasi nell'Infarto Miocardico trial (GISSI) with 2830 patients reported no effect of 300 mg vitamin E on a primary combined endpoint of death, nonfatal myocardial infarction, and stroke [58]. In 1996, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC Study) investigated the effects of 50 mg vitamin E in heavy smokers and found an increase in lung cancer but a reduced risk of and mortality from prostate cancer [59]. The Heart Outcome Prevention Evaluation (HOPE) study aimed to modify the risk for fatal and nonfatal myocardial infarct with 400 IU/d vitamin E in 4761 patients but obtained a null result [60]. In contrast, the Cardiovascular Disease in End-Stage Renal Disease (SPACE) trial on 196 hemodialysis patients receiving 800 IU/d vitamin E showed a significant reduction on a composite primary endpoint [61].

The promising secondary results of the ATBC Study on prostate cancer prompted the design of the Selenium and Vitamin E Cancer Prevention Trial (SELECT) in 35,533 healthy men [62]. Subjects were accrued and randomized to four study arms: placebo, 400 IU/d *all-rac*-α-tocopheryl acetate, 200 μg/d *L*-selenomethionine, and the combination of vitamin E and selenium supplements. As no evidence of benefit from either supplement was demonstrated in the early years of the study, the intervention was discontinued in 2008 [63]. A final data analysis revealed that dietary supplementation with vitamin E increased the risk of prostate cancer [64].

In contrast to the early optimistic hopes for vitamin E supplementation in reducing the risk for major chronic diseases, most of the clinical trials conducted to date have failed to support the promising data from many large observational studies. Nonetheless, a discordance between data from observational studies and outcomes from clinical trials always provides an opportunity to examine and understand the underlying basis for the varying results from these two research approaches.

Markedly exacerbating the diminishing enthusiasm for vitamin E research resulting from these studies was a systematic review and meta-analysis of clinical trials published in 2005 by Miller et al. along with several subsequent meta-analyses revealing an increase in cancer risk and/or total (all cause) morality from vitamin E supplementation. These meta-analyses have been extensively reviewed [65, 66]. Further details on these meta-analyses are found in Chap. 16.

Promising results were found in a randomized clinical trial of a vitamin E intervention among 561 patients with Alzheimer's disease. The patients receiving α-tocopherol at 2000 IU/d showed a reduced functional decline compared to the placebo group [67]. Similarly, significant benefits of vitamin E supplementation in clinical trials have been reported in the treatment of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis of nondiabetic patients [68, 69]. Milman et al. reported significantly reduced myocardial infarction, stroke, and cardiovascular death in type 2 diabetes patients with a haptoglobin 2–2 phenotype following vitamin E supplementation at 400 IU/d [70]; this result suggests that clinical trials of vitamin E and cardiovascular disease that did not account for genotype may have obtained a null result due to low statistical power resulting from inclusion of nonresponders (see Chap. 20).

The original hypothesis that inhibiting oxidative stress status with α-tocopherol, a single lipid-soluble antioxidant, for a few years to impact the complex, multifactorial, and decade-long patho-

physiology of chronic conditions like cancer and cardiovascular disease was undoubtedly too simple minded. However, the more recent increases in our understanding about the actions of vitamin E and its many molecular targets are shedding new light on more sophisticated nutritional approaches incorporating essential micronutrients together with other dietary bioactive components in health promotion and disease prevention.

Gene Regulatory Activity of Tocopherols and Biological Activity of Long-Chain Metabolites (2000–2018)

Following the discovery of the non-antioxidant actions of vitamin E, the search began for differential expressed genes influenced by vitamin E. mRNA expression profiles and corresponding protein concentrations have now been measured in cell cultures and animal studies (see Chap. 7).

The discovery that tocopherols and tocotrienols act as transcriptional factors and/or as ligands of nuclear receptors opened new research avenues in the 2000s. In the 1990s, hundreds of transcription factors were discovered, and tools to quantify their activation in cell systems were developed. Reporter gene assays revealed the interaction of tocopherols and tocotrienols at the transcriptional level. Several genes were found that were activated by vitamin E, including the pregnane X receptor (PXR), the sterol regulatory element-binding protein 2 (SREBP-2), and the peroxisome proliferator-activated receptor gamma (PPAR-γ) [71–74]. In addition, α-tocopherol is specifically bound by TAP and induces nuclear translocation [75].

During this period, gene array chips were developed and became commercially available for the determination of gene expression profiles of nutrients. Roy et al. investigated the role of α-tocopherol and α-tocotrienol on the gene expression profile in the development of fetal rat brain and found >200 genes were differentially expressed [76]. Vitamin E-regulated gene expression profiles have now also been investigated in several cell lines [77–79] as well as in mouse heart and brain, rat testes, and α-TTP-deficient mice [80–83].

The initial step in the catabolism of vitamin E results in the cytochrome P450 (CYP) 4F2- or CYP3A4-mediated formation of the LCMs 13′-hydroxychromanol (13'-OH) and 13′-carboxychromanol (13'-COOH), respectively [84, 85]. Aside from hepatic α-tocopherol metabolism (see Chaps. 4 and 6), emerging evidence now points to additional roles of LCMs. Synthetic α-13'-OH and α-13'-COOH have been obtained in a 2–3-step synthesis from the δ-tocotrienol derivative garcinoic acid (i.e., *trans*-13′-carboxy-δ-tocotrienol) isolated from the African bitternut *Garcinia kola* [86]. The facile synthesis of LCMs now allows intensive study of their biological properties in cell cultures [54, 87, 88]. In addition to the modulation of cellular lipid metabolism, α-LCMs exhibit anti-inflammatory features by inhibiting the lipopolysaccharide (LPS)-induced release of pro-inflammatory mediators and the production of nitric oxide (NO) [87, 88]. Interestingly, the effect of α-LCMs occurs at the transcriptional level as the LPS-induced upregulation of the expression of inducible NO synthase, cyclooxygenase-2, and interleukin-1β is efficiently blocked by α-LCMs. While the plasma concentration of LCMs is in the nanomolar range, they may have distinct biological activities comparable to vitamins A and D [54, 89].

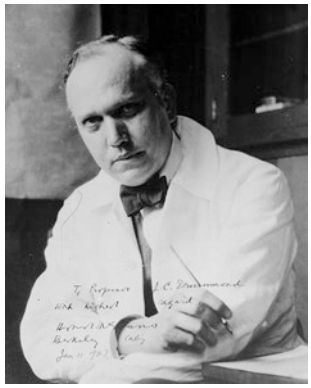
Conclusions and Lessons to Be Learned

Our brief historical overview on vitamin E is undoubtedly biased by our personal selection of the key scientific hallmarks and our own experiences in the field. Nonetheless, it is important that we recognize and value the discoveries from the early years of vitamin E research that laid the foundation for

its chemistry, metabolism, many functions, and essentiality as an established micronutrient. The coincident discovery of vitamin E as a chemical and biological antioxidant and the subsequent development of the free radical hypotheses of aging and disease by Denham Harman and others have led to a vast and robust field of research with clear implications for health promotion, disease prevention, and therapeutic application. Equipped with new molecular and analytical tools, the field of vitamin E research continues to grow and thrive with exciting and promising possibilities following almost a century of pioneering efforts by those who preceded us.

Appendix

Portrait of Herbert M. Evans in laboratory, with signed inscription to J. C. Drummond, 11 January 1927 https://de.wikipedia.org/wiki/Herbert_M._Evans#/media/File:Herbert_McLean_Evans_1927.jpg



Erhard Fernholz. Copy from: https://en.wikipedia.org/wiki/Erhard_Fernholz#/media/File:Erhard_Fernholz.jpg

Paul Karrer. Copy from: https://de.wikipedia.org/wiki/Paul_Karrer#/media/File:Paul_Karrer.jpg

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Chapter 3
Antioxidative Activity of Vitamin E

Afaf Kamal-Eldin

Keywords Vitamin E · Tocopherols · Tocotrienols · Free radicals · Antioxidant · Hydrogen donation

Key Points

- Of the eight vitamin E compounds, α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol, α- and γ-tocopherol are the most important antioxidants in nature.
- In vivo and in food systems, vitamin E protects polyunsaturated fatty acids from free radical attack.
- In vivo, this protection of polyunsaturated fatty acids from oxidation stabilizes cell membranes, where molecular organization plays a very important role for membrane integrity and functioning.
- In biological systems, α-tocopherol functions as part of an antioxidant defense network in concert with vitamin C and other hydrophilic compounds.
- The antioxidant activity of each vitamin E congener is optimum at a certain range of concentrations, above which loss of efficacy or prooxidant effects are observed.
- Recent insights into the antioxidant mechanisms revealed the importance of the microenvironment, the nature of existing lipids and interfacial compounds, temperature, local concentrations, and more, for the free radical scavenging activity of the vitamin.

Introduction

The term "vitamin E" was first used to describe dietary lipid-soluble agent(s) essential to maintain fertility in rats. Later, the name tocopherol was coined from Greek "tokos" (childbirth) and "phorein" (to bring forth) plus the suffix "ol" to highlight the alcoholic nature of this agent(s) [1, 2]. Vitamin E, currently a generic descriptor for at least eight natural tocol derivatives (four tocopherols and four tocotrienols) exhibiting the biological activity of α-tocopherol [3, 4], is based on a chromanol ring and

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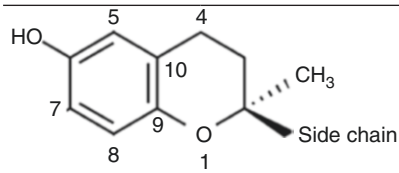
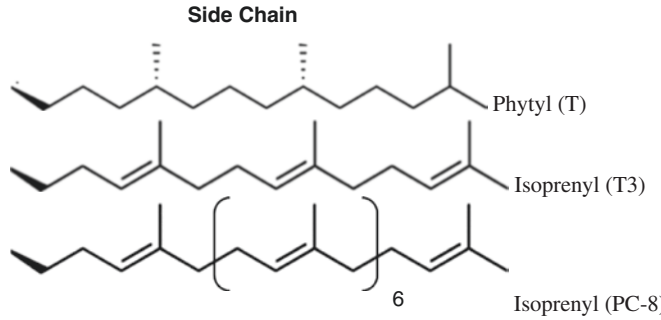
P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_3

Table 3.1 Structures and vitamin E equivalence of tocopherols and tocotrienols



Chromanol structure

Side chain



E-vitamer	*Abbreviation*	*CAS #*	*Structure*		*Vitamin E equivalence*[a]
			Chromanol substitution	*Side chain substitution*	
α-Tocopherol	α-T	59-02-9	5,7,8	Phytyl	1.0
β-Tocopherol	β-T	I6698-35-4	7,8	Phytyl	0.5
γ-Tocopherol	γ-T	54-28-4	5,8	Phytyl	0.1
δ-Tocopherol	δ-T	119-13-1	8	Phytyl	0.03
α-Tocotrienol	α-T3	493-35-6	5,7,8	Isoprenyl	0.3
β-Tocotrienol	β-T3	490-23-3	7,8	Isoprenyl	0.05
γ-Tocotrienol	γ-T3	14101-61-2	5,8	Isoprenyl	Unknown
δ-Tocotrienol	δ-T3	25612-59-3	8	Isoprenyl	Unknown
Plastochromanol-8	PC-8	4382-43-8	5,8	Isoprenyl	Unknown

[a]Determined by the rat fetal resorption assay showing the activity of 1 mg of each of the other vitamers as compared with 1 mg α-tocopherol [5]

a lipophilic side chain linked to the ring at carbon atom 2 (Table 3.1). The side chain is a saturated branched phytyl chain in tocopherols (with three chiral centers of asymmetry at C2, C4′, and C8′), while it is an unsaturated isoprenyl chain with only one chiral center at C2 and three double bonds at C-3′, C-7′, and C-11′ in tocotrienols. The four members of each subfamily (i.e., α-, β-, γ-, and δ-) differ from each other in the number and position of methyl groups on the phenolic ring of the chromanol ring. All tocopherols have the 2*R*,4′*R*,8′*R* configuration (d or *RRR*) and all tocotrienols the 2*R*,3′-*trans*, 7′-*trans* configuration [6]. Other tocochromanol derivatives exist in foods, e.g., plastochromanol-8 in flaxseed oil [7, 8], and tocomonoenols isolated from a wide range of cereals, fruits, seeds, fish, and fish eggs.

The main biological function of tocopherols and related chromanols is antioxidation, i.e., the inhibition of the autocatalytic lipid oxidation reactions by scavenging the chain-propagating lipid peroxyl radicals [9]. This role is facilitated by certain structural features of these molecules, specifically their lipid solubility and their ability to donate their phenolic hydrogens to peroxyl radicals forming relatively stable chromanoxyl radicals that will not further propagate the lipid oxidation reactions [6]. The difference between antioxidation in plants and in vitro systems such as foods, on one hand, and human and animal bodies, on the other hand, is the involvement of metabolism. Metabolism discriminates against other chromanol derivatives and favors α-tocopherol as the selected vitamer to predominate in humans and animals.

The understanding of the antioxidant mechanism of tocopherols and tocotrienols, as well as other phenolic compounds, took some time. The first and well-established biological role of vitamin E relates to its antioxidant effect, i.e., its ability to scavenge free radicals and act as a chain-breaking

antioxidant to protect unsaturated lipids from oxidative deterioration as initially suggested by Olcott and Emerson [10]. However, the observed behavior of tocopherol antioxidant action showed paradoxical results, e.g., prooxidant effects at high concentrations and synergism with other molecules, such as phospholipids. A better understanding has been reached only recently, when the hydrophilic-lipophilic balance of antioxidants has been considered along with their hydrogen-donating abilities. This chapter will discuss the antioxidant mechanism of vitamin E in light of our new understanding of the supramolecular chemistry of these reactions and how it explains peculiarities that were previously regarded as paradoxical [11].

The Antioxidant Mechanism of Vitamin E

As shown in Table 3.2, the cascade of reactions involved in the oxidation of unsaturated lipids (LH) in the absence of an antioxidant (AH), such as vitamin E, is generally described by the classical free radical mechanism consisting of initiation, propagation, and termination reactions [12]. The initial phase of the oxidation – the induction period (IP), the initiation or lag phase – is characterized by a very low transformation of unsaturated lipids, or other oxidizable materials, to hydroperoxides [13]. The overall reaction rate during the IP is usually zero order (i.e., constant) or first order (i.e., proportional to the amount of formed hydroperoxides) depending on temperature, catalysis, and nature of the substrate. The consumption of the unsaturated lipid substrates and formation of hydroperoxides are minimal during this stage. Lipid hydroperoxides are not stable and they initially decompose via monomolecular reaction (3.2) when their concentration is low. Reactions (3.1) and (3.2) will furnish a critical concentration of micelles (CMC) formed by hydroperoxides, moisture, secondary oxidation products, and other amphiphiles in the lipid mixture [14–17]. As the CMC is reached, hydroperoxides start to react with each other and decompose by monomolecular and bimolecular reactions (3.2 and 3.3) leading to the formation of $LOO^{\bullet}$, $LO^{\bullet}$, and $L^{\bullet}$ radicals and propagation reactions (3.6 and 3.7). The propagation phase is exponential and leads to fast oxidation of the unsaturated substrate(s). When the concentration of the substrate reaches a threshold concentration, the oxidation reaches the termination stage.

When tocopherols or tocotrienols are present, they intervene with the propagation reactions primarily by scavenging peroxyl radicals. Because the rate of reaction of lipid peroxyl radicals (LOO•) with tocols (reactions 3.4 and 3.5) is about 1000 times faster than their reaction with the polyunsaturated fatty acids, vitamin E intercepts the propagation of fatty acid depletion (Fig. 3.1). The tocopherol molecule and the peroxy radical approach each other and their electron clouds overlap to approach a transition state having the property of the charge transfer species ($LOO^{\delta-}$----$TOH^{\delta+}$). Then, proton

Table 3.2 List of the key reactions in lipid oxidation and its interception by tocochromanols

Initiation	$LH + X^{\bullet} + O_2 \rightarrow LOO^{\bullet} + XH \rightarrow LOOH + X^{\bullet}$ where $X^{\bullet}$ is a catalyst, such as transition metal oxides	(3.1)
Branching	$LOOH + LH \rightarrow LO^{\bullet} + L^{\bullet} + H_2O$ (first order)	(3.2)
	$LOOH + LOOH \rightarrow LOO^{\bullet} + LO^{\bullet} + H_2O$ (second order)	(3.3)
Antioxidation	$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet}$	(3.4)
	$LOO^{\bullet} + TO^{\bullet} \rightarrow$ TO-OOL	(3.5)
Propagation	$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$	(3.6)
(cyclic, autocatalytic)	$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$	(3.7)
Termination	$LOO^{\bullet} + LOO^{\bullet} \rightarrow$ radical combination products	(3.8)
	$LOO^{\bullet} + L^{\bullet} \rightarrow$ radical combination products	(3.9)
	$L^{\bullet} + L^{\bullet} \rightarrow$ radical combination products	(3.10)

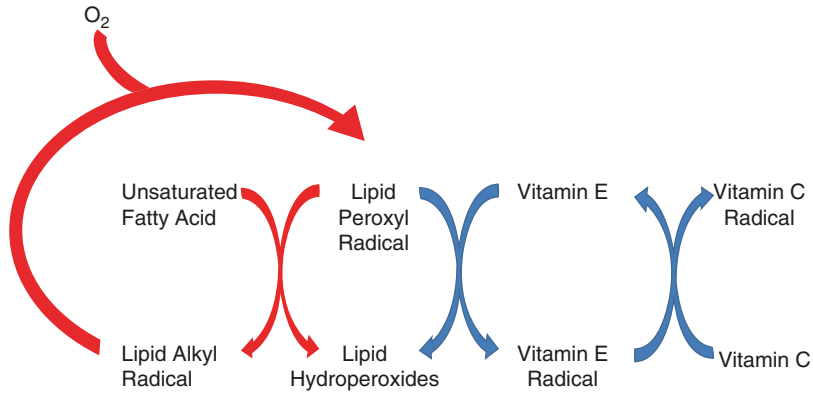


Fig. 3.1 The antioxidant effect of vitamin E (mainly α-tocopherol). In the absence of vitamin E, unsaturated fatty acids undergo autoxidation self-catalyzed by product hydroperoxides (cycle in red). The presence of vitamin E interrupts this cycle and shifts the reaction toward the formation of vitamin E radical and its recycling by vitamin C (cycle in blue)

Table 3.3 Molecular weight, bond dissociation energy (BDE), log P value, and molecular volume of α-, β-, γ-, and δ-tocopherols

Compound	M.Wt (Da)	BDE (gas phase)	LogP (miLogP)	Volume (cubic Å)
α-Tocopherol	430.71	78.2	9.043	474.499
β- and γ-tocopherols	416.68	79.5	8.982	457.938
δ-Tocopherol	402.65	81.1	8.602	441.377

Budilarto and Kamal-Eldin [11, 21], Thong and Nam [22]

tunneling takes place from the chromanol molecule to the lipid peroxy radical to form lipid hydroperoxides (LOOH) and a chromanoxyl radical [18]. The chromanoxyl radical, which is stabilized by electron delocalization in the benzene ring, is significantly more stable compared to the peroxyl radicals. Tocopherols do not only intervene with hydroperoxide formation but also stabilize hydroperoxides and prevent their decomposition to secondary oxidation products [19]. Thus, the presence of tocochromanols extends the induction period until the point where their concentration reaches a certain minimum. As a result, tocols oxidize to different products, including 4a,5-epoxy- and 7,8-epoxy-8a(hydroperoxy)-tocopherones and 5,6-epoxy-α–tocopherol quinone. After the tocols are consumed, oxidation reactions 3.6 and 3.7 commence and the oxidation enters the propagation phase.

A proof of the antioxidant effect in vivo recently came from a study on zebra fish embryos, where it was shown that vitamin E deficiency is associated with depletion of the highly unsaturated fatty acids, namely, 20:4 (arachidonic acid) and 22:6 (docosahexaenoic acid) [20]. The ability of tocochromanols to scavenge peroxyl radicals is dependent on their ability to donate their phenolic hydrogens, i.e., on the O-H bond dissociation energy (BDE), which is in the order α > β = γ > δ (Table 3.3). According to this scenario, α-tocochromanols should display higher antioxidant potential than the other homologues [23], but this is not always the case in vitro, as discussed below. Discussion of the antioxidant activity of non-alpha tocochromanol homologues may not be relevant in vivo because these chromanols are discriminated against by hepatic metabolism and selective binding proteins as is discussed in Chaps. 4 and 9 of this book.

α-Tocopherol is an important component of cell membranes and is required for protecting unsaturated fatty acids from oxidation and for maintaining membrane integrity [24–26]. The protection of the phospholipids in biological membranes from oxidative damage by α-tocopherol is supported by other, water-soluble, antioxidants such as vitamin C [27]. The concentration of α-tocopherol in membranes is small, ranging 0.1–1 mol% relative to phospholipids, necessitating some mobility of α-tocopherol and lipid free radicals to move freely and encounter each other. Flip-flop from one side of the membrane to the other side, with a half-life 10^{-3}–10^{3} s, allows efficient scavenging of free

radicals [28]. The molecular structure of α-tocopherol confers the best design for this antioxidant to scavenge free radicals across the biomembranes and at the same time to be regenerated by water-soluble reducing agents when it locates in the membrane surface [29].

The Loss of Antioxidant Efficacy

The antioxidant effect of tocochromanols, especially α-tocopherol, has been hitherto described as peculiar and paradoxical [30]. A "prooxidant" effect was reported for high concentrations of α-tocopherol in the oxidation of low-density lipoproteins [31]. This effect was explained by the reaction of the tocopheroxyl radical (α-Toc$^{\bullet}$) with unsaturated fatty acids (L-H) in the lipoprotein leading to the formation of alkyl radicals. This phenomenon, described as the loss of antioxidant efficacy of these compounds as their concentrations in oxidizable substrates increase, is commonly observed in in vitro systems [32] and was attributed to the participation of α-tocopherol in a number of side reactions [33].

A kinetic model of the oxidation of methyl linoleate and its protection by α-tocopherol was constructed of 53 individual reactions to reveal the reactions involved in the peroxidative effect of high concentrations of this antioxidant [34]. Three different reactions, involving the tocopheroxyl radical, the tocopherol molecule, and tocopherol oxidation products, were found to make the greatest and most significant contribution to this effect:

1. The chain transfer reactions involving the abstraction of a hydrogen atom from the methyl linoleate molecule and from the methyl linoleate hydroperoxides by tocopheroxyl radical suggested by Mukai et al. [35]:

$$\mathrm{TO}^{\bullet} + \mathrm{LH} \rightarrow \mathrm{TOH} + \mathrm{L}^{\bullet} \tag{3.11}$$

$$\mathrm{TO}^{\bullet} + \mathrm{LOOH} \rightarrow \mathrm{TOH} + \mathrm{LOO}^{\bullet} \tag{3.12}$$

Reaction (3.11) was later described as the α-tocopherol-mediated peroxidation (TMP) responsible for prooxidation of low-density lipoproteins [36].

2. The autoinitiation reaction of α-tocopherol with hydroperoxides, leading to the decomposition of hydroperoxides and generation of alkoxyl and tocopheroxyl radicals:

$$\mathrm{TOH} + \mathrm{LOOH} \rightarrow \mathrm{TO}^{\bullet} + \mathrm{LOO}^{\bullet} + \mathrm{H_2O} \tag{3.13}$$

3. The homolytic decomposition of quinolide peroxides, which are the combination products of tocopheroxyl radicals and lipid peroxyl radicals (TO-OOL) formed in reaction (3.8). An example of this type of reaction is shown in Fig. 3.1. These findings are in agreement with the initial suggestion of Yanishlieva and Marinova [33, 37] of the participation of both the tocopherol molecule itself and its radical in peroxidative reactions leading to the loss of efficacy of α-tocopherol. The loss of tocopherol antioxidant efficacy will be manifested to a higher degree when oxidation experiments are performed at a lower temperature, i.e., at a low initiation rate [38].

The Polar Paradox, Interfacial Phenomena, and Role of Micelles

The last 20 years have witnessed a paradigm change in our understanding of the mechanism of lipid oxidation and antioxidation [11, 39]. This paradigm change followed the publication of William Porters treatise on the *Polar Paradox* and two previous papers that highlighted the observation that

polar antioxidants are more effective in less polar oxidation environments of low surface-to-volume ratio, like bulk lipids, while nonpolar antioxidants are more effective in environments of high surface-to-volume ratio such as emulsions [30, 40, 41]. Shortly after, the *Interfacial Phenomena* was proposed as a framework to explain the reciprocal effect of antioxidants in bulk oil versus emulsions, where the partitioning of antioxidants at the interface(s) between the aqueous and nonaqueous phases exert an important effect on their potency, because their concentration at the interface allows them better accessibility to the oxidizing agents, mainly hydroperoxides, and trace metal ions, concentrated in the water phase of emulsions [42]. These works widely opened up a door to the understanding of lipid oxidation in emulsions and how it is affected by several properties of emulsion droplets and interface properties, including droplet size, and interfacial area, charge, thickness, and permeability.

Actually, Brimberg [14, 15] proposed that hydroperoxides and other surface-active compounds form micelles in the continuous lipid phase and that when these micelles reach their critical micelle concentrations, the oxidation changes kinetics from a pseudo-first order during the lag phase to exponential reactions during the propagation phase, but these works remained unnoticed. It was similarly recognized that water and other polar and amphiphilic compounds present in lipids contribute to the formation of *association colloids* in multiphases that provide the reaction site(s) for oxidation and antioxidation reactions [43–46]. According to this new understanding, the lipophilicity of antioxidants (expressed as log P value or the octanol-water partition coefficient) is another important determinant of antioxidant activity besides the dissociation energy of the phenolic O-H bond.

With log *P* values of about 9, tocopherols are surface active, and their presence will prolong the lag phase via the stabilization of micelles and delay the exponential catalysis by hydroperoxides. According to the above findings, polarity is also important for the antioxidant potency of tocopherols and tocotrienols, e.g., in bulk lipids, emulsions, and biomembranes. The hydrophilic-lipophilic balance of tocopherols and tocotrienols is determined by the degree of methylation of the aromatic ring, the degree of saturation in the phytyl side chain, and the stereochemical *RS-configuration* of the side chain [47]. Compared to α-tocopherol, the superior antioxidant potency of γ-tocopherol in bulk lipids and water-in-oil emulsions can be due to its slightly more polar nature and its less participation in the prooxidative side reactions 3.12 and 3.13. Similarly, the presence of double bonds in the side chain of tocotrienols confers increased polarity and enhances their antioxidant activity compared to tocopherols [48].

The combination of tocopherols, ascorbyl palmitate, and lecithin is known to increase the induction period and decrease the rate of oxidation during this period in the inhibition of autoxidation of fish oil [49]. It was suggested that phospholipids enhance the antioxidant activity of α-tocopherol in bulk lipids by aggregating to form microemulsions that bring the tocopherol closer to the oxidation site [50]. It was proposed that phospholipids act as synergists by enabling the antioxidants at the interface and by trapping the radicals in a cage and preventing their diffusion into the bulk oil: the so-called volume cage effect [51]. In addition to phospholipids, certain amino acids (*Tyr*, *Trp*, *Cys*, *His*, *Met*, *Phe*, and *Pro*), and peptides containing them, are known to synergize the antioxidant effects of tocopherols by contributing to the donation of the phenolic (*Tyr*), indolic (*Trp*), and sulfhydryl hydrogens (*Cys*), to interactions of the electron-dense sidechain groups of certain amino acids (*Trp*, *His*, and *Met*), and/or to the reducing power of the sulfhydryl group of *Cys* and *Met* [52]. However, the reactivity of these amino acids with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide anion radicals did not support radical scavenging mechanisms, suggesting that the synergistic effects are mainly related to the hydrophobic properties of these amino acids [53]. It was shown that amino acids (lysine, arginine, cysteine, histidine, and aspartic acid) form multi-walled carbon nanotubes with improved dispersion and enhanced antioxidant activity [54]. On the other hand, free fatty acids and monoacylglycerols exert antagonistic, or prooxidant, effects through a "micellar effect" by concentrating at the oil-water interface and accelerating the decomposition of hydroperoxides [44]. Thus, the antioxidant potency of tocols is determined not only by their ability to donate hydrogens but also by the nanostructural organization of unsaturated lipids and other species acting as synergists or antagonists.

Besides hydrogen donation and scavenging of peroxyl radicals, the antioxidant effect of tocopherols may partly be due to their action as anticoagulation agents, e.g., their effect against platelet aggregation [55] and adhesion of human erythroleukemia cells and integrin activity [56]. The lipophilicity of the molecules seems to play an important role and is determined by that of coexisting lipids and their collective organization in liquid or semiliquid crystalline structures. Synchrotron X-ray diffraction studies of model systems showed that, in an equimolar mixture of phosphatidylcholines and phosphatidylethanolamines, α-tocopherol preferentially partitions into the phosphatidylcholine phase. Differential partitioning of α-tocopherol in biomembranes may introduce stabilizing or destabilizing effects on these membranes. This area of research deserves more attention.

Tocopherols as Antioxidants for Food Stabilization

Tocopherols coexist with lipids rich in polyunsaturated fatty acids in vegetable oils (at concentrations in the range of ca. 100–1000 milligram mixed tocopherols per kilogram oil) and to a lower extent in fish oils (250–350 mg/kg) and animal fats (up to 100 mg/kg). Vegetable oils contain mainly tocopherols (Table 3.4), especially α- and γ-tocopherols, while fish oils and animal fats exclusively contain α-tocopherol. The presence of tocotrienols is limited to some oils, such as palm oil and annatto, while flaxseed oil contains significant amounts of plastochromanol-8 (see also Chap. 5 [Birringer & Frank, Minor derivatives]). Vegetable oils and animal fats are usually stabilized by their natural contents of tocopherols/tocotrienols and other synergistic molecules, mainly phospholipids, as well as by the molecular and structural organization in the natural seed or fruit. When oils and fats are extracted from their natural sources, these organizations are disturbed, and the optimal balance of antioxidants and oxidizable substrates is compromised. Lipids stabilized by tocochromanols will be stable when stored at relatively low temperatures, but these compounds are not stable at high temperatures (see also Chap. 16 [Somoza: Stability]). For example, tocopherols decompose very fast and provide limited protection to frying oils and fried foods. Foods containing very high levels of polyunsaturated fatty acids with high surface area are most susceptible to oxidative deterioration, and their oxidation follows more complicated pathways depending on several factors including structural and molecular organization [59]. In some cases, undesirable flavors and odors resulting from secondary oxidation

Table 3.4 Tocopherol and tocotrienol concentrations (ppm) and vitamin E equivalence in the major crude vegetable oils

	Tocopherol and tocotrienol levels in oils (ppm)							Vitamin E equivalent
Source	α-T	β-T	γ-T	δ-T	α-T3	γ-T3	δ-T3	(mg/kg oil)
Vegetable oils								
Corn/maize	23–573	ND-356	268–2468	23–75	ND-239	ND-450	ND-20	50–1070
Sunflower	403–935	ND-45	ND-34	ND-7	ND	ND	ND	400–960
Cottonseed	136–674	ND-29	138–746	ND-21	ND	ND	ND	150–760
Soybean	9–352	ND-36	89–2307	154–932	ND-69	ND-103	ND	25–650
Rapeseed	100–386	ND-140	189–753	ND-22	ND	ND	ND	120–530
Palm	4–193	ND-234	ND-526	ND-123	4–336	14–710	ND-377	5–470
Peanut	49–373	ND-41	88–389	ND-22	ND	ND	ND	60–430
Olive	92–259	1–3	3–10	0.2–0.4	0.3–1.0	0.4–0.9	ND	95–260
Cereal grains								

All vitamin E composition data was taken from Codex Standard 210 [57] except for olive oil [58]
Vitamin E equivalence (mg α-tocopherol per kg oil) was calculated using conversion factors from Table 3.1

products may develop at very low peroxide values at the early stage of oxidation. This situation is evident in the oxidation omega-3 fatty acids, e.g., in fish oils rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

However, results from the testing of ternary or quaternary antioxidant mixtures containing tocopherols, lecithin, and ascorbate, with or without amino acids, provide a promising starting point for further improvements based on the new understanding of the role of hydrophilic-lipophilic balance and nanostructures in lipid stability [21]. Much more work is needed to understand the roles of different functional groups and to develop models that can facilitate the optimization of antioxidant/synergist combinations also including surfactants that would improve the solubility and stability of the tocopherol in the interface and improve their antioxidant activities. For example, it is known that physical and structural differences among synergistic molecules, such as phospholipids, contribute to the net antioxidant properties [60]. It was also shown that the fatty acid profiles of oxidizing lipids influence the antioxidant properties and that the chain length and degree of saturation are critical factors in the antioxidant activity of phospholipids [61, 62].

A large scale of observations is available on the antioxidant effect of tocopherols in bulk lipids, model oil-in-water and water-in-oil emulsions, and biomembranes, but the value of these observations is limited by the analyzed variables describing primary and/or secondary oxidation products. It is only after the recent developments that scientists started to pay attention to the exact compositional and structural information. In the future, lipid oxidation studies will utilize new measurements relevant to the micro- and nanostructures of the lipids in question. The aim would be to achieve a qualitative, rather than a quantitative, difference with respect to the stabilizing power of tocopherols and selected companion synergists. It is expected that more precise knowledge and practice, based on the new paradigm, will be available during the coming decade [39].

One area of research relevant to the protection of oxidizable lipids by tocopherol preparations concerns the engineered encapsulation of the vitamin in nanoemulsions (20–200 nm), submicron-sized emulsions (50–1000 nm), and liposomes with tailored surface properties. These preparations will be able to offer controllable particle size and surface area and help to provide a relatively uniform distribution of the dose, improve the solubility, and enhance the dissolution rate and the release of the drug in the target destination [63]. Parameters such as particle size distribution, polydispersity index, zeta potential, vitamin recovery, encapsulation efficiency, and capsules microscopy are important parameters in the optimization of production technologies. The selection of the oil used, the encapsulating material, the ratio of oil to encapsulating material, and the encapsulation technique are very critical. For example, saturated fatty acids containing a small proportion of medium-chain triacylglycerols yield nanocapsules, while oils containing large amounts of long-chain mono- or di-unsaturated fatty acids yield larger particles. The structural organization of the mixed lipids, which depends on their individual and collective hydrophilic-lipophilic balance, determines the drug encapsulation efficiency [64]. Improved predictive rules and rational designs can be reached by combining empirical and theoretical approaches [65].

Tocopherols as Antioxidants in Cell Membranes

Cell membranes are described by the *fluid mosaic model* (Fig. 3.2), in which a critical barrier function allows rapid lateral diffusion of lipids and proteins within the planar membrane surface. It is well recognized that the lipids in cell plasma membranes are not regularly distributed. Membranes contain nano-domains, or rafts, enriched in sphingolipids and cholesterol, different from the surrounding

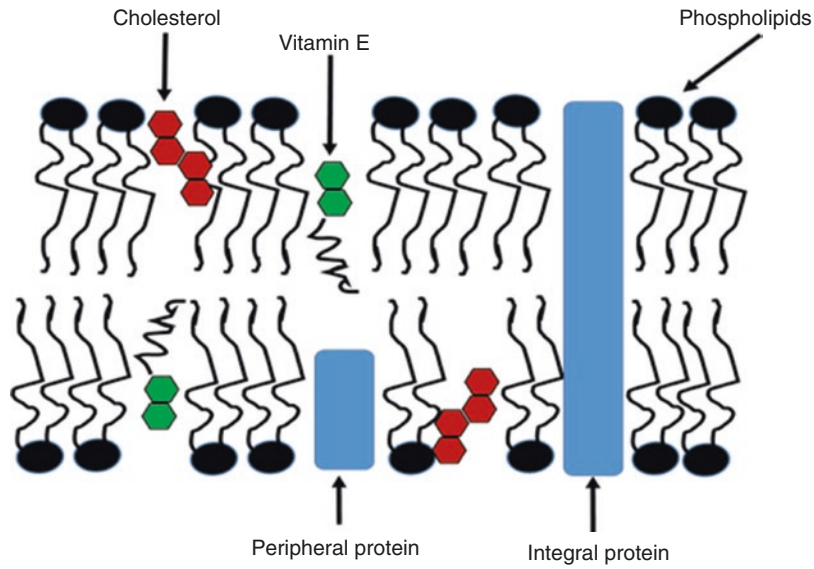


Fig. 3.2 Sketch of the fluid mosaic model of cell membranes showing component of phospholipids, cholesterol, vitamin E, and proteins

unsaturated phospholipids. Cholesterol and saturated fatty acids restrict the transport and diffusion of molecular oxygen in the membranes. α-Tocopherol, the main antioxidant in the membranes, partitions into the polyunsaturated phospholipid domains of the membranes where it is mostly needed [66]. This vitamin plays two main functions in membranes as an antioxidant and membrane stabilizer [26, 67, 68]. The chromanol head group of the tocopherol locates near the surface of the membrane, where it can capture water-soluble radicals and get recycled by ascorbic acid [69]. Since the concentration of α-tocopherol in membranes is very small, generally <1 mol % of total lipid [70], α-tocopherol swings between the two sides of the membrane by flip-flop movements that carry the chromanol group into the membrane interior and then back to the surface [29]. When the membrane reaches a high level of oxidation, the polarity and shape of the phospholipid-rich domains are altered and their acyl chains no longer be buried within the membrane interior but projects toward the surface of the membrane [71]. The *lipid whisker model* describes oxidized cell membranes where the conformation of oxidized fatty acids brings them into contact with cell surface scavenger receptors [72]. Red blood cell membranes are subject to oxidative stress, because of the high concentration of oxygen and the abundance of heme iron in these cells [73, 74]. Adequate vitamin E concentrations are required for the well-known to protection of erythrocyte cell membranes from oxidation and hemolysis [75].

Concluding Remarks

Compounds included under the collective name vitamin E are amphiphilic derivatives of chromanol and represent the primary antioxidants for polyunsaturated fatty acids in nature. The antioxidant effect of tocopherols and tocotrienols is based on their ability to donate hydrogen atoms and inhibit free radical reaction cascades. However, there is an optimum concentration for maximum protection above which a loss of efficacy or even a prooxidant effect might be observed. New findings attribute this effect either to physical modulation of the reaction environment, chemical reactions leading to the generation of radical species, or both. This knowledge will redirect research and allows for new developments in this field.

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Chapter 4
Bioavailability and Metabolism of Vitamin E

Sandra Flory, Marc Birringer, and Jan Frank

Keywords Absorption · Bioavailability · Cytochrome P_{450} · Excretion · Metabolism · Regulation Tocopherols · Vitamin E

Key Points

- Vitamin E has a high oral bioavailability of ca. 50–80% and follows the general absorptive route of dietary fats.
- All eight forms of the lipid-soluble vitamin E (α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol) are absorbed to a similar extent and reach the liver.
- The liver selectively metabolizes the non-α-tocopherol congeners to their water-soluble short-chain metabolites.
- The first and rate-limiting step in vitamin E degradation is controlled by the activity of tocopherol-ω-hydroxylase (cytochrome P_{450} 4F2) and is the most important factor regulating the retention of the different vitamin E congeners in the organism.
- The hepatic α-tocopherol transfer protein facilitates the secretion of α-tocopherol with lipoproteins into the systemic circulation and is required to maintain sufficient concentrations of α-tocopherol in blood and tissues.

Introduction

Dietary vitamin E is ingested from plant foods and fortified foods as a more or less complex mixture of tocopherols and tocotrienols and to a lesser extent tocomonoenols and other related chemical structures (see Chap. 5) in individual amounts of only a few milligrams. Intakes of higher doses of single vitamin E congeners can only be achieved by ingestion of dietary supplements. Natural sources of

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_4

vitamin E contain both free and esterified (e.g. with fatty acids) forms [1]. Because tocopheryl esters, such as αT acetate, are oxidatively more stable than free tocopherols, α-tocopherol (αT) supplements more often contain esters than free αT. The simultaneous intake and amount of dietary fat as well as the vitamin E dose ingested may affect the extent of uptake of the vitamin into the organism. Furthermore, the route of administration (oral vs. injection) may not only determine its uptake but also its fate inside the body (see below).

Vitamin E Bioavailability

Early studies in humans found that 55–79% of an oral dose of radioactively labelled α-tocopherol (αT) was absorbed, appeared in plasma 1–3 h after administration, and reached peak concentrations between 5 and 9 h after intake [2]. The high bioavailability of vitamin E was later confirmed in healthy humans given a single oral dose of 0.78 mg radioactively labelled *RRR*-αT, in whom 81% of the oral dose was absorbed [3].

In a human trial comparing the uptake of αT in the small intestine, when administered as deuterium-labelled *RRR*-αT or its ester *RRR*-αT acetate, the authors found free αT derived from the administered αT acetate in the distal jejunum, indicating that enzymes in the brush border cleave the acetyl group and release the free αT, which then is available for absorption. The uptake of the free and (upon intake) esterified form in the jejunum was similar, with ca. 6% of both being absorbed within 1 h [4].

The amount of dietary fat consumed together with vitamin E determines to some extent how much of the vitamin is absorbed. In healthy humans, ca. 10% of a 22 mg dose of deuterated *RRR*-αT acetate eaten with a fat-free breakfast were recovered in blood, whereas 33% were absorbed when the vitamin was ingested together with a breakfast containing 11 g fat [5]. In agreement, Jeanes and colleagues found a dose-dependent increase in αT bioavailability with increasing dietary fat in subjects consuming 150 mg deuterated *RRR*-αT acetate together with breakfasts providing 0, 2.7, or 17.5 g fat. Interestingly, they also found the food matrix to be an important factor in the bioavailability of vitamin E. αT was absorbed better when the 17.5 g fat were ingested from toast with butter compared to a cereal breakfast with whole milk [6].

The bioavailability of orally ingested compounds is influenced by a multitude of factors, including absorption, distribution, metabolism, and excretion, which in turn are governed by a large number of parameters themselves. The most important determinants of the bioavailability of vitamin E, absorption, metabolism, and excretion, are discussed below.

Vitamin E Absorption

The intestinal absorption of vitamin E generally parallels the absorption of dietary fat. In the acidic conditions of the stomach and subsequently by enzymatic activity of steryl-ester acylhydrolase (also known as cholesterol ester hydrolase or bile salt-dependent lipase) in the small intestine, vitamin E esters are hydrolyzed, and only the nonesterified vitamin E is absorbed in the small intestine [4, 7]. The liver secretes bile acids into the small intestine to aid the digestion of lipids and the formation of mixed micelles. Although dietary fat is required to aid the absorption of vitamin E, the amount of dietary fat is of limited importance, and even low-fat diets grant a sufficient uptake of the vitamin [8]. The micelles and micellar lipids enter the enterocytes either by receptor-mediated transport or passive diffusion [9]. The scavenger receptor class B type I (SR-BI), scavenger receptor cluster-determinant 36 (CD36), and the Niemann-Pick C1-like protein 1 (NPC1L1) have been identified as receptors involved in the intestinal uptake of free vitamin E [7, 10, 11]. Unlike other lipid-soluble vitamins,

vitamin E has no specific plasma transport protein. In order to be transported in the aqueous environment of the circulation, vitamin E is incorporated into chylomicrons, which are secreted into the lymphatic system by enterocytes with the involvement of the ATP-binding cassette transporter A1 (ABCA1) [9]. The chylomicrons pass through the thoracic duct into the systemic circulation where a fraction of the transported vitamin E is transferred to high-density lipoproteins (HDL), from where it is distributed to all circulating lipoproteins or tissues. Chylomicron degradation, catalyzed by lipoprotein lipase, ultimately results in the formation of chylomicron remnants, which are taken up into the liver facilitated by the hepatic low-density lipoprotein receptor-related protein 1 (LRP1) [12]. Up to this point, there appears to be no discrimination between vitamin E congeners [13].

Our present understanding of the intracellular trafficking of vitamin E, or more specifically αT, in liver cells (see Chap. 9) is as follows: αT is internalized by endocytosis and accumulates in the late endosome, from where it is transported to the plasma membrane and secreted with lipoproteins into the systemic circulation. A cytosolic protein in liver cells, α-tocopherol transfer protein (αTTP), binds αT (and probably, albeit to a smaller degree, the non-αT congeners) in the outer leaflet of the endosomal membrane and expedites its transport to the plasma membrane, where the binding to phosphatidylinositol 4,5-bisphosphate induces a conformational change that results in the release of αT and its integration into the membrane [14–16]. The secretion of αT from liver cells involves ABCA1, which is required for the incorporation of vitamin E into lipoproteins that deliver the vitamin to extrahepatic tissues. The fraction of the lipid-soluble vitamin E congeners that is not secreted into the bloodstream is degraded to water-soluble carboxyethylhydroxychromanol (CEHC) metabolites by side-chain degradation without modification of the chromanol head and excreted via bile and urine [9] (see next paragraph). The hepatic secretion of vitamin E favors the (natural) *RRR*-α-tocopherol, probably as a result of the selectivity of αTTP toward the two *R*-congeners (*R* configuration at carbon 2) [15, 17] in combination with the preferential metabolism of the non-αT congeners (see below) [18, 19].

Vitamin E Metabolism

In the 1950s, tocopheronic acid and tocopheronolactone, the so-called Simon products (Fig. 4.1), were discovered in human urine as the first vitamin E metabolites [20]. These Simon metabolites were thought to be degradation products of vitamin E that had exerted its antioxidant function, as ring opening occurs when the tocopheroxyl radical is formed [21]. Approximately 30 years later, this theory was rejected when a new metabolite derived from δ-tocopherol was found in human urine. This side-chain shortened metabolite, which was later called δ-carboxyethylhydroxychromanol (δCEHC), had an intact chromanol ring structure that was conjugated with sulfuric acid [22]. The detection of this metabolite unmasked the Simon metabolites as oxidative artifacts generated during sample extraction and cleanup and was the start of further research into vitamin E metabolism [20].

The metabolism of vitamin E, which occurs mainly in the small intestine and the liver [23], comprises a number of enzymatic steps that are identical for all four tocopherol respectively four tocotrienol congeners (Fig. 4.2). The first and rate-limiting step is the terminal ω-hydroxylation of the side chain by cytochrome P_{450} enzymes (CYP) in the endoplasmic reticulum [18], resulting in the formation of the long-chain metabolite 13′-hydroxychromanol (13′-OH) [9]. CYP4F2 appears to be the main enzyme responsible for catalyzing this reaction in humans [24]. However, other CYP, including CYP3A4, appear to have minor ω-hydroxylation activity [25].

The side chain of 13′-OH then undergoes α-oxidation to 13′-carboxychromanol (13′-COOH) in peroxisomes. Subsequently, β-oxidation in the mitochondria results in the stepwise removal of 2-carbon units and thereby the formation of the intermediate-chain metabolites (ICM) and eventually the water-soluble short-chain metabolite (SCM) CEHC (Fig. 4.2) [9].

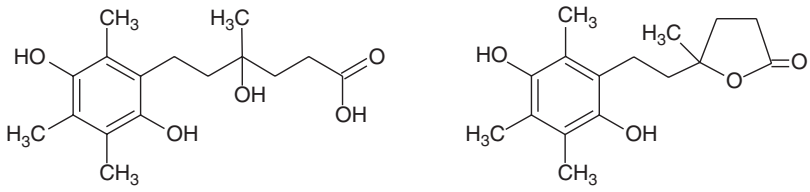

Fig. 4.1 Chemical structures of the originally isolated Simon products, which were later shown to be artifacts produced by oxidation reactions during sample isolation and cleanup

Bardowell et al. investigated the role of a murine orthologue of the human *CYP4F2* gene in vitamin E metabolism in Cyp4f14-knockout mice and found two new metabolites, namely, 12′-hydroxy-tocopherol (12′-OH: γ- and δ-12′-OH) and 11′-hydroxy-tocopherol (11′-OH: γ- and δ-11′-OH), in fecal pellets of mice fed a diet rich in γ-tocopherol [23, 26]. The metabolites derive from ω-1 and ω-2-hydroxylation and were excreted via bile into feces.

Tocotrienols are metabolized similar to tocopherols except that their unsaturated side chain undergoes saturation reactions, similar to the metabolism of unsaturated fatty acids (Fig. 4.2). Consequently, some additional metabolites are generated, which have been found in mouse and human feces and urine [27, 28]. In addition, tocotrienols are metabolized to a larger extent than their corresponding tocopherols [29, 30]. Carboxymethylbutylhydroxychromanol (CMBHC), not CEHC, seems to be the quantitatively major metabolite derived from tocotrienols in cultured liver cells (see also section "Regulation of Vitamin E Metabolism") [30].

All generated vitamin E metabolites are excreted via feces and urine. In feces, all of the metabolites, including the lipid-soluble long-chain metabolites, can be detected, whereas in urine, only the more water-soluble short-chain metabolites are present [28]. Similar to xenobiotics, around 90% of the urinary metabolites are conjugated by the phase II enzymes UDP-glucuronosyltransferase and sulfotransferase [9, 31]. Hence, vitamin E metabolites in rat urine are mainly sulfate conjugates and in human urine mainly glucuronidated conjugates [32].

Regulation of Vitamin E Metabolism

The first and rate-limiting step of vitamin E metabolism, which therefore regulates the formation of the vitamin's degradation products, is controlled by CYP, mainly CYP4F2, but perhaps to a limited extent also CYP3A4, in humans [18, 24, 25, 33]. The nuclear pregnane X receptor (PXR) is a central point of regulation for the expression of CYP as well as other phase I and II enzymes. Tocotrienols, and to a much smaller degree tocopherols, activated PXR and consequently the expression of CYP in cultured liver cancer cells (HepG2) [34]. In another study, only the four tocotrienols (α, β, γ, δ) but none of the four tocopherols activated PXR in HepG2 and human colon cancer cells (LS 180) [35]. The induction of PXR by tocotrienols and the lack thereof by tocopherols were confirmed by other independent experiments in LS 180 and HEK cells [36, 37]. The latter trial also found no PXR-activating activity of CEHC but for the first time observed a potent induction of PXR by the long-chain metabolite α-13′-COOH (see also Chap. 6) [36].

In vivo studies also show conflicting results with regard to the activation of PXR and the induction of CYP in different animal models. Mice given high doses of *all rac*-αT acetate (1000 mg/kg diet) for 4 months had a small but statistically significant increase in the mRNA expression of Cyp3a11, the murine homologue to human CYP3A4, in the liver compared to control animals fed 35 mg *all rac*-αT acetate per kilogram diet [38]. In mice fed *RRR*-α-tocopheryl acetate for 3 months

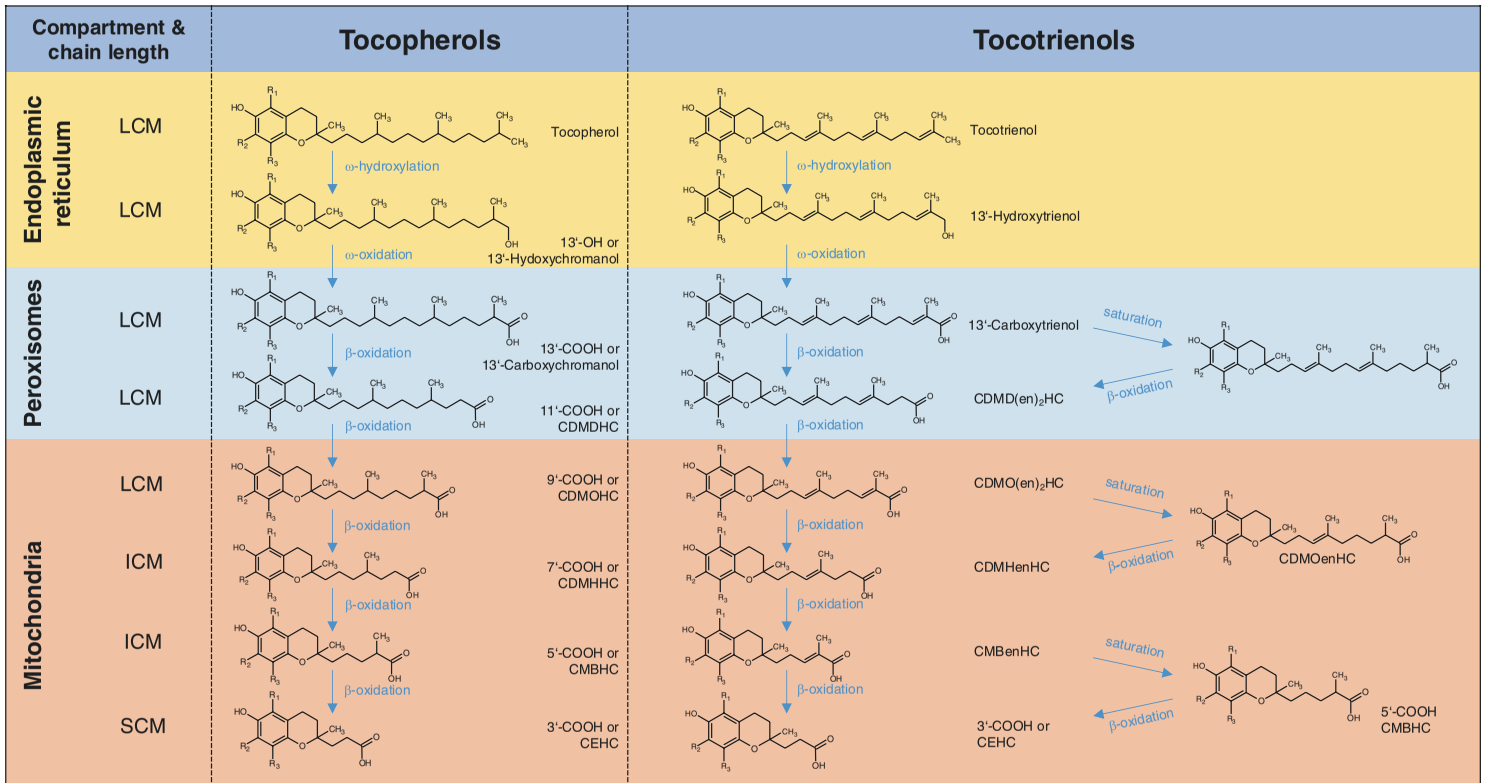

Fig. 4.2 Steps in the side-chain degradation of tocopherols and tocotrienols to their side-chain shortened water-soluble carboxyethyl hydroxychromanol metabolites
In the first and rate-limiting reaction, the side chain of the tocopherols and tocotrienols (α, R1 = CH_3, R2 = CH_3; β, R1 = CH_3, R2 = H; γ, R1 = H, R2 = CH_3; δ, R1 = H, R2 = H) is terminally hydroxylated in the endoplasmic reticulum and then terminally carboxylated in the peroxisomes. After five cycles of β-oxidation, the short-chain metabolites CEHC are formed. The steps in the metabolism of tocopherols and tocotrienols are largely similar, but some additional enzymes are required for the saturation of the side chain of tocotrienols (see right side of the figure)
Abbreviations: LCM long-chain metabolite, ICM intermediate-chain metabolite, SCM short-chain metabolite, α-CDMDHC α-carboxydimethyldecylhydroxychromanol, α-CDMOHC α-carboxymethyloctylhydroxychromanol, α-CDMHHC carboxymethylhydroxychromanol, α-CMBHC α-carboxymethylbutylhydroxychromanol, α-CEHC α-carboxyethylhydroxychromanol, α-CDMD(en)2HC α-carboxydimethyldecadienylhydroxychromanol, α-CDMO(en)2HC α-carboxydimethyloctadienylhydroxychromanol, α-CDMOenHC α-carboxydiemethyloctenylhydroxychromanol, α-CDMHenHC α-carboxymethylhexenylhydroxychromanol, α-CMBenHC α-carboxymethylbutadienylhydroxy chromanol

at 2 (deficient), 20 (sufficient), or 200 mg/kg diet (supplemented), a reduced mRNA expression of Cyp3a11 was observed in the deficient group, but no significant differences were seen between sufficient and supplemented animals [39]. The addition of γ-tocotrienol to these diets did not result in an increased mRNA expression of Cyp3a11 [39]. In rats, the feeding of diets deficient or adequate in vitamin E (<2 or 60 mg *RRR*-αT) for up to 9 months did not change the mRNA of any of the 33 hepatic CYP expressed [40]. In guinea pigs fed 20 or 250 mg *RRR*-αT per kg diet for 6 weeks, the hepatic protein expression of CYP3A4 and CYP20A1 and the serum concentrations of products of the enzymatic activity of CYP3A4 were similar in both groups [41]. The differences between the study by Mustacich et al. [38] and the other animal studies reviewed above [39–41] might be explained by the significantly higher oral dose of vitamin E administered; 1000 mg/kg diet of synthetic αT acetate in mice is 33 times more than the recommended dietary dose and corresponds to ca. 7–10 g per day for an average human, which would be >450–660-fold higher than the recommended dietary allowance of 12 mg/d. This is further supported by studies using subcutaneous injection of high doses of αT in rats, which results not only in very high hepatic concentrations of the vitamin but also an induction of CYP mRNA [42].

The notion that vitamin E does not induce PXR and CYP at dietary doses that can be reasonably achieved in humans is in agreement with studies in humans addressing the effects of αT supplementation on CYP3A4-mediated drug metabolism. In patients treated with drugs that are CYP3A4 substrates and simultaneously supplemented with 400 mg/d *RRR*-α-tocopheryl acetate for 8 weeks, CYP3A4 activity was unchanged [43]. In another human trial, healthy volunteers were given the CYP3A4 substrate midazolam intravenously prior to and following a 3-week oral supplementation with 503 mg *RRR*-αT per day. No differences in CYP3A4 activity were observed between placebo- and vitamin E-treated subjects [44].

In summary, there is no convincing evidence to suggest that orally ingested tocopherols or tocotrienols may induce CYP activity in animals or humans, even when consumed at high but reasonably achievable doses.

Quantitative Differences in the Retention and Excretion of Vitamin E Congeners in Humans

Although γ-tocopherol (γT) is the main vitamin E form in the human diet in some populations, such as the United States, αT is the predominant congener in human and animal tissues [45]. Plasma concentrations of αT (11–37 μmol/L) are higher than those of γT (2–5 μmol/L) [9]. The extent of the retention in and excretion from the organism differs between the different vitamin E forms.

Findings in human subjects show that the urinary excretion and plasma concentrations of γCEHC exceed those of αCEHC by a factor of ~4–5 and ~14–16, respectively, [46]. In agreement, up to ~50% of ingested γT was excreted in urine as the corresponding γCEHC metabolite in humans [47], while only 1–3% of the consumed αT dose was converted to urinary αCEHC [48]. Furthermore, the urinary excretion of deuterated αCEHC derived from natural *RRR*-α-tocopherol and synthetic *all rac*-α-tocopherol was 2–4 times higher for the synthetic vitamin E [49].

Because the extent of vitamin E absorption and transport to the liver is similar for all vitamers [13], there must be a regulation of the different rates of retention inside the liver. The two potential key factors regulating the retention and excretion of vitamin E congeners are the rate-limiting steps in their metabolism, namely, the binding to the hepatic αTTP and the ω-hydroxylation of the side chain.

The first key factor that was proposed to be responsible for the preferential retention of αT over the non-αT congeners was the αTTP (Chap. 9). The hepatic protein facilitates the secretion of αT from the liver into the bloodstream [15]. Hosomi and co-workers determined the binding affinities of αTTP for some vitamers relative to that for *RRR*-α-tocopherol and observed the following lower values:

RRR-β-tocopherol, 38%; *RRR*-γ-tocopherol, 9%; *RRR*-δ-tocopherol, 2%; *SRR*-α-tocopherol, 11%; and α-tocotrienol, 12% [50]. Interestingly, the binding affinity values for the tocopherols reflect, at least in part, their plasma concentrations, which led to the original proposal that αTTP may be the primary determinant of the discrimination between the congeners [50], a notion that has been challenged by more recent findings (see below).

If αTTP were the key factor regulating the discrimination against non-αT congeners, a knockout of the αTTP gene would be expected to result in increased retention (concentrations) of γT. In conflict with this expectation, the concentrations of γT in αTTP knockout mice were lower than in wild-type mice after feeding a γT-enriched diet or a diet containing equimolar concentrations of both αT and γT [19, 51]. The assumption now is that vitamin E metabolism and not αTTP is the major factor responsible for the regulation of the organismal concentrations of the congeners [19]. This is emphasized by observations in the model organism *Drosophila melanogaster*, an organism that does not express a protein equivalent to αTTP but shows the overall discrimination against non-αT forms. In addition, tocopherol-ω-hydroxylase activities for the individual vitamers in *Drosophila melanogaster* are comparable to the ones in rat and human microsomes [29, 52]. In line with these observations, lower plasma concentrations of γT than αT were observed in αTTP knockout mice [19, 53]. Generally, decreased plasma concentrations of all vitamers in αTTP knockout mice indicate that αTTP is important for their general retaining in the body, but not the discrimination between them, which is more likely facilitated by vitamin E metabolism [19].

The first and rate-limiting step in the metabolism of vitamin E is catalyzed by CYP4F2, an enzyme which has also been called tocopherol-ω-hydroxylase [18]. In vitro, CYP4F2 exhibited similar binding affinities for αT and γT but much higher catalytic activity toward γT [18, 24]. Sontag and Parker identified the following structural features as important factors favoring a rapid conversion by CYP4F2: absence of methyl groups at the chromanol ring, particularly at carbon 5 (γ and δ congeners), and unsaturation of the side chain (tocotrienols). This is in agreement with earlier observations that HepG2 cells produce less CEHC and CMBHC from αT and α-tocotrienol than from γT and γ-tocotrienol [30].

Not only the position but also the number of methyl groups is another factor influencing the enzyme activity, which is higher for the dimethyl γT than for the trimethyl αT [29]. Tocopherols with fewer methyl groups are able to penetrate deeper into the membranes of the endoplasmic reticulum, where the CYP4F2 is positioned. Consequently, the dimethyl congener γT may be more easily available for interactions with the enzyme than the trimethyl αT, as previously reported for interactions with the membrane-localized enzyme phospholipase A2 [54]. Furthermore, an inhibitory effect on the metabolism of γT was observed in HepG2 cells co-incubated with αT and γT [19, 29] and may be explained by competition for enzymatic degradation by CYP4F2 [29].

The central role of the ω-hydroxylase activity of CYP for the retention and excretion of vitamin E congeners is furthermore supported by experiments with phytochemicals with CYP inhibitory activity [55]. The cereal lipids alkylresorcinols greatly increased the concentrations of γT, but not αT, in rats by competitive inhibition of tocopherol-ω-hydroxylase [56]. The sesame lignan and potent tocopherol-ω-hydroxylase inhibitor sesamin played an important role in the elucidation of the role of vitamin E metabolism for the retention and excretion of different vitamin E forms. In HepG2 cells [19, 33, 56], mice [19], rats [57–62], and humans [46, 63], administration of sesamin increases the concentrations of γT and reduces the production of its metabolite γCEHC. Consumption of sesamin with sesame oil muffins by human volunteers significantly inhibited γT metabolism and resulted in a reduced urinary excretion of γCEHC, proving the importance of the in vitro findings for human nutrition [46, 63, 64].

The central role of the ω-hydroxylase activity in the regulation of vitamin E metabolism was confirmed using knockout mice without ω-hydroxylase activity. The knockout leads to significantly reduced concentrations of urinary metabolites of α-, γ-, and δ-tocopherol and higher excretion of unmetabolized tocopherols; the disruption of the enzyme activity furthermore had a larger impact on the metabolism of γT than that of αT [23, 26].

However, the processes underlying the selective retention of αT and the preferential excretion of the non-αT congeners as metabolites may not be as black and white, as an interaction between the αTTP and ω-hydroxylase activities has been proposed [19]. Grebenstein and colleagues observed an inverse relationship between the expression of αTTP in hepatocytes and their ability to convert γT to γCEHC. The authors hypothesized that αTTP may bind γT, albeit to a lesser extent than αT, and thereby prevent the contact of γT with CYP4F2, which would protect the congener from side-chain degradation [19].

In summary, the above findings indicate that the tocopherol-ω-hydroxylase activity is the most important factor regulating the degradation of vitamin E to its short-chain metabolites destined for excretion. This activity also appears to be the main determinant controlling the retention of the different vitamin E congeners in humans. αTTP is required for the secretion of vitamin E from the liver and appears to limit the excretion of mainly αT, but to a smaller extent also the non-αT congeners from the body, thus making sure that sufficient vitamin E is retained for its essential biological functions.

Research Gaps/Conclusion

Around 70 years ago, the short-chain metabolites of vitamin E were detected for the first time. By now, the main steps of vitamin E metabolism have been discovered and characterized and the regulating factors described. Nevertheless, some gaps in our understanding of these processes exist, such as how the different metabolites are transported from one cellular compartment to the other (e.g., endoplasmic reticulum to peroxisomes to mitochondria). Furthermore, the rate-limiting step, which is mainly catalyzed by CYP4F2, may also be facilitated by additional as yet undiscovered enzymes. Last but not least, an important question that still awaits a conclusive answer is: Is the main aim of vitamin E metabolism the prevention of excessive accumulation of all or certain vitamin E congeners by facilitating their elimination from the body? Or does the formation of the long-chain metabolites represent the conversion of vitamin E into the actual biologically active molecules (see also Chap. 6)?

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Chapter 5
Occurrence and Bioactivities of Minor Vitamin E Derivatives

Marc Birringer and Jan Frank

Keywords Vitamin E · 6-hydroxy-chromanols · Sesquiterpenes · Meroditerpenes · Biological activity

Key Points

- Terrestrial and marine organisms produce a large variety of tocopherol derivatives.
- More than 70 sesquiterpenes with a 6-hydroxy-chromanol moiety are described.
- Tocopherol derivatives show anti-inflammatory and cytotoxic properties.
- Side chain modifications of the derivatives increase their biological activity.

Introduction

Since the discovery of the fertility properties of tocopherols by Bishop and Evans in 1922 (see Chap. 2), numerous structurally related 6-hydroxy-chromanols and 6-hydroxy-chromenols have been discovered in nature. In terrestrial plants, tocochromanols of the vitamin E class represent the most widely distributed and predominant chromanols. The term vitamin E is used for eight structurally related congeners α-, β-, γ-, and δ-tocopherol (**1**,**2**,**3**,**4**) and α-, β-, γ-, and δ-tocotrienol (**5**,**6**,**7**,**8**), with *RRR*-α-tocopherol being the compound with the highest activity in rat gestation-fetal resorption assays [1, 2].

Only photosynthetic organisms, such as plants, algae, and cyanobacteria as well as fungi, corals, sponges, and tunicates, are able to perform the biosynthetic steps leading to a chromanol ring system. Mammals, however, rely on these resources (esp. plant oils), since vitamin E is essential for a wide range of higher organisms including humans (see Chap. 9) [3].

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_5

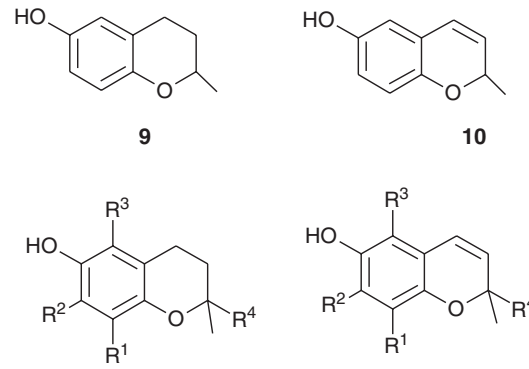


Fig. 5.1 Parent structures of tocochromanols and tocochromenols. 2-Methyl-3,4-dihydro-2*H*-chromen-6-ol (**9**) and 2-methyl-2*H*-chromen-6-ol (**10**)

This chapter will focus on the structural diversity and bioactivity of minor vitamin E derivatives, meroditerpenes that belong to the class of chromanols and chromenols. Recently, Birringer et al. summarized a database structure search for 6-hydroxy-chromanols and 6-hydroxy-chromenols with more than 230 compounds. The authors include natural products from terrestrial and marine origin comprising di-, sesqui-, mono-, and hemiterpenes [4]. Out of this collection, 135 meroditerpenes were described. Here we present the most important minor vitamin E derivatives. The biosynthesis, bioactivity, and chemical properties of tocopherols and tocotrienols are summarized in several outstanding reviews [5] and will be discussed only briefly.

In general, tocochromanols derive from the parent structure 2-methyl-3,4-dihydro-2*H*-chromen-6-ol (**9**) and tocochromenols from 2-methyl-2*H*-chromen-6-ol (**10**) that are formed by cyclisation of substituted (R_1–R_3) 1,4-benzoquinones (Fig. 5.1).

The highest structural variability of the mother compounds (**9** and **10**) is observed in the side chain modifications at R_4 [4]. The unsaturated (tocotrienols) and saturated (tocopherols) side chains, respectively, are prone to (partial) reduction of the double bonds or oxidation of the methyl groups by cytochrome P450-dependent hydroxylases and oxidases. As a consequence, the formation of oxidation products, such as alcohols, ketones, aldehydes, and carboxylic acids, and truncations of the side chain occur. In addition, intramolecular cyclisation and rearrangements of the isoprene units can produce cyclic ring systems.

For a discussion of the complex antioxidant and redox chemistry of tocopherols forming corresponding radicals, quinones, dimers, or polymers, the reader is referred to Chap. 3 and excellent reviews [6, 7].

Biosynthesis in Plants and Structural Diversity

To facilitate an understanding of the structural variability of tocochromanols in plants and other photosynthetic organisms, their biosynthesis is briefly introduced. The biosynthetic pathways of tocotrienols, tocomonoenols, tocopherols, and plastochromanol-8 are depicted in Fig. 5.2 and consist of four main steps. First, the geranylgeraniol diphosphate chain that originates from the 1-deoxy-D-xylulose-5-phosphate pathway in plastids is synthesized and stepwise reduced by geranylgeraniol reductase to dihydro-geranylgeraniol diphosphate, tetrahydro-geranylgeraniol diphosphate, and phytyl diphosphate. In a second step, the diphosphates serve as substrates for transferases that catalyze the alkylation of homogentisic acid, leading to benzoquinol derivatives, such as methyl-geranylgeraniol benzoquinol, methyl-tetrahydro-geranylgeraniol benzoquinol, methyl-phytyl benzoquinol, and methyl-solanesyl benzoquinol.

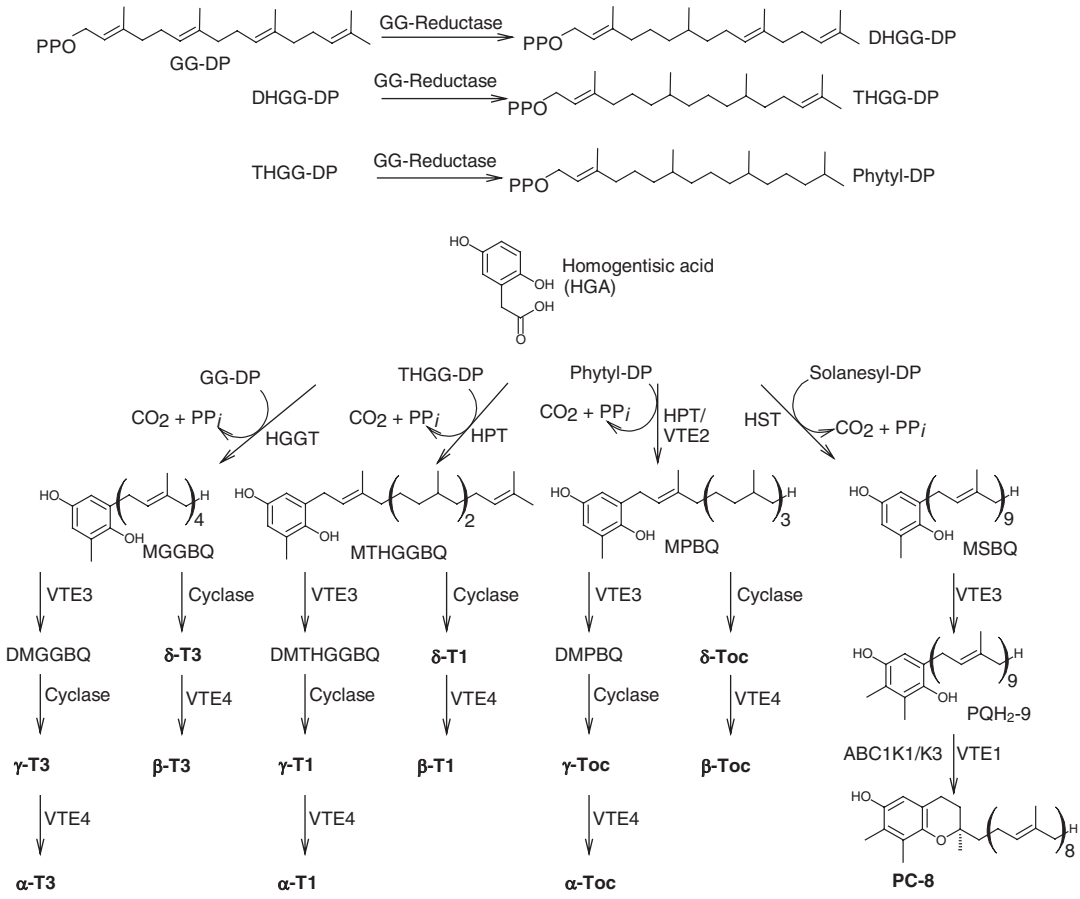
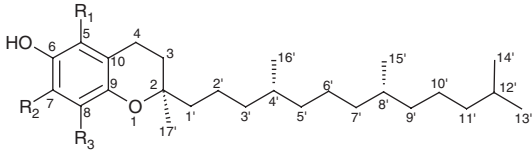


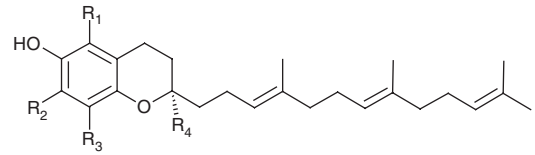
Fig. 5.2 Biosynthetic pathway toward the formation of chromanols within photosynthetic organisms. Abr.: HPP, *p*-hydroxyphenylpyruvate; HGA, homogentisic acid; HPPD, hydroxyphenylpyruvate dioxygenase; DOXP, 1-deoxy-D-xylulose-5-phosphate; GG-reductase, geranylgeraniol reductase; DHGG-DP, dihydro-geranylgeraniol diphosphate; THGG-DP, tetrahydro-geranylgeraniol diphosphate; MGGBQ, methyl-geranylgeraniol benzoquinol; MTHGGBQ, methyl-tetrahydro-geranylgeraniol benzoquinol; MPBQ, methyl-phytyl benzoquinol; MSBQ, methyl-solanesyl benzoquinol

Depending on step 3 and 4, tocochromanols will be partly or fully methylated. In the case of δ- and β-tocochromanol, ring closure (step 3) is followed by S-adenosyl methionine-dependent methylation of the chromanol ring (step 4), whereas γ-tocochromanols are built by methylation (step 3) followed by cyclization (step 4). α-Tocochromanols are synthesized by methylation of γ-tocochromanols. The cyclization of the prenylated quinones to chromanols by tocopherol cyclase occurs within plastoglobules. The latter biosynthetic step yields *R*-configuration at C-2 atom and thus seems to be unique for plant species. For in-depth details of the biosynthetic pathways, the reader is referred to previously published excellent reviews [8–12].

Tocopherols are ubiquitously found in most plant oils, whereas tocotrienols occur only in non-photosynthetic organs of higher plants, mainly eudicots and monocots [12, 13]. For example, α-tocotrienol (**5**) was found in barley, γ-(**7**) and δ-tocotrienol (**8**) in palm oil (Fig. 5.3) [5, 13, 14]. In contrast to tocopherols, tocotrienols exhibit higher bioactivity in vertebrates. Ashan et al. recently reviewed the bioactivity of tocotrienols, which may act as anticancer, antidiabetic, anti-inflammatory, antioxidant, immune-stimulatory, cardio-, neuro-, hepato-, and nephro-protective molecules [15].



-tocopherols	-R_1	-R_2	-R_3
α **1**	-CH_3	-CH_3	-CH_3
β **2**	-CH_3	-H	-CH_3
γ **3**	-H	-CH_3	-CH_3
δ **4**	-H	-H	-CH_3



-tocotrienols	-R_1	-R_2	-R_3	-R_4
α **5**	-CH_3	-CH_3	-CH_3	-CH_3
β **6**	-CH_3	-H	-CH_3	-CH_3
γ **7**	-H	-CH_3	-CH_3	-CH_3
δ **8**	-H	-H	-CH_3	-CH_3
desmethyl **11**	-H	-H	-H	-CH_3
didesmethyl **12**	-H	-H	-H	-H

Fig. 5.3 Structures and substitution patterns of tocopherols (**1–4**) and tocotrienols (**5–8** and **11**–**12**)

A minor fraction of rice bran contained desmethyltocotrienol (3,4-dihydro-2-methyl-2-(4,8,12-trimethyltrideca-3′(*E*),7′(*E*),11′-trienyl)-2*H*-1-benzopyran-6-ol) (**11**) and didesmethyltocotrienol [3,4-dihydro-2-(4,8,12-trimethyltrideca-3′(*E*),7′(*E*),11′-trienyl)-2*H*-1-benzopyran-6-ol) (**12**) [16] (Fig. 5.3). These two compounds show cholesterol-lowering activity in chicken, most likely by inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme in the biosynthesis of cholesterol. The two desmethyltocotrienols reduced total serum cholesterol by 26% and 31% relative to a control diet and reduced LDL cholesterol by 41% and 48%, respectively. Similar to tocotrienols, both compounds suppress the proliferation of B16 melanoma cells [16, 17].

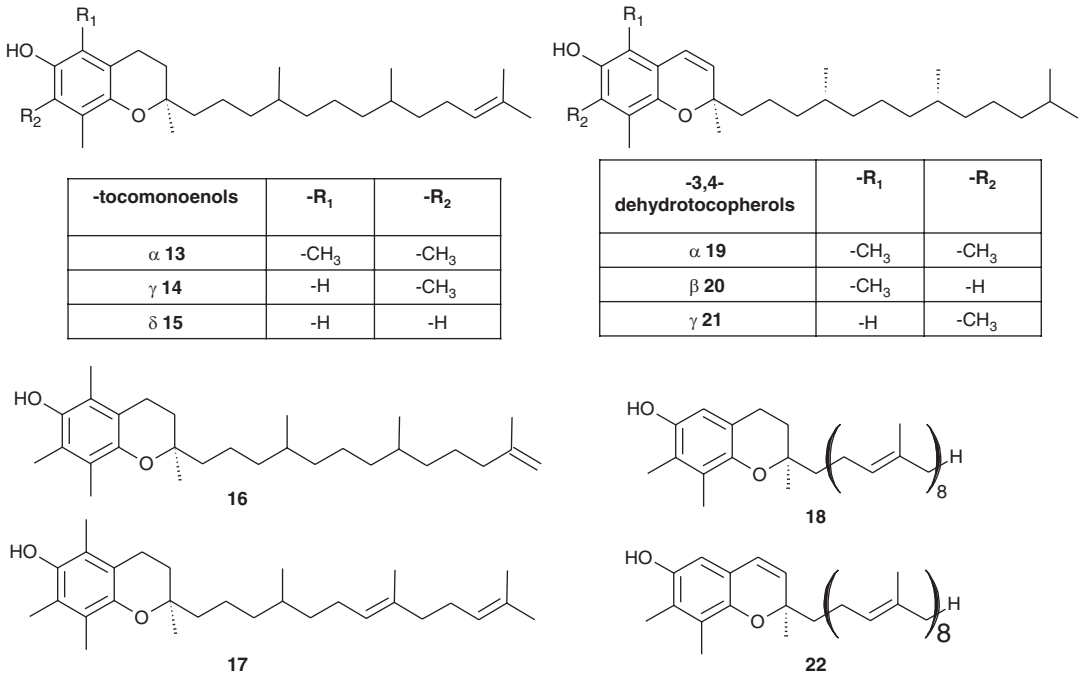
By partial reduction of the prenyl side chain, tocodienols and tocomonoenols are formed in several plant species (Fig. 5.4) [12]. α-Tocomonoenol (**13**) was found in palm seed and pumpkin seed oils [18, 19], γ-tocomonoenol (**14**) in pumpkin seed oil as well as green leaves and etiolated beans of *Kalanchoe daigremontiana* and *Phaseolus coccineus* [12, 18], and δ-tocomonoenol (**15**) was found in kiwi fruits (*Actinidia chinensis*) (Fig. 5.4) [20].

Interestingly, marine-derived α-tocomonoenol (**16**) (MDT) is a structural isomer of α-tocomonoenol with a terminal double bond at C-13′. As reported by Yamamoto et al., cold-water fish contain a substantial amount of MDT [21, 22]. Since tocochromanols are only synthesized by photoactive organisms, the authors suggested a dietary source for MDT in fish. In fact, phytoplankton contains up to 21% (of total tocopherol) MDT. Also Antarctic krill (*Euphasia superba*) contains up to 8% (of total tocopherols) MDT [23].

α-Tocodienol (**17**) has recently been discovered as a trace compound (0.2% of the total vitamin E content) in palm oil [24].

Some higher plant species produce plastochromanol-8 (**18**), a γ-tocochromanol with eight isoprenoid units in the side chain. The biosynthesis of the polyterpene follows that of tocotrienols except of the use of solanesyl-diphosphate synthase to form the elongated side chain of plastochromanol-8 (Fig. 5.4) [25]. Plastochromanol-8 was first discovered in leaves of the rubber tree (*Hevea brasiliensis*) and since then in many higher plants, where it acts as a fat-soluble antioxidant [25–27]. Dietary sources, such as rapeseed and linseed oil, accumulate between 5.6 and 18.5 mg/100 g, respectively [27].

Dehydrotocopherols derive from the biochemical elimination between C-3 and C-4 of the chromanol ring and were first isolated as α-, β-, and γ-dehydrotocopherols (**19**, **20**, **21**) from wheat germ oil [28] and from various *Stemona* species, such as Korean Stemonae Radix (Fig. 5.4) (*Stemona tuberosa*) [29, 30]. γ-Dehydrotocopherol shows proliferative effects on mouse fibroblasts, and a potential use as wound healing agent has been suggested [29]. Solanachromene (**22**) (plastochromenol-8) contains a double bond in the chromanol ring and was found in aged flue-cured tobacco leaves [26, 31].



-tocomonoenols	$-R_1$	$-R_2$
α **13**	$-CH_3$	$-CH_3$
γ **14**	-H	$-CH_3$
δ **15**	-H	-H

-3,4-dehydrotocopherols	$-R_1$	$-R_2$
α **19**	$-CH_3$	$-CH_3$
β **20**	$-CH_3$	-H
γ **21**	-H	$-CH_3$

Fig. 5.4 Structures and substitution patterns of tocomonoenols (**13**–**16**), tocodienol (**17**), dehydrotocopherols (**19**–**21**), and plastochromanols (**18** and **22**)

Although described in textbooks, primary literature on tocopherylesters at C-6 is scarce [32, 33]. Acylesters of saturated fatty acids (C12:0, C14:0, C16:0, and C18:0) and tocopherols were found in *Nuphar luteum* and *Nymphaea alba* [33] and in the pulp of yellow bell pepper (*Capsicum annuum*) [32].

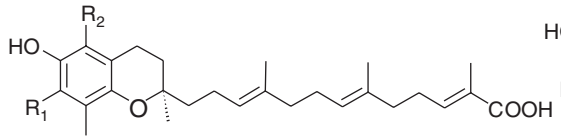
Garcinoic Acid, Amplexichromanols, and Litchtocotrienols

In the last decade, garcinoic acid (**23**) (*E*-13′-carboxy-δ-tocotrienol, δ-garcinoic acid), has been intensively studied (Fig. 5.5) [34]. The oxidation product of δ-tocotrienol was first isolated from *Clusia grandiflora* by Delle Monache et al. and later from the African bitter nut *Garcinia kola* and was further characterized for its chemical and physiological properties [34–39]. Garcinoic acid has been detected in different amounts within the Clusiaceae family including *Tovomitopsis psychotriifolia, Clusia obdeltifolia, Clusia burlemarxii, Clusia pernambucensis,* and *Garcinia kola* and together with γ-garcinoic acid (**24**) in the bark of *Garcinia amplexicaulis* [40, 41].

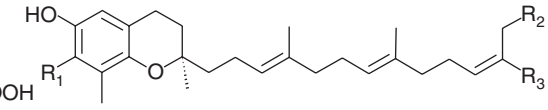
δ-Garcinoic acid exerts potent anti-inflammatory, antiproliferatory, and antibacterial properties [4, 34]. Recently, microsomal prostaglandin E_2 synthase has been identified as a possible target for its anti-inflammatory action [41]. δ- and γ-garcinoic acid inhibited the enzyme with IC_{50} values of 6.7 and 2.0 μM, respectively.

δ-Garcinoic acid reduced the growth of C6 cells and RAW264.7 mouse macrophages with an EC_{50} of 10 μM and 5 μM, respectively [34, 38]. As demonstrated by Maloney and Hecht, δ-garcinoic acid inhibits DNA polymerase β with an IC_{50} of about 4 μM [42].

As a by-product of the isolation of garcinoic acid, garcinal (**25**) (δ-(*E*)-garcinal), with a terminal aldehyde group, was found in the *G. kola* nut [37].



- garcinoic acid	-R_1	-R_2
δ **23**	-H	-H
γ **24**	-CH_3	-H



- amplexichromanols	-R_1	-R_2	-R_3
δ **26**	-H	-OH	-CH_2OH
γ **27**	-CH_3	-OH	-CH_2OH
δ-deoxy **28**	-H	-OH	-CH_3
γ-deoxy **29**	-CH_3	-OH	-CH_3

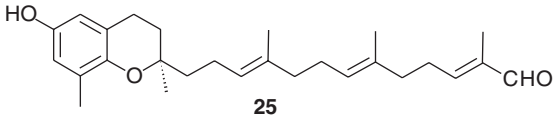


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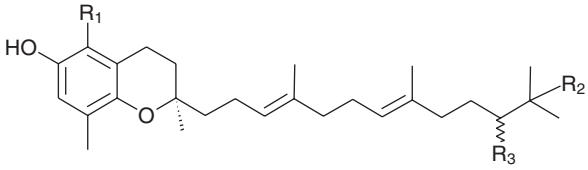
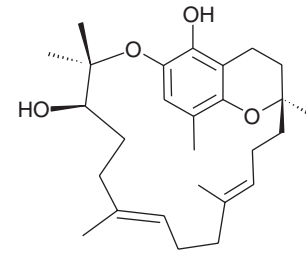
Fig. 5.5 Structures and substitution patterns of garcinoic acids (**23**–**24**), amplexichromanols (**26**–**29**), and garcinal (**25**)



Litchtocotrienol	-R_1	-R_2	-R_3
A **30**	-H	-OH	-OH (*R*)
B **31**	-OCH_3	-OH	-OH (*R*)
C **32**	-H	-OCH_3	-OH (*R*)
D **33**	-OCH_3	-OCH_3	-OH (*R*)
E **34**	-H	-H	=O
F **35**	-OCH_3	-H	=O
G **36**	-OCH_3	-OH	=O



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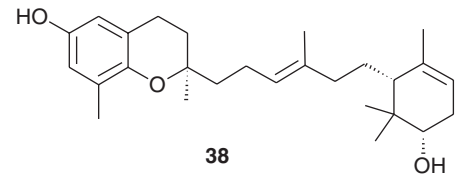

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Fig. 5.6 Structures and substitution patterns of litchtocotrienols (**30**–**38**) from *Litchi chinensis*

Several side chain-modified compounds with remarkable structural variability were isolated from the bark of *Garcinia amplexicaulis*, an endemic shrub from New Caledonia. δ- and γ-amplexichromanol (**26**) and (**27**) are terminal-hydroxylated δ- and γ-tocotrienols, respectively [40]. Both compounds inhibited capillary formation of vascular endothelial growth factor-induced human primary endothelial cells at 25 nM. Besides δ-(*E*)-deoxy-amplexichromanol (**28**) and γ-(*E*)-deoxy-amplexichromanol (**29**), several minor compounds such as two terminal aldehydes and a dihydroxy derivative were isolated from *Garcinia amplexicaulis* (Fig. 5.5) [40, 43].

The extraction of leaves of *Litchi chinensis* (Sapindaceae) resulted in the isolation of several side chain- and chromanol-modified δ-tocotrienols with anti-cancerogenic potential [44]. All litchtocotrienols were isolated in low yield. Litchtocotrienols A-G (**30**–**36**) are hydroxylated at C-11′, and E-F (**34**, **35**) contain a ketone group at C-11′ (Fig. 5.6). In addition, for litchocotrienols B, D, F, and G, a methoxy group is introduced at position C-5 of the chromane ring. The ansa-chromane macrolitchtocotrienol A (**37**) derives from an intramolecular condensation between C-12′ and C-6. Finally, cyclo-

litchtocotrienol A (**38**) with a cyclohexene ring within the side chain was isolated. All litchtocotrienols showed moderate cytotoxicity in HepG2 liver cells and gastric epithelial cells, with IC_{50} values ranging from 10 to 50 μM.

All isolated compounds from *Garcinia amplexicaulis* and *Litchi chinensis* show high structural similarity to side chain-modified tocochromanols from *Sargassum* species of marine algae.

Vitamin E Derivatives from Marine Organisms

Tocopherols and tocotrienols are well known to be produced by algae as well as marine invertebrates and microorganisms [45, 46]. Most interestingly, δ-tocotrienol (**8**) is widely distributed, especially in algae and sponges, and appears as the lead structure of most of the diverse compounds described in this and the following paragraph.

Among them, sargachromanols, sargachromenols, cystoseira metabolites, chromarols, epitaondiols, smenochromenes, and strongylophorines constitute the largest and best studied groups [4]. Antibacterial, antiviral, anti-inflammatory, and cytotoxic properties were attributed to these compounds, making them potential lead structures for drug development [4, 47].

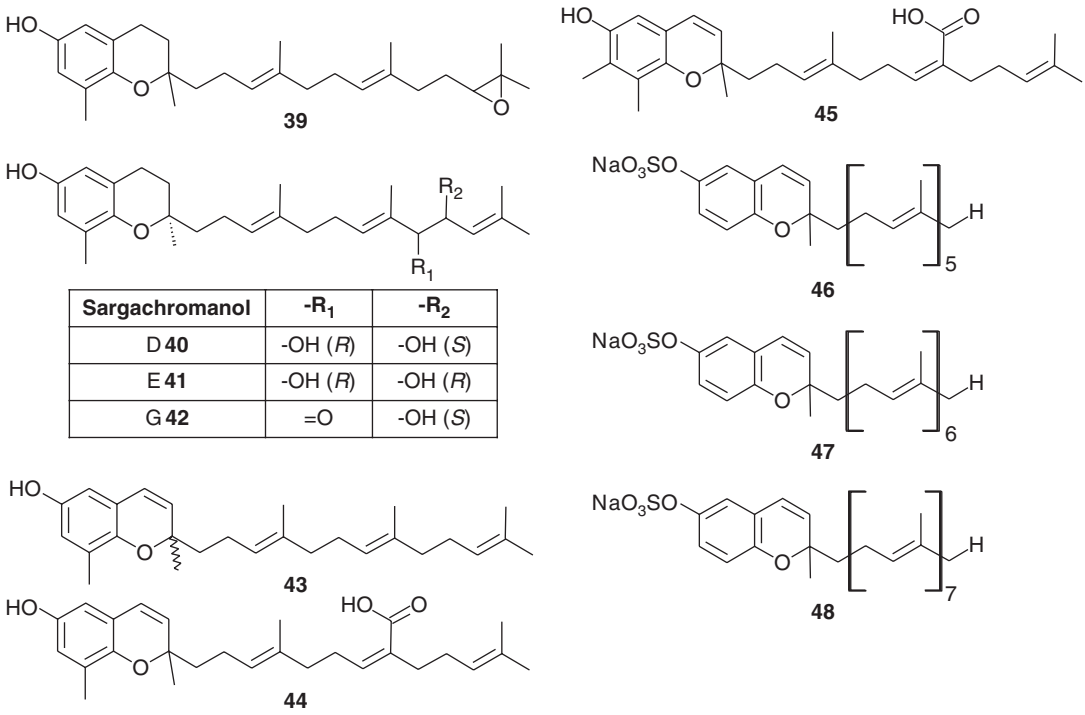
Sargachromanols, Sargachromenols, and Sarcochromenols

Sargachromanols and sargachromenols exhibit the largest structural diversity among meroditerpenes. The core structure of both congeners derives from δ-tocotrienol and δ-dehydro-tocotrienol, respectively. As an important biosynthetic precursor, δ-tocotrienol-11′-12′-epoxide (**39**) was the first sargachromanol discovered in brown algae (*Sargassum tortile)* by Kato et al. in 1975 [48]. It has been suggested that the activation of the terminal double bond leads to hydroxyl-, oxo-, and cyclic derivatives, respectively. Studies showed that δ-tocotrienol-11′-12′-epoxide induced the settling of swimming larvae of the hydrozoa *Coryne uchidai*, thus obviously acting as an intercellular signaling molecule [49].

Jang et al. isolated a series of sargachromanols (A to P) from *Sargassum siliquastrum* and characterized them by extensive two-dimensional nuclear magnetic resonance experiments [50]. Later, Lee et al. isolated the structures Q to S from the same species [51]. We only highlight the structures of the sargachromanols D, G, and E, since the biological properties of these compounds are well-studied. Sargachromanols D (**40**) and E (**41**) possess two hydroxyl groups at C-9′ and C-10′ and are diastereomers of each other (Fig. 5.7). Sargachromanol G (**42**) shows a C-9′ carbonyl and a C-10′ hydroxyl group. For further description of the chemical properties of other sargachromanols, the reader is referred to a recent review [4].

Sargachromanol D is a strong Na^+/K^+-ATPase ion pump inhibitor, with an IC_{50} value of 3.6 μM [52]. The study revealed that the hydroxyl group at C-9′ and/or C-10′ is important for the inhibitory activity. Similar to other side chain-modified tocochromanols, such as garcinoic acid or sargachromenol, the molecule reduced lipopolysaccharide (LPS)-induced production of nitric oxide and prostaglandin (PG) E_2 in murine RAW 264.7 macrophages and inhibited the expression of the pro-inflammatory enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [53]. In addition, the production of pro-inflammatory cytokines TNF-α, interleukin-1β (IL)-1β, and IL-6 was reduced by sargachromanol D. Since sargachromanol D showed dual antagonistic activity toward an L-type Ca^{2+}-channel and endothelin A/B_2 receptor, it was suggested as an antihypertensive agent [54].

Sargachromanol E and G were isolated from *Sargassum siliquastrum* for bioactivity studies [55–60]. Similar to sargachromanol D, both compounds inhibited the expression of pro-inflammatory



Sargachromanol	-R_1	-R_2
D **40**	-OH (*R*)	-OH (*S*)
E **41**	-OH (*R*)	-OH (*R*)
G **42**	=O	-OH (*S*)

Fig. 5.7 Structures and substitution patterns of δ-tocotrienol-11′-12′-epoxide (**39**), sargachromanols (**40**–**42**) from *Sargassum* species, sargaol (**43**), and sargachromenols (**44**–**48**)

cytokines in LPS-stimulated murine RAW 264.7 macrophages [55, 58, 59]. Sargachromanol E induced apoptosis of promyelocytic HL-60 leukemia cells via caspase-3 activation and inhibited ultraviolet A-induced aging of human dermal fibroblasts [60] [61]. Sargachromanol G (**42**) showed anti-osteoclastogenic effects on the expression of IL-1β-induced osteoclastogenic factors in the human osteoblast cell line MG-63 and suppressed the activation of nuclear factor κ B (NF-κB) and mitogen-activated protein kinase in receptor activator of NF-κB ligand (RANKL)-induced RAW264.7 cells [55, 56].

Sargaol (**43**) or dehydro-δ-tocotrienol is the potential biosynthetic precursor for most of the sargachromenols found in brown algae and was originally isolated from *Sargassum tortile* collected at the Japanese Tanabe Bay and showed high cytotoxic activity [62]. In contrast to sargachromanols, which were only found in algae, sargachromenols have been found in the fruits of the Amazonian Myristicaceae *Iryanthera juruensis* and *Iryanthera grandis* [63–65] and in the Mexican Asteraceae *Roldana barba-johannis* [66]. Interestingly, *Iryanthera* leaves were used by the indigenous population to treat infected wounds and cuts, and the latex of the bark was used against infections [65]. δ-Sargachromenol (**44**) was found as the active ingredient and was isolated in 0.4% and 0.8% yields (dry mass) from *Roldana* and *Iryanthera* species, respectively. δ-Sargachromenol is a δ-dehydrotocotrienol derivative bearing a carboxyl group at C-15′ of the side chain and is thus structurally related to δ-garcinoic acid (**23**) (Fig. 5.7). 7-Methyl-sargachromenol (**45**) (γ-sargachromenol) was isolated from the fruits of *Iryanthera juruensis* by Silva et al. [64].

δ-Sargachromenol is one the most investigated meroditerpenoid since it received attention in drug research because of its inhibitory activity against enzymes related to Alzheimer's disease, its strong anti-inflammatory activity, and its anti-hyperproliferative properties in skin cells. It was obtained from marine organisms and was named after the brown algae *Sargassum serratifolium*, from which it was first isolated by Kusumi et al. [67]. Sargachromenol is widely distributed in *Sargassum* species [4].

Several studies found sargachromenol to inhibit acetylcholinesterase and butyrylcholinesterase activity [68, 69]. In addition, the authors found that sargachromenol is a non-peptidic, noncompetitive inhibitor of β-site amyloid precursor protein-cleaving enzyme 1 with an IC_{50} value of 7.0 μM and a K_i value of 2.9 μM [69]. In line with these results, sargachromenol promotes neurite outgrowth and survival of rat PC12D pheochromocytoma cells via activating phosphatidylinositol-3 kinase [70]. Rengasamy et al. evaluated the drug-likeness of several natural products isolated from algae and found δ-sargachromenol (**44**) as good fit to Lipinski's "rule of five" [47], thus making δ-sargachromenol an interesting drug candidate for treating Alzheimer's disease and other neurodegenerative diseases.

Sargachromenol is a potent anti-inflammatory compound that prevented TPA-induced ear edema in mice and inhibits lipoxygenase (LOX) and cyclooxygenase (COX)-1 and − 2 [65, 66]. In addition, sargachromenol inhibited LPS-induced inflammation markers in murine RAW 264.7 macrophages and the production of PGE_2 and nitric oxide accompanied by a reduced protein expression of inducible nitric oxide synthase and COX-2 [71]. A similar study reported the inhibition of nitric oxide formation in LPS-stimulated murine microglial BV-2 cells and suppression of the release of TNF-α, IL-1β, and IL-6 [72]. Several markers of vascular inflammation were also decreased in primary endothelial cells by δ-sargachromenol, namely, TNF-α-induced ICAM-1 and VCAM-1 expression, adhesion of monocytes to HUVEC, and decreased production of monocyte chemoattractant protein-1 and matrix metalloproteinase-9 [73]. Sargachromenol binds to human farnesoid X receptor and inhibits its transactivation, which finally could lead to decreased plasma triacylglycerols and increased HDL cholesterol [74]. In conclusion, δ-sargachromenol (**44**) is a promising anti-atherogenic drug candidate.

Sarcochromenols A (**46**), B (**47**), and C (**48**) are a group of long-chain tocochromenols with five, six, and seven isoprene units, respectively (Fig. 5.7). They were isolated from the Pacific Ocean sponge *Sarcotragus spinulosus* (Schmidt) (family of Thorectidae) and showed Na^+/K^+-ATPase inhibitory activity similar to that of the sargachromanols D, F, H, and L (IC_{50} value for sarcochromenol A of 1.6 μM) [52, 75]. The compounds have also been isolated from the Indian sponge *Ircinia fasciculata* (Spongillidae) [76]. In addition, an un-sulfated form of sarcachromenol B was isolated in 0.1% yield.

Cyclic Meroditerpenes

Reports of cyclic meroditerpenes in plants are rare. To the best of our knowledge cyclolitchtocotrienol A (**38**) and walsurol (**49**) obtained from the bark of the Yunnan tree *Walsura yunnanensis* (Meliaceae) are the only meroditerpenes in higher plants that form a six-membered ring structure within the side chain [77]. In contrast, cyclic tocochromanols are widely distributed in marine species [4]. A fraction-guided screening for selective human 15-LOX inhibitors from an extract of the Papua New Guinean sponge *Psammocinia* (order of Dictyoceratida, family of Irciniidae) revealed chromarols A to D (**50**–**53**; Fig. 5.8) as potent inhibitors [78], with IC_{50} values of 0.6, 4.0, 0.7, and 1.1 μM, respectively. The authors observed a high selectivity for 15-LOX, since the IC_{50} values for 12-LOX were above 100 μM for all four chromarols. The biosynthesis of the cyclohexene ring system in the side chain of chromarols presumably derives from an acid-catalyzed cyclization as described for cyclolitchtocotrienol A (**38**) and walsurol (**49**) [4].

Taondiol (**54**) (Fig. 5.9) was the first polycyclic side chain derivative of tocotrienol that was isolated in 0.05% yield from *Taonia atomaria* (order Dictyotales) [79]. Its isomer, epitaondiol (**56**), was isolated from *Stypopodium zonale* and *Stypopodium flabelliforme*, and its bioactivity was intensively studied [80–89]. The polycyclic compound shows ichthyotoxic and antiviral activity and acts as an anti-inflammatory agent in vitro and in vivo [85, 88, 89]. Epitaondiol inhibited cell proliferation of several cancer cell lines and murine macrophages (RAW.267) [83]. Its epimer, 2β,3α-epitaondiol (**57**), exhibited moderate neurotoxicity toward mouse neuro-2a cells [90].

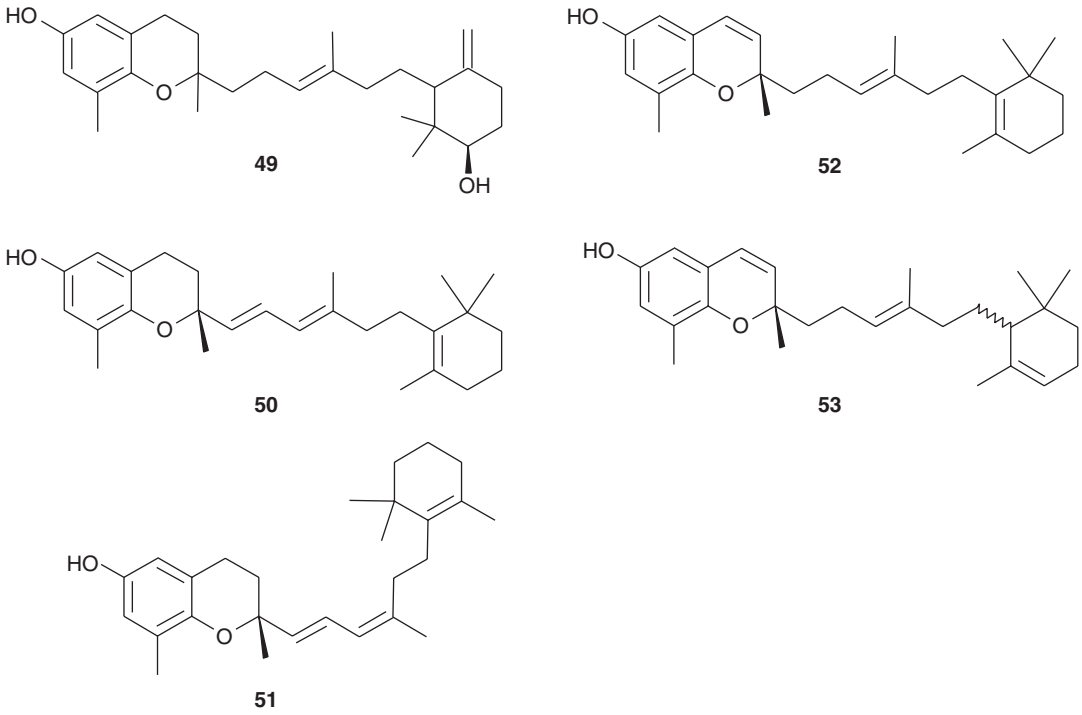


Fig. 5.8 Structures of cyclic meroditerpenoids walsurol (**49**) and chromarols A to D (**50**–**53**)

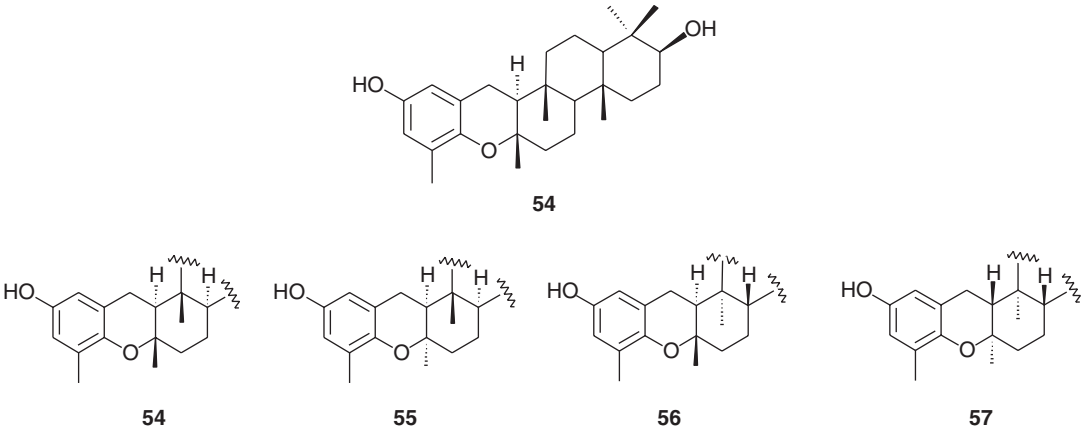


Fig. 5.9 Structures of cyclic meroditerpenoids taondiol (**54**), isoepitaondiol (**55**), epitaondiol (**56**), and 2β,3α-epitaondiol (**57**)

Areche et al. assigned the stereochemistry of isoepitaondiol (**55**), isolated from *Stypopodium flabelliforme*, to the formerly described isotaondiol [91]. By now, the structures of taondiol, isoepitaondiol, epitaondiol (**56**), and 2β,3α-epitaondiol (**57**) (Fig. 5.9) have been unambiguously assigned [80, 81, 91].

Bifurcarenone (**58**) was suggested to be the precursor of another class of cyclic diterpenes that were isolated from *Cystoseira mediterranea,* namely, the cyclic diterpenes, mediterraneols, cystoseirols, cystoketal chromanes, and bifurcarenone chromanes (Fig. 5.10).

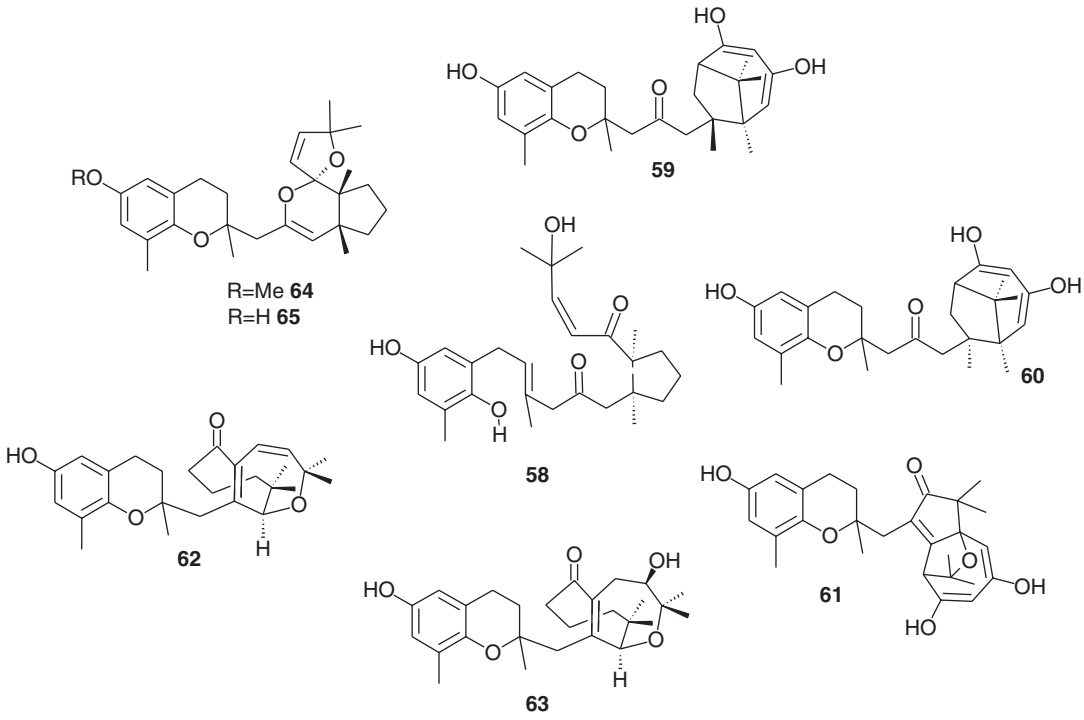


Fig. 5.10 Structures of bifurcarenone (**58**); mediterraneols C (**59**), D (**60**), and E (**61**); cystoseirols A (**62**) and B (**63**); and two cystoketal chromanes (**64**) and (**65**) from *Cystoseira* species

Mediterraneols C (**59**), D (**60**), and E (**61**) have been isolated as their trimethoxy derivatives from *Cystoseira mediterranea* in high yield [92, 93]. The complex structures of stereoisomers mediterraneols C and D comprise a bridged cyclooctane structure with two dienol moieties. Mediterraneol E (**61**) is a tricyclic oxygen-bridged diterpene with antineoplastic activity [92]. Mediterraneols have been found to inhibit the mobility of sea urchin sperm and the mitotic cell division of fertilized urchin eggs [93].

Bicyclic cystoseirols were isolated from *Cystoseira stricta*, *Cystoseira mediterranea*, and *Cystoseira tamariscifolia*. Cystoseirols A (**62**) and B (**63**) possess a oxabicyclo [5, 4, 1]dodecane ring that results from a single methyl group displacement, supplementary bridges, and ring fissions [92, 94–96]. Cystoseirol A (**62**) inhibited plant tumor formation in a crown-gall potato bioassay [94].

Two cystoketal chromanes (**64**) and (**65**) were isolated from the Sicilian brown alga *Cystoseira balearica* and *Cystoseira amentacea*, respectively. Structural elucidation revealed a tricyclic ring system within the side chain (Fig. 5.10) [97–99]. Demethoxy cystoketal chromane (**65**) showed cytotoxic activity with high selectively towards HepG2 cells (IC_{50} = 14.77 μg/ml) [98].

Besides algae, several sponges produce a large number of tocochromanols and tocochromenols. Sponges from *Strongylophora* species produce a group of polycyclic strongylophorines that resemble taondiol structural motives (Fig. 5.11). Strongylophorines contain a demethylated aromatic ring and modifications at the methyl groups at C-13′ and/or C-15′. They were discovered by Braekman et al. because of their ichthyotoxic activity [100]. The biosynthesis follows that of taondiol and is an enzyme-catalyzed cyclization cascade. Strongylophorines 2 (**66**), 3 (**67**), 4 (**68**), and 5 (**69**) were isolated from *Strongylophora* species from the Philippines [101, 102]. The molecules contain a cyclic lactone, a carboxy, an aldehyde, or a hydroxyl group moiety at C-13′, respectively. Strongylophorines 3, 9 (**70**), and 11 (**71**) were isolated from the Taiwanese species *Strongylophora durissima*. The 6-methoxy (**70**) and 6-acetyl (**71**) derivatives are structurally related to strongylophorine 2, which

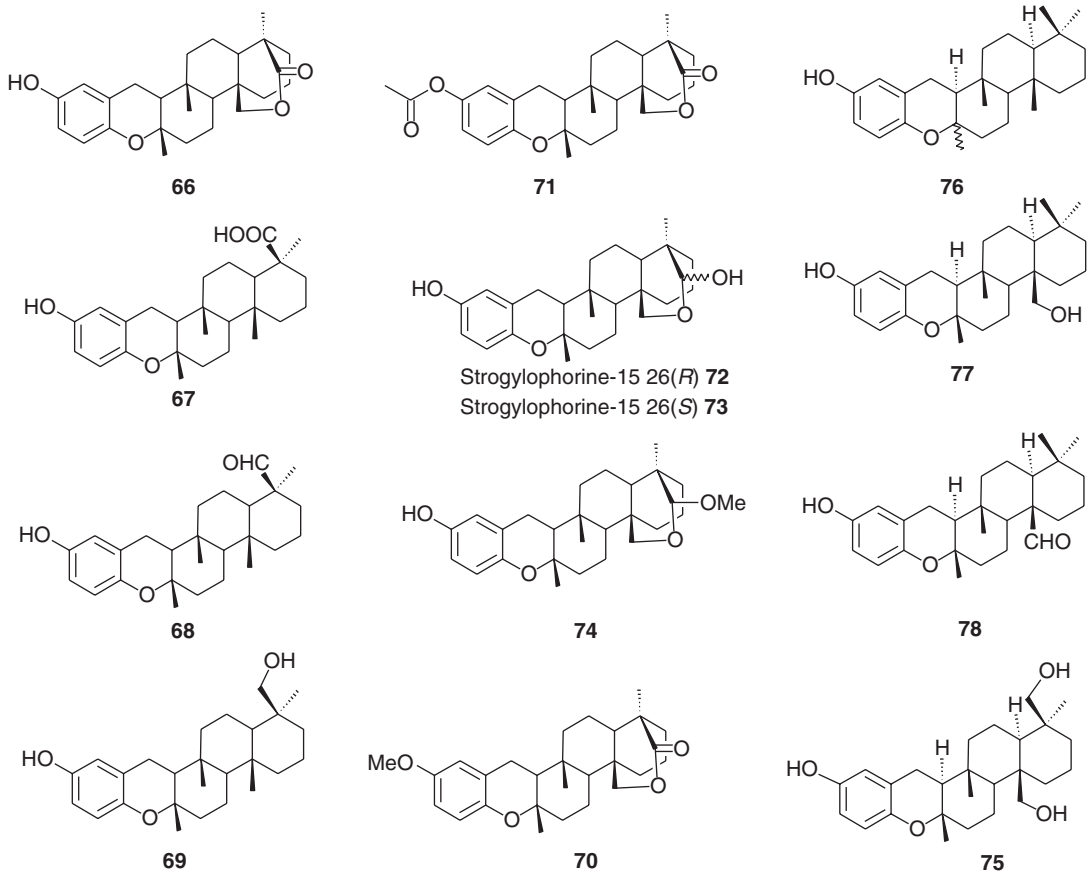


Fig. 5.11 Structures of strongylophorines (**66**–**78**) from *Strongylophora* species

contains a cyclic lactone moiety [103]. Strongylophorines 15 (26*R*) (**72**) and 16 (26*S*) (**73**) were isolated from the Okinawan sponge *Strongylophora strongylata* as epimers at the hemiacetal carbon [104]. Noda et al. found a mixture of strongylophorines 15 and 16 to be strong inhibitors of the proteasome with IC_{50} values of 3.6 μM [105]. Lee et al. found inhibitory activity for protein tyrosine phosphatase 1B for **74**, **66**, **67**, **72**, and strongylophorine 17 (**75**) with IC_{50} values of 8.5, 24.4, 9.0, 11.9, and 14.8 μM, respectively [106]. Strongylophorines 2 and 3 inhibited hypoxia-inducible factor-1-dependent luciferase expression in engineered U251-HRE glioma cells with EC_{50} values of 8 and 13 μM [107]. Strongylophorines 22 (**76**), 23 (**77**), 24 (**78**), and 17 (**75**) were isolated from the Okinawan sponge *Petrosia corticata* and displayed moderate cytotoxic activity against human cervical carcinoma epithelial (HeLa) cells [108]. All strongylophorines exhibited ichthyotoxic, insecticidal, antibacterial, fungicidal, and cytotoxic properties.

Conclusions

With some exceptions, all higher plants produce side chain-saturated tocopherols with the typical methylation pattern α-, β-, γ-, and δ-, respectively. Tocopherols and their chemical and biological properties are dominantly described in the literature. The meroditerpenes presented here show a remarkable structural variability. Side chain modifications by oxidation and/or cyclization occur

widely, especially in marine organisms. Cytochrome P_{450} enzymes are most likely responsible for the initial oxidation to epoxy, hydroxy, and carboxy derivatives, respectively, although the corresponding enzymes were studied only in animal vitamin E metabolism and are not fully understood yet [109, 110]. Marine organisms, led by brown algae (Phaeophyceae), cover most of the minor derivatives described here, followed by plants and sponges. Interestingly, sponges (Porifera) produce mainly cyclic di- and sesquiterpenes. 8-Methyl- or desmethyltocotrienol moieties were found in most of the diterpenes isolated from marine organisms.

The primary biological function of the side chain modifications remains unclear. On the one hand, ichtyotoxicity, algicidal, and anti-macroalgal activities were found for several metabolites in marine species, and, thus, side chain-modified metabolites are presumably used as chemical protectants. However, recent advances in the research on human vitamin E metabolites suggest specific biological activities [111, 112]. Most of the compounds described in this chapter influenced arachidonic acid metabolism and the synthesis of pro-inflammatory cytokines. Inhibition of COX-1 and COX-2 expression, respectively, reduced prostaglandin metabolite formation and inhibition of 5- and/or 12-LOX blocked leukotriene synthesis. Further studies will have to reveal if meroterpenoids have the potential to be developed into anti-inflammatory drug candidates. In line with the anti-inflammatory activities, side chain-modified chromanols showed the strongest cytotoxic activity against cancer cells. Antiproliferative and cytotoxic properties were modulated by the presence of hydroxyl and carboxy groups [4].

Rengasamy et al. evaluated the drug-likeness of several natural products isolated from algae and found δ-sargachromenol (**44**) and epitaondiol (**56**) as good fits to Lipinski's "Rule of Five" [47]. This rule estimates the potential of a drug candidate based on physiochemical properties, such as molecular weight, number of hydrogen bond acceptors and donors, and distribution coefficient (logP) [47, 113]. We and others tested several vitamin E metabolites for their biological activities in vitro and in vivo and found them to have antibacterial, antiviral, anti-inflammatory, and cytotoxic properties. In general, any modification of the prenyl side chain increased their biological activity.

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Chapter 6
Bioactivity of Vitamin E Long-Chain Metabolites

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Keywords Vitamin E · Long-chain metabolites of vitamin E · 13′-hydroxychromanol (13′-OH) 13′-carboxychromanol (13′-COOH) · Vitamin E metabolism · Biological activity

Key Points

- Metabolic activation of vitamin E precursors by hepatic catabolism
- Unknown distribution mechanisms and storage of LCMs (i.e., occurrence in organs and tissues)
- Detection of both α-LCMs in complex biological matrices
- Involvement of the LCMs in various regulatory processes
- Evidence for a general concept of metabolic activation for fat-soluble vitamins

Abbreviations

Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
APCI	Atmospheric pressure chemical ionization
α-TTP	α-Tocopherol transfer protein
ABCA1	ATP-binding cassette transporter A1
13′-COOH	Carboxychromanol

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_6

CEHC	Carboxyethylhydroxychromanol
CD36	Cluster of differentiation 36
CYP	Cytochrome P450
ECD	Electrochemical detector
ESI	Electrospray ionization
FID	Flame ionization detector
FLD	Fluorescence detector
GC	Gas chromatography
HPLC	High-pressure liquid chromatography
13′-OH	Hydroxychromanol
ICM	Intermediate-chain metabolite
LCM	Long-chain metabolite
LDL	Low-density lipoprotein
LDLR	LDL receptor
LRP	LDL receptor-related protein
MS	Mass spectrometry
LC3	Microtubule-associated protein 1 light chain 3
PBMC	Peripheral blood mononuclear cell
P-gp	P-Glycoprotein
PARP	Poly-ADP ribose polymerase
Q-TOF	Quadrupole time-of-flight
ROS	Reactive oxygen species
SR-B1	Scavenger receptor B1
SCM	Short-chain metabolite
SPE	Solid phase extraction
TOH	Tocopherol
T3	Tocotrienol
UV-Vis	Ultraviolet visible spectroscopy
VLDL	Very low-density lipoprotein

Formation of Vitamin E Long-Chain Metabolites

The metabolism of vitamin E is initiated by an ω-hydroxylation via cytochrome P450 (CYP) 4F2/3A4, resulting in the formation of 13′-hydroxychromanols (13′-OH). The subsequent ω-oxidation forms 13′-carboxychromanols (13′-COOH). Subsequent cycles of β-oxidation shorten the side chain and finally result in water-soluble carboxyethylhydroxychromanols (CEHCs or 3′-COOH). A more detailed description is here provided in the chapter "Vitamin E metabolism." The knowledge about the regulatory processes of vitamin E metabolism is sparse, since the responsible enzymes are largely unknown (except CYP4F2 and CYP3A4) or have not yet been experimentally confirmed. Torquato et al. provided first hints by the observation of the upregulation of CYP4F2 protein by α-13′-OH in human HepG2 liver cells, indicating a positive regulatory feedback loop [1]. Whether this holds true for the other long-chain metabolite (LCM) forms, i.e., β-, γ-, or δ-13′-OH, or even for other metabolites (e.g., α-13′-COOH), is subject of further investigations. The enzymes that are responsible for the ω- and β-oxidation have not yet been experimentally validated, but Mustacich et al. described the organelles that are responsible for the metabolism of vitamin E and suggested the respective enzyme classes [2]. Based on this work, the identification of the aldehyde and alcohol dehydrogenases that are responsible for the ω-oxidation of 13′-OH is possible. Furthermore, the enzymes that are responsible for the degradation of branched-chain fatty acids have been suggested to degrade also the side chain

of vitamin E. However, this also requires validation in future studies. The successful identification of the respective enzymes is a vital starting point for unraveling regulatory mechanisms of vitamin E metabolism.

Distribution of Vitamin E Long-Chain Metabolites in the Human Body

Hepatic Formation and Transport of Vitamin E Long-Chain Metabolites

Storage and metabolism of vitamin E are strictly balanced in healthy humans to ensure constant and sufficient supply. Therefore, hepatic metabolism of vitamin E is regulated by a physiological feedback loop to avoid excessive accumulation of vitamin E and to bridge periods of vitamin E deficiency. All ingested vitamin E forms are first transferred via chylomicrons to the liver, but α-tocopherol (TOH) is selectively bound by the α-tocopherol transfer protein (α-TTP) [3] to direct it either for release or metabolism in different cellular compartments, the endoplasmic reticulum, the peroxisomes, and the mitochondria (for details, the reader is referred to the chapter "Vitamin E metabolism"). Hence, the intermediate metabolites of vitamin E must be transported between these compartments during their metabolic degradation. But, no experimental data is available whether this inter-compartment transport occurs by passive diffusion or is conducted by active transport via specific proteins. However, non-metabolized vitamin E is released from the liver, and the contribution of very low-density lipoproteins (VLDL) [4], oxysterol-binding proteins [5], and the ATP-binding cassette transporter A1 (ABCA1) [6] to this process has been discussed. The chemical structure of the α-13′-long-chain metabolites (α-13′-LCMs) and α-TOH is similar, except for the terminal oxidation of the aliphatic side chain. Therefore, the lipophilic nature of these molecules may be similar, and their transport via VLDL is conceivable but needs to be verified in future studies. In addition, the intracellular transport of the LCMs may occur via specific LCM-binding and LCM-transport proteins or via binding and transport proteins jointly used for α-TOH (e.g., α-TTP [3], tocopherol-associated protein [7]) or fatty acids (e.g., fatty acid-binding proteins [8]), since α-TOH follows the general transport pathway of lipids [9]. However, whether the transport of the LCMs is realized by specific (i.e., binding and transport proteins) or unspecific (i.e., via lipoproteins) mechanisms remains unclear.

Extrahepatic Transport and Storage

Only limited information about the transport of the LCMs in blood and their bioavailability in extrahepatic tissues is yet available. α-13′-OH [10] and α-13′-COOH [11] have been found in human serum, and increased serum concentrations after supplementation with 1000 IU α-TOH/d were observed [10, 11]. It might be possible that their extrahepatic transport in blood occurs via lipoproteins as it has been described for α-TOH [12]. Vitamin E in general is packed into chylomicrons after intestinal absorption and later transferred to other lipoproteins like HDL via phospholipid transfer protein, or it remains in chylomicron remnants. After the hepatic uptake, discrimination for α-TOH and resecretion into the blood via VLDL take place. According to the known fate of VLDL, α-TOH also occurs in LDL and HDL particles [13]. However, further studies are needed to clarify the involvement of lipoproteins or specific binding proteins in the distribution of the LCMs. The cholesteryl ester transfer protein, another member of the protein family of serum lipid transfer proteins, is also discussed to contribute to vitamin E transport and metabolism [14] and may also be involved in the transport of the LCMs. Until today, no specific plasma transport proteins for α-TOH [12, 15] nor the LCMs have been described.

It has been shown that normal vitamin E plasma levels of 25 μM can be increased about threefold by vitamin E supplementation, whereas α-13′-COOH serum levels increased even about eightfold. Preliminary studies in mice showed that α-13′-COOH reaches its highest concentrations in plasma 6 h after injection (unpublished data). Based on the increase of the LCMs after supplementation and the fast rise of plasma concentration and clearance from blood, LCMs might reflect the current nutritional status with respect to vitamin E. However, this hypothesis needs confirmation under non-supplemented normal conditions. As adipose tissue and skeleton muscles have been identified as long-term storage depots for vitamin E and the LCMs exhibit structural similarities to their respective vitamin E precursors, similar storage characteristics are conceivable [16, 17]. Hence, continuous release of the LCMs from these depots is likely. Nevertheless, a reliable analysis of tissue distribution and tissue-specific concentration of the LCMs is needed to draw conclusions about their bioavailability, stability, overall physiological relevance, and relevance in single organs. Thereby, the potential of these molecules for the therapeutic treatment of organ-related diseases such as nonalcoholic fatty liver disease should be studied. Next, investigations of putative regulatory feedback loops for the uptake of the LCMs are needed. Beside the major storage organs and tissues known for α-TOH (i.e., fat and muscles), the accumulation of α-TOH and its corresponding metabolites has also been shown for the kidney and small intestine [18]. Further, the analysis of the concentrations of the LCMs in the brain, since vitamin E deficiency is known to cause cognitive dysfunctions [19], as well as the heart, where the concentration of α-TOH increases after supplementation [20], is of interest. In addition, α-TTP is expressed in the placenta, indicating the importance of α-TOH for preventing fetus resorption [15]. However, to date there is no data available on the contribution of α-TTP to the tissue distribution of the LCMs. Crucial for the binding specificity of α-TTP is the substitution of the chroman ring system of possible ligands as well as the *R*-configuration at the 2′-position [3]. The phytyl side chain is very flexible, thus barely contributing to the specificity [21]. As the α-LCMs differ only in the terminal modification of the phytyl-like side chain from α-TOH, they may be likely bound by α-TTP with good affinity. Supporting this assumption, α-TOH analogues with terminal side chain modifications, like nitrobenoxadiazyl (NBD)-α-TOH and anthroyloxy (AO)-α-TOH, bind α-TTP and are in use to investigate functions of α-TPP [22, 23]. Hence, terminal modification of the α-TOH side chain does not prevent binding to α-TTP. However, the final confirmation of the binding of the α-LCMs to α-TTP is pending. Apart from human plasma or serum [11, 24], the concentrations of the LCMs in different organs or tissues have not been investigated. Given that only 1% of total body TOH is located in the blood [25], most of the vitamin E is stored in other parts of the body. To date, only serum concentrations of the LCMs are known, and it is possible that other tissues display higher concentrations of the LCMs than the blood.

Intercellular Transport and Intracellular Distribution

In addition to information about the accumulation of the LCMs in organs, investigations of uptake mechanisms for the LCMs into cells and cellular compartments will provide valuable information about intracellular distribution patterns. Initial experiments in human skin fibroblasts, human THP-1 macrophages, and human HepG2 liver cells revealed similar uptake kinetics for the LCMs (unpublished data), but further experiments are needed to characterize the transport mechanisms. As indicated above, the transport via lipoproteins, as known for α-TOH, has not yet been shown for the LCMs. The data on LCM uptake from in vitro experiments indicate for an independent transport mechanism but do not exclude lipoprotein-specific mechanisms. Hence, internalization of particles carrying the LCMs might involve among others the LDL receptor (LDLR) or the LDL receptor-related protein (LRP), as known for α-TOH [13]. In addition, the scavenger receptor B1 (SR-B1) and ABCA1 are of particular importance within the distribution pathways of vitamin E [14]. SR-B1 mediates the uptake of vitamin E into the intestine and in peripheral tissue, whereas LDLR and LRP

mediate the uptake into the liver. The efflux of vitamin E via ABCA1 takes place in the intestine and the liver. In addition, ABCA1 regulates the cellular efflux of vitamin E in macrophages and fibroblasts [6]. Based on its lipophilic nature, vitamin E and thus most likely also the corresponding LCMs are present in the endoplasmic reticulum as well as the peroxisomes [26]. Besides α-TTP, further intracellular binding proteins, such as the tocopherol-associated protein and the tocopherol-binding protein, are known to be involved in intracellular trafficking of α-TOH [27]. Further, the multidrug resistance transporter P-glycoprotein (P-gp) has been identified to mediate α-TOH transport across the plasma membrane and its secretion into bile [28]. All members of this protein family bind α-TOH with lower affinity than α-TTP [13]; however, their relevance as transporters of the LCMs remains unclear. Further experiments are needed to clarify the importance of α-TOH-related binding proteins, such as afamin [29], as well as the uptake mechanisms and kinetics that are responsible for the cellular and tissue distribution of the LCMs and hence their regulatory effectiveness.

Excretion

The metabolism of vitamin E finally forms the hydrophilic end-product α-CEHC, which is excreted via urine or secreted into the circulatory system [18]. In contrast, free α-13′-OH has been found to be excreted with feces [30], as shown earlier for γ-TOH and α-TOH [31]. Therefore, the bioavailability-to-excretion ratio of the LCMs needs to be investigated in further studies. A useful experimental tool to observe and analyze the formation of metabolites in humans is to use deuterium-labeled α-TOH [32]. As shown by Freiser et al., the γ-LCMs are mainly conjugated with sulfates [33] and glucuronides in plasma and are likely excreted. However, biological activity is only possible if the LCMs are available in unconjugated form [34]. To gain deeper insights into the pharmacokinetics of vitamin E, in particular into the formation of the metabolites as well as their bioavailability and distribution in tissues and excretion, robust and reliable analytical tools are indispensable.

Analytical Approaches for Vitamin E Metabolites

In the early days of vitamin E research, simple chromatographic methods were used to separate the different forms of vitamin E from other lipids and lipophilic molecules such as triglycerides and phospholipids. Thin-layer chromatography was one of the early approaches to detect vitamin E in biological samples [35]. Next, the development of gas chromatography (GC) and high-pressure liquid chromatography (HPLC) approaches allowed for better separation of the different vitamers [36, 37]. By coupling of either GC or HPLC to high-sensitive mass spectrometry (MS), vitamin E forms could be detected even in nanomolar concentrations within different matrices [38, 39]. Next, the discovery of vitamin E metabolism in animals and humans and emerging evidence for important biological functions of vitamin E metabolites made it necessary to enhance the existing analytical procedures for vitamin E [40]. All vitamin E forms undergo enzymatic modification (sulfation and glucuronidation) during their metabolic degradation, complicating their detection in biological samples. Thus, only a few research groups were able to establish valid methods for the determination of vitamin E metabolites in human matrices (reviewed in [40]), and so far only a few detected the LCMs in human blood [10, 11, 24, 41].

The different physicochemical properties of the vitamin E metabolites are major criteria for the development of appropriate analytic strategies. Short-chain metabolites (SCMs) and LCMs were found as sulfates, glucuronides, and glucosides or as unconjugated carboxychromanols in different matrices [40]. Therefore, the quantitative extraction of these metabolites from biological samples requires enzymatic deconjugation using sulfatase and β-glucuronidase or chemical hydrolysis.

Table 6.1 Subset of analyzable metabolites in biological samples with their corresponding preparation and detection methods

Deconjugation	Extraction	Chromatography	Sample	Metabolite	Ref.
–	LL	GC-MS	Cell culture supernatant (HepG2)	γ-7′,9′,11′,13′-COOH γ-13′-OH	[43]
S	LL	HPLC-FLD LC-MS (ESI(−))	A549 cells	γ-9′,11′13′-COOH γ-13′-OH γ-9′S,11′S,13′S-COOH δ-9′,11′,13′-COOH δ-9′S,11′S,13′S-COOH	[44]
			Rat plasma (200 µl)	γ-9′S,11′S,13′S-COOH γ-13′-COOH, γ-13′-OH α-TOH, γ-TOH	
			Rat liver (homogenate of the entire organ)	γ-9′S,13′S-COOH γ-13′-COOH, γ-13′-OH α-TOH, γ-TOH	
–	–	HPLC-UV-Vis	Cell culture supernatant (HepG2)	α-5′,13′-COOH α-13′-OH	[48]
S + G	LL	HPLC-ECD LC-MS (ESI(−))	Human serum (20 µl)	ICM, SCM, TOH	[49]
			Human urine (20 µl)	ICM, SCM	
			Human feces (10 mg)	α-3′,5′,13′-COOH γ-3′,11′-COOH δ-3′,11′-COOH α-TOH, γ-TOH, δ-TOH α-T3, γ-T3	
S + G	LL	Q-TOF-LC-MS (ESI(+))	Human serum (500 µl)	α-13′-COOH	[11]
–	LL	HPLC-ECD GC-MS	Human serum (1 ml)	α-13′-OH	[10]
n.i.	LL	LC-MS/MS (APCI)	Human serum (1 ml)	α-13′-COOH α-13′-OH	
S + G	LL	LC-MS/MS (ESI(+))	Human plasma or serum (500 µl)	α-3′,13′-COOH γ-3′-COOH α-13′-OH	[24]
			Human plasma or serum (100 µl)	α-TOH, γ-TOH	

Abbreviations used are the following: *n.i.* no information, *LL* liquid-liquid, *GC* gas chromatography, *HPLC* high-pressure liquid chromatography, *LC* liquid chromatography, *MS* mass spectrometry, *FLD* fluorescence detector, *S* sulfatase, *G* β-glucuronidase, *ESI* electrospray ionization, *APCI* atmospheric pressure chemical ionization, *SCM* short-chain metabolite, *ICM* intermediate-chain metabolite, *T3* tocotrienol, *TOH* tocopherol, *OH* hydroxychromanol, *COOH* carboxychromanol

The SCMs have been measured in urine, plasma, feces, cell extracts, and other biological fluids (e.g., bile), with analytical strategies adopted to their chemical characteristics, including water solubility and chemical conjugation. In contrast, the LCMs exhibit a more lipophilic nature and thus occur in feces, cells, tissues, and blood but not in urine. Hence, analytical strategies for the LCMs focus on their lipophilic properties and their occurrence as sulfated derivatives (especially the carboxychromanols) [40]. A brief overview of a subset of analyzable metabolites and the corresponding methods for preparation and detection is provided in Table 6.1.

Specifications of Long-Chain Metabolites

The vitamin E metabolites with a side chain length between 13 and 9 carbon units are summarized as LCMs, with 13′-OH and 13′-COOH being the first metabolites formed from their metabolic precursors, the TOHs or tocotrienols (T3s) [42]. The first analytical approach for the detection of γ-13′-OH,

γ-13′-COOH, γ-11′-COOH, and γ-9′-COOH has been published in 2002 by Sontag and Parker, who used a GC-coupled MS-based approach for the determination of these metabolites in the culture supernatant of HepG2 liver cells that have been incubated with *RRR*-γ-TOH [43]. This finding was confirmed by Jiang and coworkers who detected γ- and δ-LCMs in the cell culture supernatant of human lung epithelial A549 cells. Especially the carboxychromanols were detected in their sulfated form, indicating that acid metabolites are preferred for this type of chemical modification [44]. The sulfate modification can be removed by enzymatic deconjugation, leading to facilitated detection and improved analytical recovery of the acid metabolites [41]. Hence, Jiang and coworkers detected for the first time the γ-LCMs in complex matrices, such as rat liver and plasma [44]. At about the same time when Sontag and Parker published their first analytical approach for the detection of the γ-LCMs, Azzi and coworkers discovered gene regulatory actions of α-TOH, a finding that was also confirmed for the corresponding LCMs [45, 46]. Further, recent insights into the metabolism of vitamin E showed that *RRR*-α-TOH is the preferred form for hepatic uptake, rendering α-TOH as the most relevant vitamin E form with biological activity in humans [42, 47]. Based on these findings, the focus of analytical interest switched from the γ- to the α-TOH LCMs. In 2010, Birringer and coworkers detected for the first time α-13′-OH and α-13′-COOH in the culture supernatant of HepG2 liver cells enriched with *RRR*-α-TOH [48].

In the same year, Mustacich and coworkers used a LC-MS technique to analyze α-13′-OH in rat liver microsomes, presenting the first determination of an α-LCM in a complex matrix [2]. Further, the first determination of the α-LCMs in humans was done by the group of Zhao et al., who detected α-13′-COOH in human feces [49]. In 2014, 12 years after Azzi and coworkers published their hypothesis outlining a gene regulatory role for α-TOH, Wallert et al. found α-13′-COOH in human serum, indicating a systemic relevance in humans. In this study, 500 μl serum of a healthy, middle-aged (39 years), nonsmoking male, who received a balanced diet with no additional supplementation of vitamin E, was used for the detection of α-13′-COOH via quadrupole time-of-flight (Q-TOF) LC-MS [11]. This study provided first evidence that the α-LCMs are transferred into blood circulation after α-TOH has been metabolized in the liver. Further, Wallert and coworkers showed in a cell model that α-13′-OH and α-13′-COOH are more potent regulators of gene expression than their metabolic precursor [11]. Taken together the results of Wallert et al. indicate that the LCMs are a more active form than their precursor molecules that might promote regulatory effects in peripheral tissues of the human body. Only 1 year later, α-13′-OH was also found in human serum using a GC-MS approach [10]. Based on these two analytical approaches, current analytical research is focused on the development of analytical strategies enabling the simultaneous determination of all LCMs and their respective metabolic precursors. The first attempt was made in 2016, when Torquato et al. used LC-MS/MS combined with atmospheric pressure chemical ionization (APCI) (−) for the detection of α-13′-OH and α-13′-COOH in the same analytical session [41]. This analytical approach has been confirmed by Giusepponi and coworkers by using LC-MS/MS with a different type of ionization (electrospray ionization (ESI) (+)) [24]. In 2012, Bardowell and coworkers were able to determine 12′-OH and 11′-OH in the feces of mice fed a γ-TOH-enriched diet [50]. The detection of these metabolites in feces provided evidence for ω-1 and ω-2 hydroxylation activity and that 12′-OH cannot undergo oxidation followed by side chain truncation. Therefore, this metabolite is excreted via bile and can be found in the feces of mice and humans [50].

Sample Preparation

In the early days of vitamin E research, solid extraction of lipid fractions from biological matrices was the only challenge in a preparation procedure. With the growing knowledge about chemical properties, metabolic pathways, and tissue distribution of vitamin E metabolites, the challenges got more difficult and complex. Today, issues such as conjugation, oxidation, chemical differences between the single compounds, and even their appearance in various matrices must be considered. While the LCMs can be detected in feces, cells, tissues, and blood, the SMCs are mostly found in biological

fluids, such as bile, urine, and plasma, but also in feces, isolated cells, and solid tissues [40]. To increase the recovery of vitamin E metabolites, cell and solid tissue samples must be homogenized, and complex lipids need to be hydrolyzed before lipid extraction. During these processes, antioxidants like butylated hydroxytoluene, ascorbic acid, and pyrogallol can be added to avoid autoxidation of the metabolites [11, 51].

The use of analytical standards is essential to assess metabolite recovery during workup and analysis. Therefore, authentic compounds or stable isotope-labeled synthetic analogues need to be added at the beginning of the sample preparation. In the case of the 13′-OH and 13′-COOH LCMs, no analytical standards are commercially available, and the molecules must be synthesized or semi-synthesized from natural compounds [34, 48, 52]. Therefore, Wallert and coworkers used garcinoic acid, a natural compound occurring in the nuts of the African plant *Garcinia kola* (reviewed in [53]) for the semi-synthesis of α-13′-COOH and α-13′-OH as well as δ-13′-COOH and δ-13′-OH [11].

Another important step of the preparation procedure is the enzymatic deconjugation of the vitamin E metabolites. The LCMs as well as the SCMs mostly appear as sulfated or glucuronidated conjugates in biological samples [11, 51]. These conjugates are a result of the chemical modification during the hepatic metabolism and can lower the recovery of their corresponding metabolites. Freiser and coworkers reported that especially the acid forms of the LCMs are conjugated, with a predominance of the sulfate conjugates [54]. This mismatch between sulfation and glucuronidation has been confirmed in various studies, indicating that enzymatic sulfation could be the predominant phase II reaction in vitamin E metabolism [55–57]. The enzymatic hydrolysis of conjugated metabolites with β-glucuronidase and sulfatase appeared as a reliable method for the workup of biological fluids or tissues in different publications [11, 51]. Wallert and coworkers incubated 500 µl human serum for 30 min (at 34 °C) with a combination of 1500 IU β-glucuronidase and 26 IU/ml sulfatase for enzymatic deconjugation. This procedure led to a higher recovery of the unconjugated acid LCMs, enabling the first determination of α-13′-COOH in human serum [11]. In addition, the application of methanolic HCl for the deconjugation of CEHCs in urine samples appeared to be more efficient than enzymatic hydrolysis [56]. Beneath the strategy of enzymatic deconjugation, Pope and coworkers tried to analyze the conjugates directly using MS (ESI) to avoid artificial production of Simon metabolites by deconjugation steps. This method showed promising results for CEHC but has to be improved further for the general determination of vitamin E metabolites [58].

In most analytical studies on vitamin E, liquid-liquid extraction was used for the purification of the metabolites and their metabolic precursors from different matrices [10, 11, 24, 44, 49]. Wallert and coworkers performed liquid-liquid extraction with a mixture of hexane and dichloromethane (ratio 5:2) containing 1% butylated hydroxytoluene. The serum samples were mixed with solvent for 1 min at room temperature and were then centrifuged (2000 × g, 15 min, 10 °C) to achieve the separation of organic and inorganic layers. The upper organic phase was collected in glass tubes, dried under N_2, and resuspended in 50 µl methanol [11]. Solid-phase extraction (SPE) is another way to extract vitamin E metabolites from biological matrices. Yang et al. isolated γ-13′-OH, γ-13′-COOH, γ-11′-COOH, and γ-9′-COOH from cell culture medium of A549 cells with a C_{18}-SPE cartridge, using acetic acid for metabolite elution [59]. Next, Wallert and coworkers are currently working on a SPE-based method for the extraction of the LCMs from human blood (unpublished data). Here, only 100 µl plasma will be used for metabolite extraction, providing a significant advantage compared to the liquid-liquid extraction-based alternatives, which require 500 µl plasma.

Detection of Vitamin E Long-Chain Metabolites

LC-/HPLC Analysis

HPLC-based analysis of vitamin E metabolites is performed with either normal phase or reverse phase columns coupled to electrochemical (ECD), fluorescence (FLD), UV-Vis, or evaporative light scattering detectors, with ECD being the most sensitive for vitamin E determination. Therefore,

HPLC-ECD has been used by Zhao and coworkers to detect TOH metabolites in human feces [49]. Fluorescence detection has lower sensitivity than ECD, and the response for some of the physiological metabolites is too low for applications involving human samples. However, FLDs have been also used for the determination of TOHs, T3s, and their corresponding metabolites in cell culture supernatants [44], rat plasma and liver [44, 54], and fetal bovine serum [57]. LC-MS/MS is the most widely used technique for the determination of the LCMs, providing an accurate quantitative analysis of these compounds in various biological matrices [24, 41, 57, 60]. Hence, LC-MS/MS enabled the first simultaneous detection of α-13′-OH and α-13′-COOH in human serum [1]. Further, Jiang et al. showed that negative polarity (ESI(−)) LC-MS/MS can be also used to quantify conjugated and unconjugated vitamin E metabolites in rodent blood [57].

GC Analysis

GC-based methods for the analysis of vitamin E are either coupled to flame ionization detectors (FID) or MS detectors. In contrast to HPLC-based methods, GC-based analysis of vitamin E metabolites requires an additional derivatization step for TOH and carboxychromanols [43, 51]. The purified extracts are heated and silylated with N-methyl-N-trimethyl-silyltrifluoroacetamide (MSTFA) or N,O-(bis-trimethylsilyl) trifluoroacetamide (BSTFA) to accomplish derivatization [61]. This additional procedure is required for the detection of TOH and its metabolites by GC-based separation and detection. Traditionally, FID is the most often used detector in GC, due to its high response to organic molecules, but nowadays this detection technique is more and more replaced by MS, allowing a more sensitive detection of vitamin E metabolites. Therefore, GC-MS-based methods have been used for metabolite detection in cell culture supernatants [43, 44], the simultaneous detection of α-TOH and its oxidation product α-tocopherolquinone in human blood [61], and the first determination of α-13′-OH in human serum [10]. Further, GC-MS procedures were also used to investigate Simon's metabolites and α-CEHC in plasma and urine of animals and humans [62, 63], as well as for the analysis of α- and γ-TOH with their corresponding metabolites in human plasma [51].

Features of Long-Chain Metabolite Analysis in Human Blood

In recent years, the LCMs have emerged as a new class of signaling molecules with possible relevance for the regulation of physiological functions. This change of direction in vitamin E research resulted in the development of new analytical methods to assess the LCMs in human matrices. Unfortunately, the determination of these compounds, especially in blood, appeared to be very difficult. The first detection of the α-LCMs in human serum was in 2014 by Wallert and coworkers. This group detected α-13′-COOH, but not its metabolic precursor α-13′-OH, in the serum of a healthy volunteer, receiving 1000 IU of *RRR*-α-TOH/day over 1 week. Before the measurement, the LCMs undergo enzymatic deconjugation with a mixture of sulfatase and β-glucuronidase and were extracted with hexane and dichloromethane. Based on the results of Wallert et al., the LCMs seem to appear in low nanomolar concentrations in human serum, indicating that detection sensitivity could be a major problem for metabolite analysis in future studies [11]. Only 1 year later, Ciffolilli et al. determined α-13′-OH in the same serum sample with a GC-MS-based method. Again, the applied method could not be used to detect also the second α-LCM (α-13′-COOH) [10]. To overcome these drawbacks, Torquato and coworkers tried to optimize the proposed methods by using LC-MS/MS. First, APCI and ESI sources were compared in positive and negative acquisition mode for the simultaneous determination of the TOHs and the LCMs with APCI (−) providing the best signal intensity for the α-LCMs [41]. As a result of the optimized protocol, Torquato and coworkers were able to detect α-13′-OH and α-13′-COOH simultaneously in one serum sample [41]. Only 1 year later, Giusepponi and colleagues obtained the same results using an ESI (+) source [24]. Interestingly, both groups were for the first time able to separate α-13′-OH and α-13′-COOH from several unknown compounds with identical

masses. An accurate mass investigation performed by Giusepponi et al. identified these unknown compounds as possible structural isomers of α-13′-OH and α-13′-COOH. If this holds true, the blood concentrations of α-13′-OH and α-13′-COOH would be detected as a bulk parameter, comprised of up to three different isomers [24, 41].

Regulatory Actions of Vitamin E Long-Chain Metabolites

Anti-inflammatory Actions

For studies on the anti-inflammatory actions of the LCMs, (i) cells were treated with the respective LCM in conjunction with a pro-inflammatory stimulus, or (ii) isolated enzymes were used to study the influence of the LCMs on their activity. Several LCMs (α-, γ-, δ-13′-COOH; δ-9′-COOH; α-13′-OH) affected the inflammatory response, i.e., expression (mRNA or protein) or the activity of various pro-inflammatory enzymes, including cyclooxygenase 2 (COX2) [10, 34, 64, 65], inducible nitric oxide synthase (iNOS) [10, 65–67], or 5-lipoxygenase (5-LO) [64, 68], as well as inflammatory mediators such as chemokines and cytokines. In general, the 13′-COOH metabolites are more potent than the shorter LCMs, and the conjugation of the LCMs with sulfate abrogates their anti-inflammatory actions [34, 67].

The first study on the anti-inflammatory actions of the LCMs was carried out in 2008 by Jiang and coworkers in human adenocarcinomic alveolar basal epithelial cells. This cell line is capable to metabolize vitamin E and showed an inhibition of the arachidonic acid-stimulated COX activity after treatment with TOH [34]. The inhibitory effect was less effective after pre-treatment with sesamin, a known suppressor of the metabolism of vitamin E, indicating an involvement of the LCMs as regulatory substances. In addition, the LCMs were extracted from the cell culture supernatant of the A549 cells to confirm their inhibitory capacity on COX activity (IC_{50}: δ-13′-COOH: 4 μM; δ-9′-COOH: 6 μM). The same experiments were performed with the sulfated LCM conjugates, which did not exert anti-inflammatory effects, indicating that only unconjugated LCMs can act as anti-inflammatory compounds [34]. Anti-inflammatory actions on lipopolysaccharide (LPS)-stimulated COX2 mRNA and protein expression as well as release of COX-derived prostaglandins PGE_2 for α-13′-OH [10] and PGE_2, PGD_2, and PGF_{2a} for α-13′-COOH [65] were also shown in murine RAW264.7 macrophage-like cells.

In addition, the α- and δ-LCMs (α- and δ-13′-OH, α- and δ-13′-COOH) mediated the inhibition of iNOS mRNA and protein expression, as well as release of NO in response to LPS in RAW264.7 macrophages [10, 65–67]. Interestingly, the observed inhibitory effects depended on the structure of the LCMs, with the 13′-COOH metabolites being more effective than the 13′-OH metabolites, while the substitution of the chroman ring (α- vs. δ-LCMs) had no detectable influence.

The inhibitory effects of the LCMs on 5-LO activity have been shown in (i) human promyelocytic HL60 leukemia cells, where the LCMs blocked the ionophore-induced release of leukotriene B_4, as well as (ii) on the isolated 5-LO, where δ-13′-COOH was more effective than zileuton, a synthetic antagonist for 5-LO [68]. The inhibition of 5-LO activity by δ-13′-COOH was also reported by Jang et al. [64].

Cellular Lipid Homeostasis

Until now, only a few aspects of lipid homeostasis have been studied regarding their modulation by the LCMs. Hence, merely these aspects can be discussed in the following. These include the regulation of cluster of differentiation 36 (CD36), uptake of oxidized LDL, phagocytosis, and the

intracellular storage of lipids. Taken together, these mechanisms represent key processes in macrophage foam cell formation, a significant hallmark of the pathogenesis of atherosclerosis [69]. The human monocytic THP-1 cell line, which can be differentiated to macrophage-like cells, was used to study the effects of the LCMs on foam cell formation by Wallert et al. in 2014 [11]. Under basal conditions (i.e., without the stimulation with oxidized LDL), the LCMs α-13′-OH and α-13′-COOH induced the expression of CD36 mRNA as well as CD36 protein. Interestingly, this result contrasts with the effects of the precursor α-TOH, which downregulated the expression of CD36 at a concentration of 100 μM in the THP-1 macrophage model. Thus, the metabolites likely function in a different mode than their natural precursors. In addition, the LCMs appear to be several times more effective than α-TOH, as they exert their effect on CD36 expression in concentrations as low as 5 and 10 μM for α-13′-OH and α-13′-COOH, respectively. To confirm these findings in a more physiological model, the LCMs were also applied to peripheral blood mononuclear cell (PBMC)-derived primary macrophages in this study. Here, the effects of both LCMs on CD36 protein expression were confirmed [11].

The scavenger receptor CD36 is a receptor binding oxidized LDL in macrophages and mediates the uptake of this modified lipoprotein [70]. In a feed-forward mechanism, oxidized LDL induces the expression of CD36, leading to an increased uptake of oxidized LDL [71]. This mechanism promotes foam cell formation; thus, Wallert et al. examined whether the LCMs interrupt or support oxidized LDL-induced CD36 expression. As expected, the incubation with oxidized LDL induced the expression of CD36 in human THP-1 macrophages [11]. The TOH precursor diminished the induction by oxidized LDL, resembling the findings under basal conditions. Likewise, the effect of the LCMs resembled the initial findings. The preincubation with the LCMs augmented the induction of CD36 protein expression by oxidized LDL significantly. In contrast, naïve LDL did not induce CD36 expression in THP-1 macrophages. In combination with naïve LDL, α-TOH downregulated CD36 protein expression, while the LCMs induced the expression. Given the augmented expression of CD36 by the LCMs, increased oxidized LDL uptake by LCM-treated macrophages can be expected. Interestingly, incubation of THP-1 macrophages with the LCMs for 24 h before challenging the cells with oxidized LDL leads to a decrease of about 20% in the uptake of oxidized LDL compared to untreated control cells. Again, PBMC-derived macrophages were treated in a similar fashion, and the findings were confirmed. The LCM α-13′-OH decreased the uptake by 24% and α-13′-COOH by 20%, respectively. In foam cell formation, the consequence of an increased uptake of oxidized LDL is an increase in intracellular lipid content. Thus, the THP-1 macrophage model reacted with an increase of intracellular neutral lipids in response to the incubation with oxidized LDL. Concomitant with the decreased uptake of oxidized LDL in response to the LCM preincubation, the neutral lipid content of THP-1 macrophages was not increased in cells treated with the LCMs and oxidized LDL in combination [11].

However, the induced expression of CD36 is contradictory to the observed inhibitory effects of the LCMs on the uptake of oxidized LDL. Consequently, the LCMs likely act through a distinct mechanism. Results by Wallert et al. suggest that phagocytosis, as a major uptake pathway for oxidized LDL [72], is also affected by the LCMs. Experiments with fluorescence-labeled microbeads revealed that the LCMs significantly decreased the phagocytic activity of THP-1 macrophages. Here, α-13′-COOH seems to be more potent than α-13′-OH, with 41% inhibition vs. 16% inhibition, respectively [11]. This finding is not perfectly in line with the equal inhibitory effect of the two LCMs on the uptake of oxidized LDL. However, the inhibition of phagocytosis by the LCMs provides a good explanation for the discrepancy between CD36 regulation and uptake of oxidized LDL.

Taken together, the LCMs modulate macrophage lipid metabolism on the level of lipid uptake and storage. Different pathways implicated in foam cell formation, a hallmark of atherosclerosis, are affected by the LCMs. In total, the treatment of macrophages with the LCMs leads to a reduced uptake of oxidized LDL and concomitantly reduced lipid accumulation, a desirable effect in terms of the prevention of atherosclerosis. However, the underlying molecular mechanisms are not fully understood. Thus, further studies on the modes of action of the LCMs are needed.

Cancerogenesis and Chemoprevention

Antiproliferative Effects of Tocopherol Long-Chain Metabolites

Abnormal proliferation is a characteristic of cancer cells and represents a crucial element of cancer development and progression. Thus, cancer therapy is based in part on drugs that kill cells with high rates of proliferation and regeneration. However, such substances cause severe side effects as they also affect rapidly proliferating healthy tissues like the skin, hair, or parts of the gastrointestinal tract [73]. Hence, natural compounds with antiproliferative activities are regarded as beneficial in the prevention and treatment of cancers, as they generally exert less side effects. Several natural compounds have been identified that inhibit pathways contributing to cell proliferation. Among them are the promising constituent of *Curcuma longa*, namely, curcumin, which has been shown to affect Wnt, NF-κB, and mTOR signaling inter alia and resveratrol, a constituent of grapes, with blocking activity on mitogen-activated protein kinases and tyrosine kinases inter alia [73]. The chemopreventive properties of curcumin [74] and resveratrol [75] have been found in several studies. Cancer-preventing properties have also been reported for TOHs and T3s. This effect can be attributed at least in part to the LCMs as outlined below.

First studies on the TOH metabolites in this context were carried out with a focus on the SCMs. Here, the SCMs exerted comparable effects to their precursors with respect to the inhibition of cell proliferation. Interestingly, the γ-forms appeared to be more potent in inhibiting cell proliferation than the α-forms, a finding that was earlier reported for the T3s [76]. Accordingly, it was found that γ-TOH as well as γ-CEHC reduced the proliferation of human PC3 prostate cancer cells in a concentration of 1 μM by about 30–40%. Almost maximal inhibition of cell proliferation, i.e., 70–80%, was obtained with 10 μM. However, α-CEHC and α-TOH inhibited cell growth by 40–45% in concentrations of 50 μM [77]. Interestingly, the antiproliferative effects seem to be cell type-dependent, as PC3 cells showed higher inhibition compared to human HTB-82 rhabdomyosarcoma cells and human endothelial vascular cells (HEVC) [77]. Given that the precursors and the SCMs exert antiproliferative effects, Birringer et al. were interested in the effects of the LCMs on cell proliferation. Therefore, α-13′-COOH and δ-13′-COOH as well as α-13′-OH and δ-13′-OH and their respective precursors were applied to HepG2 liver cells [48]. While the LCMs with carboxy function potently led to cell growth arrest, the hydroxy metabolites failed to exert antiproliferative effects. Again, the α-forms were less potent than their δ-counterparts. Neither the hydroxy metabolites nor the TOHs inhibited cell growth in the concentrations tested. Thus, the authors concluded that the carboxylation of the TOH side chain is essential for the antiproliferative effects of the LCMs [48]. However, in PC3 cells, not only α- and γ-CEHC impeded cell proliferation but also δ-13′-COOH and α-13′-OH. All compounds inhibited cell proliferation by about 60% at concentrations of 10 μM [52]. Thus, the effect of the hydroxy metabolites seems to be cell type-dependent. This might be explained by differences in the cellular metabolism of the TOHs and the TOH metabolites. Different responsiveness of cell types to vitamin E metabolites was also reported in colon cells [64]. The δ-13′-COOH metabolite reduced the proliferation of human HCT-116 colon carcinoma and human HT-29 colorectal adenocarcinoma cells with IC_{50} values of 8.9 μM and 8.6 μM, respectively, whereas the T3 metabolite δ-T3-13′-COOH (i.e., δ-garcinoic acid) was less potent with IC_{50} values of 16 μM and 17 μM. Interestingly, normal colon epithelial cells were less affected by the metabolites. While 10 μM of δ-13′-COOH suppressed cell viability of HCT-116 and HT-29 cells by around 60%, normal human colon epithelial cells showed reduction of merely 10–20%. In line with this, 20 μM of δ-T3-13′-COOH (δ-garcinoic acid) reduced the viability of the cancer cells by 70–80%, but the viability of normal colon cells was affected only by 10–20% [64].

Taken together, the precursor molecules, i.e., the TOHs and T3s, apparently exert antiproliferative effects depending on the methylation pattern of the chroman ring. The γ-forms of T3 and TOH have antiproliferative properties [76, 77], while the α- and δ-forms have not [48, 64]. It should be noticed that Jang et al. found no effects of γ-T3 in their setting [64]. However, the metabolic conversion leads to

LCMs and SCMs with antiproliferative properties, independent of the methylation pattern of the chroman ring. While the action of the hydroxy LCMs is controversial [48, 52] and likely depends on the cell type, the carboxy LCMs reliably affect the proliferation of different cancer cell lines [48, 52, 64]. Thus, a key determinant of the antiproliferative properties is likely the carboxy function, a notion that is further supported by the reported actions of the SCMs carrying a carboxy group (α- and γ-CEHC) [52, 77]. A promising finding with respect to anticancer properties of the LCMs is the resistance of normal colon cells to the LCMs, while the proliferation of colon cancer cells is strongly reduced [64]. If this effect is reproducible, vitamin E and its metabolites might be useful in cancer prevention and treatment.

Pro-apoptotic Effects of the Tocopherol Long-Chain Metabolites

Apoptosis is a coordinated cellular process, ultimately leading to programmed cell death. The balance of cell division and cell death is crucial for the homeostasis of organisms. Inappropriate rates of apoptosis are implicated in several pathological conditions, such as neurodegenerative diseases, autoimmune disorders, and cancers [78]. The rate of apoptosis is usually lower in cancer cells, leading to malignant cells, tumor metastasis, and resistance to anticancer drugs. Thus, apoptosis is part of the problem as well as a possible solution. Several therapeutic strategies based on the targeting of apoptosis pathways have been developed [78].

In addition to the antiproliferative effects, Birringer et al. also analyzed the apoptotic effects of the LCMs in HepG2 liver cells [48]. Flow cytometric analyses using annexin V staining revealed a significant induction of apoptosis, when HepG2 cells were treated with 20 μM α-13′-COOH, δ-13′-COOH, or δ-13′-OH. In line with this, α-13′-COOH and δ-13′-COOH strongly induced the cleavage of caspases 3, 7, and 9. The hydroxy LCM δ-13′-OH leads to an activation of the same caspases but less effectively. In contrast, α- and δ-TOH as well as α-13′-OH were not able to induce caspase cleavage. Accordingly, poly-ADP ribose polymerase (PARP)-1 cleavage as a downstream effect of caspase activation followed a similar pattern. The α- and δ-carboxy metabolites showed strong induction, while merely a slight effect for δ-13′-OH and no effect for α-13′-OH and the TOHs were observed. Further, mitochondrial apoptosis, a process accompanied by the increased production of reactive oxygen species (ROS), was examined. On that account, ROS production in the HepG2 cells in response to TOHs and their metabolites was analyzed. Here, in contrast to the precursors and the hydroxy metabolites, the carboxy metabolites significantly induced ROS formation. Not only intracellular but also intramitochondrial ROS levels were induced by the carboxychromanols. Again, the other substances tested did not induce ROS production. With these findings, evidence was provided for mitochondrial-derived apoptosis. Further, alterations in the mitochondrial membrane potential in TOH- and LCM-treated cells were found. Significant reductions in the mitochondrial membrane potential were observed for α-13′-COOH, δ-13′-COOH, and δ-13′-OH in concentrations of 20 μM. Here, α-13′-COOH was again more effective (60% reduction) than the δ-LCMs (20% reduction for both the hydroxy and the carboxy metabolites) [48].

Taken together, the carboxy LCMs reliably induce apoptosis in HepG2 cells, and evidence was provided that a pathway leading to mitochondrial apoptosis is involved in this effect. Interestingly, the authors have shown that δ-13′-OH is efficiently metabolized to δ-13′-COOH by HepG2 cells, while the conversion of α-13′-COOH to α-13′-OH is less effective [48]. This finding provides a nice explanation for the discrepancy in the effects of α-13′-OH and δ-13′-OH. The δ-metabolite leads to apoptosis through a rapid conversion to the pro-apoptotic carboxy metabolite, while the α-metabolite is slowly converted and thus unable to induce apoptosis. In conclusion, as shown for the antiproliferative actions of the LCMs, the carboxy function of the metabolite seems to be crucial for the observed pro-apoptotic effect.

The apoptotic actions of the long-chain vitamin E derivatives with carboxy function were confirmed in a study on colon cancer cells [64]. Here, δ-13′-COOH and δ-T3-13′-COOH (δ-garcinoic acid) induced early and late apoptosis. In line with the findings of Birringer et al., induction of

caspase-9 activation and PARP cleavage by the carboxy metabolites was found. Further, the autophagy marker microtubule-associated protein 1 light chain 3 (LC3)-II was increased by the treatment with the carboxy LCMs. Interestingly, the TOH metabolite was more effective than the T3 metabolite in the induction of apoptosis and autophagy. Based on previous findings on the metabolic precursors, Jang et al. examined whether an alteration in sphingolipid metabolism by the LCMs is causing the induction of apoptosis. It was found that δ-13′-COOH increased dose-dependently the total content of ceramides, dihydroceramides, and dihydrosphingosines. In contrast, the content of all sphingomyelins was decreased. Similar effects were observed for the T3 metabolite. Thus, both carboxy LCMs modulate sphingolipid metabolism when apoptosis and autophagy are induced. An inhibition of sphingosine biosynthesis by myriocin treatment partly inhibited the induction of LC3-II expression but not the induction of PARP cleavage by the metabolites. Hence, elevated levels of dihydroceramides and dihydrosphingosines likely contribute to LCM-induced autophagy [64].

The LCMs have been shown to induce apoptosis in different cell types. Interestingly, evidence for two different modes of action has been provided by the studies on the LCMs so far. Birringer et al. have shown the induction of mitochondrial apoptosis by the LCMs in HepG2 cells, while Jang et al. have reported that an altered sphingolipid metabolism contributes to LCM-induced apoptosis in colon carcinoma cells. Treatment strategies targeting apoptosis aim at different signaling pathways, including B-cell lymphoma proteins, p53, or caspases. Effects of the LCMs on caspases have been shown in both studies on LCM-induced apoptosis. However, based on these findings, the applicability of the LCMs for chemoprevention or inhibition of cancerogenesis can hardly be assessed. Further studies are required to confirm desired properties like specificity for malignant cancer cells or to unravel distinct apoptosis signaling pathways [78].

Interaction with Pharmaceuticals

The cellular uptake of molecules is tightly regulated by several mechanisms, one of which is the excretion of, for example, pharmaceuticals from the cells via exporter proteins, such as the multidrug resistance protein P-gp. P-gp is a well-known representative of these exporters [79]. A specific inhibition in tumor cells is helpful, when antitumor therapies are applied, since the activation of these exporters may lead to a reduced cellular net uptake and efficiency of the pharmaceuticals.

Podszun et al. studied the effects of vitamin E (α-TOH, α-T3, γ-TOH, and γ-T3) and their metabolites (α-13′-COOH, α-CEHC, γ-CEHC) as well as plastochromanol-8 on the expression of P-gp in LS 180 Dukes' type B colorectal adenocarcinoma cells and found an induction of the expression and activity for α-13′-COOH and γ-T3 [80]. Furthermore, pregnane X receptor activity was induced by α-T3, α-13′-COOH, and γ-T3, as assayed by a reporter gene assay. The authors summarized that an increased uptake of vitamin E via supplements could lead to interactions with pharmaceuticals due to an increased activity of the P-gp exporter.

Structure-Specific Effects

A structure-function relationship study of the LCMs revealed a highly specific regulation of target genes by the LCMs (α-13′-OH, α-13′-COOH, δ-13′-OH, and δ-13′-COOH). Neither the precursors (α- and δ-TOH) nor their substructures (pristanic acid and α-CEHC) were able to cause the same effects on the expression of scavenger receptor CD36 or inducible nitric oxide synthase (iNos) as the 13′-hydroxy or 13′-carboxy LCMs. Furthermore, the regulation was almost independent of the substitution pattern of the chromanol ring system (α- vs. δ-LCMs) but dependent on the modification of the side chain (TOH vs. 13′-OH and 13′-COOH, respectively), with the 13′-COOH being most potent. Hence, this specific regulation might suggest the existence of receptor-specific pathways for the LCMs [67].

Conclusions and Outlook

With the demonstration of the occurrence of the LCMs in human serum, Wallert and coworkers provided evidence for their possible role as systemic signaling molecules [11]. This concept was supported by several studies, characterizing the involvement of the LCMs in the regulation of inflammatory processes, lipid metabolism, cancerogenesis, and chemoprevention as well as xenobiotic metabolism. Interestingly, the LCMs act more potent and in part even contrary to their precursors. Thus, some of the controversial effects found for vitamin E might be explained by the actions of the LCMs. Nevertheless, large parts of their mode of action are still unrevealed and need further characterization. Although the analysis of the LCMs made great progress over the last several years, especially the distribution of the LCMs in extrahepatic tissues beside human serum needs further investigation. Taken together, the LCMs could be regarded as the active forms of vitamin E, as it has already been shown for the metabolites of vitamin A and D (reviewed in [81]). If this concept of a general mechanism for metabolic activation of fat-soluble vitamins holds true, the LCMs of vitamin E could comprise a new class of signaling molecules in the human body. This concept sheds new light to the field of vitamin E research and may help for better understanding the complex mode of action of vitamin E as well as its function as a vitamer. A brief overview about the current knowledge on LCMs in the human body and issues for future investigations is provided in Fig. 6.1.

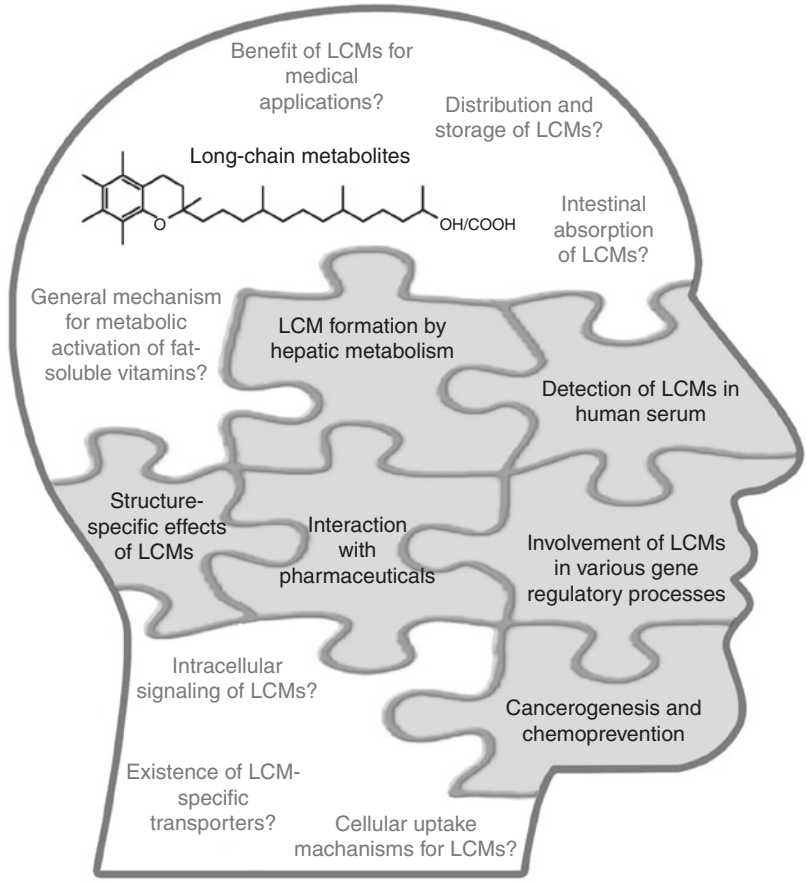

Fig. 6.1 Completing the puzzle of the activities of the LCMs in the human body

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Chapter 7
Gene Regulatory Activity of Vitamin E

Alexandra Fischer and Gerald Rimbach

Keywords Vitamin E · Gene expression · Inflammation · Lipid metabolism · MicroRNA · Immune response

Key Points

- Tocopherol and tocotrienols exhibit gene regulatory properties.
- Vitamin E modulates immune response.
- Vitamin E affects hepatic gene expression.
- Vitamin E induces changes in steroidogenesis and affects cholesterol homeostasis.
- Vitamin E influences miRNA levels.

Introduction and Chemistry of Vitamin E

In 1922, Evans and Bishop (University of Berkeley, California) discovered vitamin E to be an essential factor for the reproduction of rats [28]. Other important milestones in vitamin E research include its structural elucidation, synthesis and description of its biological functions which are summarized in Chap. 1 [31, 34, 50, 62, 99]. Furthermore the identification as well as the crystal structures of vitamin E trafficking proteins, including α-tocopherol transfer protein (α-TTP) and supernatant protein factor (SPF), has been solved [19, 70, 96, 97].

Vitamin E naturally occurs in at least eight biologically active isoforms [79]. Research has mainly focused on its antioxidant properties; however, in recent years, the cell signalling and gene regulatory aspects of tocopherols and tocotrienols have also been reported [16]. Through technological advances (e.g. gene chip technology), novel vitamin E-sensitive molecular targets that are controlled through signalling pathways have been discovered [16, 90].

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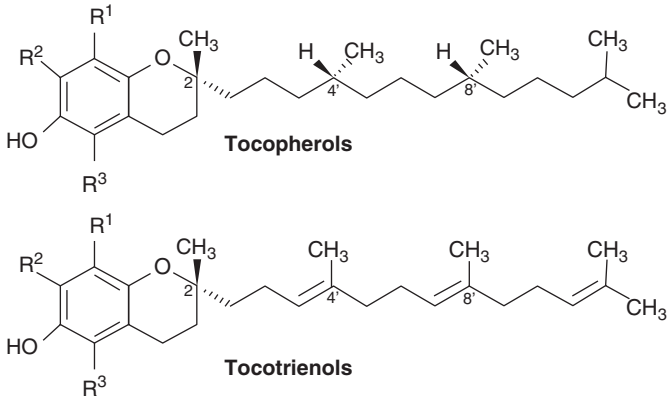
P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_7



	R1	R2	R3
α-	CH_3	CH_3	CH_3
β-	CH_3	H	CH_3
γ-	CH_3	CH_3	H
δ-	CH_3	H	H

Fig. 7.1 Molecular structure of vitamin E stereoisomers

Tocopherols and tocotrienols are generated by plants from homogentisic acid. All are derivatives of 6-chromanol with an aliphatic 16-carbon side chain attached to the chromanol ring [89]. The four tocopherol isoforms (α-, β-, γ-, δ-) consist of a fully saturated isoprenoid side chain, whereas the isoprenoid side chain of the four tocotrienol homologues (α-, β-, γ-, δ-) contains three double bonds. Accordingly, tocopherols have three chiral centres at positions 2′, 4′ and 8′, while tocotrienols have only one at position 2′ [84]. The individual tocopherols and tocotrienols differ in the number and position of the methyl groups on the phenol ring, with the α-, β-, γ- and δ-isoforms containing three, two, two and one methyl groups, respectively [79, 89] (Fig. 7.1).

These structural characteristics define the biological activity of the isoforms. Each methyl group attached to the chromanol ring confers additional antioxidant capacity. Therefore, the α-isoforms are supposed to be the most biologically active forms of vitamin E, regarding their antioxidant properties [89].

Although humans absorb all forms of vitamin E [79], α-tocopherol represents ~90% of total vitamin E in the body [106]. This is partly due to the higher affinity of α-TTP for α-tocopherol relative to the other tocopherols and tocotrienols [42]. Recently, we have found that self-assembled α-TTP nanoparticles promote the delivery of vitamin E across the endothelial barrier [3]. This offers new strategies to deliver drugs into tissues protected by endothelial barriers (e.g. the brain).

Gene Regulatory Activity of Vitamin E

Overview

Vitamin E is involved in cell signalling processes, independent of its antioxidant properties [5, 10] (Fig. 7.2). It interacts with cell receptors (e.g. low-density lipoprotein receptor (LDL-R) [80]) and redox-regulated transcription factors (e.g. pregnane X receptor (PXR) [55]), thereby driving gene expression (e.g. scavenger receptor (SR), cluster of differentiation (CD) 36 [6, 82, 109]). The first non-antioxidant properties of vitamin E have been described by Azzi and co-workers, indicating that α-tocopherol suppresses the activity of protein kinase C (PKC) and 5-lipoxygenase (5-LOX) and stimulates protein phosphatase 2A and diacylglycerol kinase [11, 12, 24, 62]. Furthermore, α-tocopherol has an influence on the transcription of some genes, including α-TTP [30], α-tropomyosin (TPM1) [4] and matrix metalloproteinase MMP1 [86].

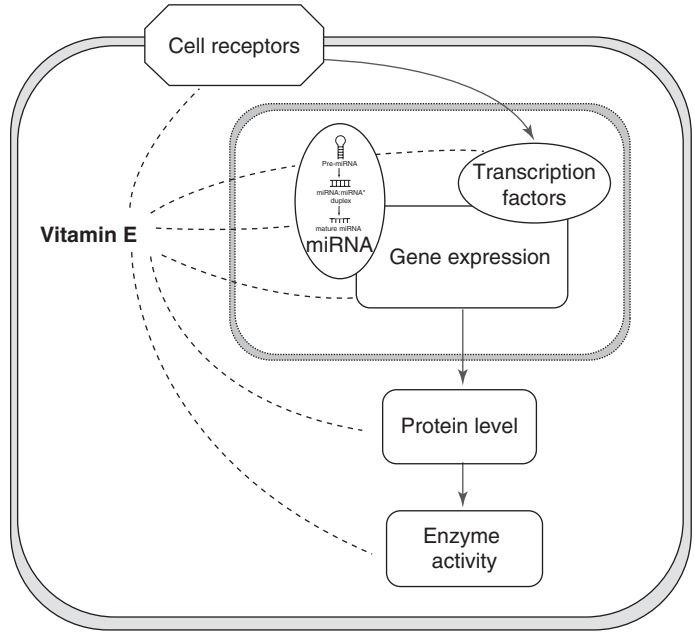


Fig. 7.2 Vitamin E is a cell signalling molecule that affects cell receptors, transcription factors, miRNAs, gene expression, protein levels, and enzyme activities of vitamin E-specific molecular targets

Gene Regulatory Activity of Different Vitamin E Isoforms

Notably, the various isoforms of vitamin E exhibit different gene regulatory activities [26]. In a comparative gene expression profiling study by Berbeé et al. [8], the differences in gene expression between γ-tocotrienol, γ-tocopherol and α-tocopherol in pretreated human endothelial cells after radiation injury were evaluated. γ-Tocotrienol was more potent in inducing gene expression changes than α-tocopherol or γ-tocopherol. Multiple changes in functional pathways, such as the response to oxidative stress, response to DNA-damaging stimuli, cell cycle phase, regulation of cell death and cell proliferation, haematopoiesis and blood vessel development, were affected by γ-tocotrienol. In a study by Zingg et al. [119], the in vivo regulation of gene transcription by α- and γ-tocopherol in murine T lymphocytes was evaluated by gene array analysis. It was shown that dietary supplementation with α- or γ-tocopherol influenced the immune response in a dose- and structure-dependent manner. Genes were found to uniquely respond to either high α-tocopherol (e.g. induction of CD40 ligand, tumour necrosis factor (TNF) ß) or γ-tocopherol (e.g. repression of poliovirus receptor-related-2). In prostate cancer cells, tocopherylquinone, the oxidation product of α-tocopherol, decreased androgen-responsive gene expression, including transmembrane-4-L-six-family-1, kallikrein-2 and kallikrein-3, whereas α-tocopherol did not [29]. γ-Tocopherol supplementation inhibits protein nitration and ascorbate oxidation in rats with induced inflammation by zymosan injection; however, by contrast, α-tocopherol did not significantly affect protein nitration and ascorbate oxidation in response to zymosan treatment [46]. γ-Tocopherol inhibits human prostate cancer cell proliferation to a greater extent than α-tocopherol through the upregulation of transglutaminase 2 (TGM2) and the downregulation of cyclins (CCNs) [105]. Tocopherols, including α-, β-, γ- and δ-tocopherols, inhibited cyclooxygenase-2 (COX2) gene expression in RAW 264.7 macrophages after exposure to macrophage activators. Compared with α-tocopherol, β-, γ- and δ-tocopherols exhibited significantly greater inhibitory properties [72]. Abdala-Valencia et al. [1] showed that the activation of PKCα via intercellular adhesion molecule-1 (ICAM1) is differentially regulated by various vitamin E isoforms in human microvascular endothelial cells. ICAM1 activation of PKCα was inhibited by d-α-tocopherol,

and this inhibition was ablated by the addition of d-γ-tocopherol. These tocopherols regulated ICAM1 activation of PKCα without altering the upstream signal extracellular signal-regulated kinases 1/2 (ERK1/2).

Gene Expression in Vitamin E Deficiency

To gain a global view of the molecular role of action of vitamin E, our group has performed global gene expression profile experiments, both in rat liver in vivo and in hepatocellular liver carcinoma (HepG2) cells in vitro.

To study the influence of a short-term (49 days) [33] or long-term (290 days) vitamin E deficiency in rats [6], animals were maintained on semisynthetic diets either supplemented with or deficient in vitamin E (DL-α-tocopheryl acetate [33]; *RRR*-α-tocopheryl acetate [6]). Furthermore, HepG2 cells were supplemented with vitamin E (*RRR*-α-tocopheryl acetate) concentrations corresponding to those that were obtained in the in vivo study [88]. Differential gene expression in rat liver and in HepG2 cells was monitored by Affymetrix GeneChip technology covering 7000 transcripts. Vitamin E deficiency generated via the diet over a 7-week period did not induce any significant changes in the expression profile amongst the assessed genes. Likewise, in another study, a 7-week vitamin E deficiency did not change the expression pattern of 465 genes evaluated in the liver of rats [33]. A combined vitamin E and selenium deficiency, however, altered the expression of genes encoding proteins involved in inflammation, acute phase response, inhibition of apoptosis, cell cycle and antioxidant defence. Furthermore, long-term vitamin E deficiency in rats induced the expression of hepatic coagulation factor IX (FIX), 5-α-steroid reductase type 1 (5α-R1) and CD36 mRNA levels.

FIX is a blood-clotting protein that plays an essential role in the activity of the intrinsic coagulation pathway [61]. α-Tocopherol is proposed to decrease the levels of FIX by function as an antagonist of the vitamin K-dependent γ-carboxylase, which is necessary to activate enzymes of the blood-clotting cascade (e.g. FIX). As a consequence of the reduced FIX levels, the activated partial thromboplastin (APT) time was increased in our rat study [6] (Fig. 7.3a).

5α-R1, which is widely distributed in numerous tissues, catalyses the irreversible conversion of testosterone to 5-α-dihydrotestosterone [92]. Both testosterone, the primary androgen to be synthesized and secreted by the testes, and 5-α-dihydrotestosterone, which is the major androgen in the prostate, are potent natural androgens that are present in all mammals [118]. It has been demonstrated that dietary vitamin E decreases 5α-R1 mRNA levels, resulting in a changed ratio of 5-α-dihydrotestosterone/testosterone [6] (Fig. 7.3b). In addition, we could show that the rate-limiting enzyme of glutathione synthesis, γ-glutamyl-cysteinyl-synthetase (γGCS), as well as glutathione synthase, was downregulated in the liver of vitamin E-deficient rats. Reduced levels of glutathione in the liver have been associated with reduced tolerance to oxidative stress and xenobiotics [108] and decreased regeneration of vitamin E radicals via ascorbic acid [39] (Fig. 7.3c). Overall, measurement of the corresponding functional endpoints such as APT time, plasma 5-α-dihydrotestosterone and hepatic glutathione confirmed the gene chip data, suggesting that dietary vitamin E exhibits an important part in numerous metabolic processes within the rat liver [6]. Similar to our rat experiment, it was shown in vitro that adding vitamin E to HepG2 cells altered the expression of FIX and CD36 [88] and therefore confirmed partly our in vivo data.

In another set of experiments, two groups of male rats were fed with either a vitamin E-deficient diet or a control diet enriched with *RRR*-α-tocopheryl acetate for 430 days. Differential gene expression in skeletal muscle was controlled at five time-points, with all animals individually profiled [75]. The differentially expressed genes consist of muscle structure and extracellular matrix genes, as well as antioxidative, anti-inflammatory and anti-fibrotic genes. Our data suggest that molecular transcription possibly provides a very initial indicator to identify forthcoming degenerative conditions in vitamin E deficiency. They give additional information into potential molecular

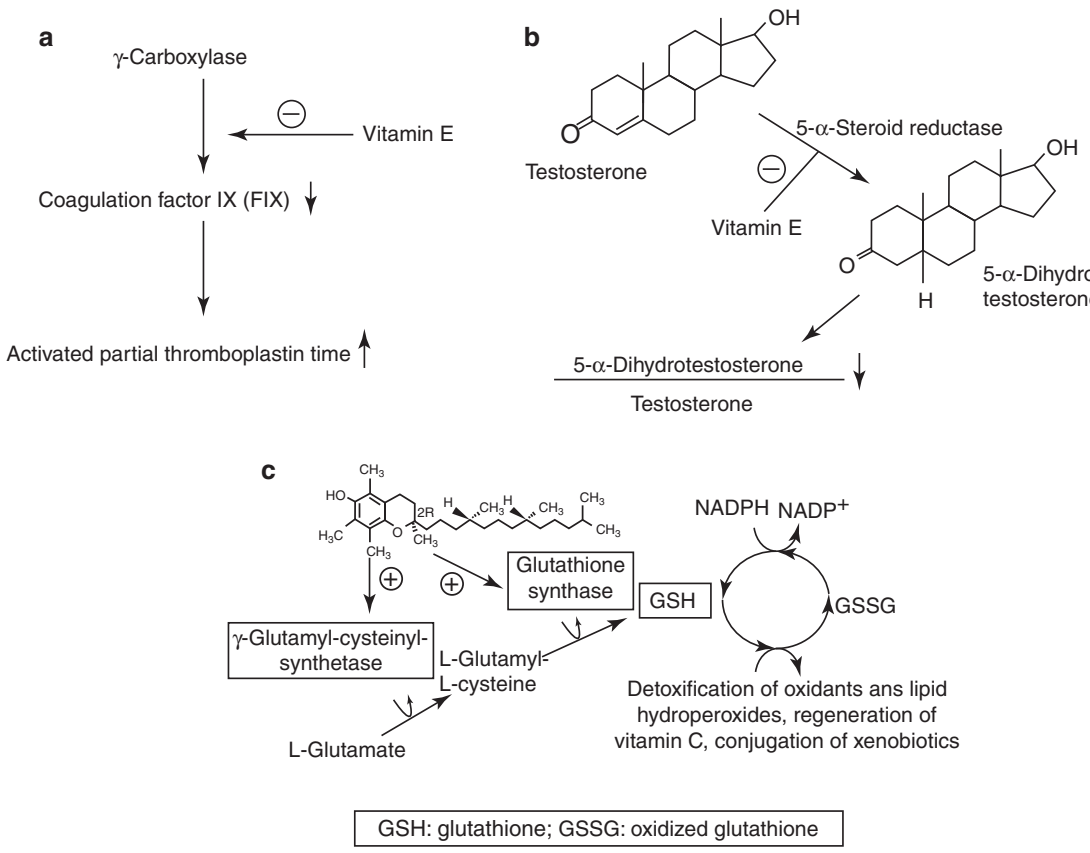


Fig. 7.3 Overview of the effects of vitamin E on (**a**) the inhibition of the activation of coagulation factor IX and the increase in the activated partial thromboplastin time, on (**b**) the inhibition of 5-α-steroid reductase type 1 and on (**c**) the induction of hepatic glutathione synthesis

mechanisms due to vitamin E deficiency in skeletal muscle and display the stimulation of a profound protection programme that may explain the long preservation of muscle structure during vitamin E deficiency.

Lipid Metabolism

Our in vivo experiments in rats also show that vitamin E causes alterations in steroidogenesis, thereby having an effect on cholesterol homeostasis in testes and adrenal glands. Genes encoding proteins regulating the uptake (LDL-R) and de novo synthesis (e.g. 7-dehydrocholesterol reductase (DHCR7), 3-hydroxy-3-methylgluteryl coenzyme A synthase (HMGCS), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), isopentenyl diphosphate δ-isomerase (IPPI) and farnesyl pyrophosphate synthetase (FPPS)) of cholesterol, the precursor of all steroid hormones, have been found to be α-tocopherol sensitive molecular targets [7]. Furthermore, HMGCR and CD141 have also been identified as γ-tocotrienol sensitive molecular targets [81].

Recently, genome-wide expression profiling in the muscle and subcutaneous fat of lambs in response to dietary vitamin E supplementation revealed the upregulation of genes (e.g. sterol regulatory element-binding transcription factor 1 (SREBF1), LDL-R, HMGCS1), encoding proteins centrally involved in

lipid metabolism including sterol, steroid and cholesterol biosynthesis [57]. In muscle, vitamin E supplementation led to the downregulation of genes related to the intracellular signalling cascade and metabolic processes (e.g. cytokine-inducible SH_2-containing protein, insulin-like growth factor 1 receptor and acetyl-CoA acetyltransferase 1 (ACAT1)). In differentiated preadipocytes, tocotrienol modulates crucial lipid metabolism-related genes, e.g. genes that code for lipid biosynthesis (fas cell surface death receptor (FAS), stearoyl-CoA desaturase 1 (SCD1), acetyl-CoA-carboxylase 1 (ACC1), adiponectin receptor 2 (ADIPOR2) and LDL-R) and ß-oxidation (carnitine palmitoyltransferase 1 (CPT1) and uncoupling protein 2 (UCP2)) [18]. In addition, transcription factors such as sterol regulatory element-binding protein (SREBP) 1c and peroxisome proliferator-activated receptor (PPAR) γ were markedly regulated by tocotrienol. The addition of α-tocopheryl phosphate (but not α-tocopherol) to preadipocytes transcriptionally activates a set of genes (tribbles homologue 3, sestrin-2 and insulin-induced gene 1), potentially preventing fat accumulation in these cells [60]. By contrast, the authors could show that, in differentiated adipocytes, α-tocopheryl phosphate is responsible for the transcriptional inhibition of the same genes, possibly facilitating fat uptake and storage. In the aortae of rabbits fed with a cholesterol-rich diet, vitamin E supplementation affords protection by decreasing MMP1 and increasing PPARγ, glutathione S-transferase (GST)-α and ATP-binding cassette transporter 1 (ABCA1) levels [15]. Likewise, using proteomics techniques, the expression levels of apolipoprotein (Apo) AI and ApoE, which are involved in lipid metabolism, and peroxiredoxin (PRDX) 1, PRDX2 and thioredoxin, which are involved in the antioxidant system, were significantly different in the aortae of rabbits fed with a cholesterol- and vitamin E-rich diet, compared with those of control rabbits [48]. In addition, 14-3-3 protein zeta/delta and 14-3-3 protein beta/alpha in cell signalling, and biglycan, vimentin, TPM1 and smooth muscle α-actin (ACTA2) as structural and contractile proteins, were differentially expressed. In ApoE knockout mice, vitamin E conditionally inhibits atherosclerosis by anti-oxidation and regulation of vasculature gene expression [100]. At the ages of 6, 14 and 22 weeks, vitamin E downregulated the vasculature mRNA expression of scavenger receptor CD36 and upregulated the mRNA expression of PPARγ, liver X receptor α and ABCA1, which are involved in reverse cholesterol transportation; however, vitamin E had no significant effects on these genes when given at an older age (30 and 38 weeks). In rat primary hepatocytes, γ-tocotrienol reduces the triacylglycerol level through the regulation of fatty acid metabolism, thereby increasing the expression of CPT1A mRNA and suppressing the gene expression of C/EBP homologous protein (CHOP), SREBP1c and IL1ß [73].

Antioxidative Enzymes and Cellular Ageing, Inflammation and Immune Response

Dietary vitamin E, at various doses, increased the mRNA and protein expression of the antioxidative enzymes glutathione peroxidase 3 (GPX3) and GSTα1 in sheep testes [114] and modulated the gene expression of superoxide dismutase (SOD) 1 and 2, chopper chaperone for SOD (CCS1), PRDX6, catalase (CAT) and forkhead-box-protein (FOXO3A) in vitro [25].

In addition, vitamin E has been suggested to modulate age-associated changes by altering the redox balance, resulting in altered gene and/or protein expression. γ-Tocotrienol has been shown to prevent the cellular ageing of human diploid fibroblasts by the modulation of gene expression [65, 66]. Following the incubation of senescent cells with γ-tocotrienol, global gene expression analysis was performed and revealed 100 genes that were differentially expressed by at least 1.5-fold. Amongst these genes were interleukin 1 receptor-associated kinase 3, selenoprotein S, heat shock protein family A5, homocysteine-responsive ER-resident ubiquitin-like domain 1, DNAJ HSP member B9 and methionine sulfoxide reductase B1. Furthermore, translocase of inner mitochondrial membrane, ADP ribosylation factor 4, RAD50-interacting protein 1, NTF2-related export protein 1, calcium-dependent secretion activator 2, component of oligomeric Golgi complex 6 and glutaredoxin 5 were also differentially expressed.

Enrichment scores revealed that biological processes such as inflammation, protein transport, apoptosis and cell redox homeostasis were affected in these cells treated with γ-tocotrienol. In human lymphocytes from young and old individuals challenged with H_2O_2 and treated with the tocotrienol-rich fraction, 24 proteins were found to be affected. Amongst these were several proteins involved in the stress response (PRDX2, PRDX3 and PRDX6), which were shown to be downregulated with H_2O_2 exposure, and the effect was reversed following treatment with the tocotrienol-rich fraction [22].

Vitamin E modulates the immune response, in part by reducing inflammation. LPS-induced inflammation in chickens was significantly lower in birds fed with vitamin E, as indicated by lower IL6 mRNA steady-state levels [49]. In chickens receiving either α- or γ-tocopherol or both isoforms after inducing oxidative stress by feeding with n-3 polyunsaturated fatty acids, a chicken-specific genome microarray analysis was performed in the liver [53]. The effect of γ-tocopherol was evident in the expression of genes involved in inflammatory processes and the immune response (e.g. interferon regulatory factors (IRFs), lymphocyte antigen 96 encoding gene (LY96), toll-like receptor 2 (TLR2), leukocyte ribonuclease A-2 (RSFR)), while α-tocopherol affected genes involved in lipid and cholesterol metabolism (e.g. SREBP2, PPARα). In old (22–24 month) mice infected with *S. pneumonia*, α-tocopherol supplementation enhanced the resistance of aged mice to bacterial pneumonia and altered the expression of multiple epithelial and neutrophil adhesion molecules involved in migration, including CD55, CD47, CD18/CD11b and ICAM1 [13].

In vitro assays in LPS-stimulated mouse bone marrow cells in the presence or absence of vitamin E revealed enhanced klotho transcript levels and hormone secretion and inhibited IL12p70 protein expression by vitamin E [115]. The TNFα-induced changes in secretion and gene expression of monocyte chemotactic protein 1, IL6, and adiponectin in 3T3-L1 adipocytes were attenuated by γ-tocotrienol [68]. Furthermore, TNFα-mediated IκB-α phosphorylation and nuclear factor κ-light-chain-enhancer of activated B-cell (NF-κB) activation were significantly suppressed by γ-tocotrienol treatment.

In tocotrienol-treated MCF-7 human breast cancer cells, global gene expression analysis revealed an upregulation of genes responsible for modulating the immune response [85]. Interferon-induced transmembrane protein (IFITM) 3 expression was induced by the tocotrienol-rich fraction; IFITM2 was induced by α-tocopherol, tocotrienol-rich fraction, α-tocotrienol and γ-tocotrienol; ferritin heavy polypeptide 1 (FTH1) was induced by γ-tocotrienol; and COL IVα3 was induced by δ-tocotrienol.

Pretreatment of cardiomyocytes with vitamin E under heat stress conditions increased the expression of metallothionein, PPARγ coactivator 1A (PGC1A), nuclear respiratory factor 1 and mitochondrial transcription factor A [110]. γ-Tocotrienol has been shown to inhibit cytokine-triggered activation of NF-κB, and its upstream regulator transforming growth factor ß (TGF-β) activated kinase-1 in murine RAW 264.7 macrophages and primary bone marrow-derived macrophages [111]. In these cells, γ-tocotrienol induced the upregulation of A20, an inhibitor of NF-κB, B-cell lymphoma 2 (BCL2), C-X-C motif chemokine receptor 4, vascular endothelial growth factor and MMP9 [67].

Cytochrome P450

α-Tocopherol is transported to the liver and, through a series of oxidation reactions, converted to α-carboxyethyl hydroxychromane, which involves an initial ω-oxidation step [47]. There is evidence that cytochrome (CYP) P450 enzymes induced after α-tocopherol supplementation perform this ω-oxidation step [9]. Unlike α-tocopherol, other vitamin E forms are significantly metabolized to carboxychromanols via CYP P450 F2-initiated side-chain ω-oxidation (see Chap. 4) [9, 17, 95].

In a study to examine the short- and long-term effects of natural versus synthetic vitamin E on CYP P450-dependent gene expression in vitro, HepG2 cells were incubated with increasing concentrations of *RRR*-tocopheryl acetate (natural vitamin E) or *all-rac*-tocopheryl acetate (synthetic vitamin E). After 1 week, the mRNA levels of several CYP genes were measured applying GeneChip technology.

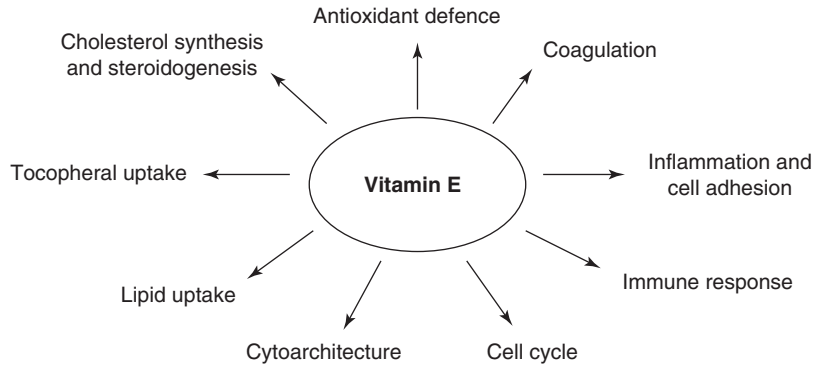


Fig. 7.4 Potential targets of transcriptional regulation by vitamin E

Interestingly, no significant changes in gene regulatory activity were monitored between *RRR*- and *all-rac*-α-tocopheryl acetate. In order to evaluate the function of vitamin E in CYP gene expression in vivo, male albino rats were allocated to either a vitamin E-enriched (*RRR*-tocopheryl acetate) or a vitamin E-deficient semisynthetic diet. However, neither in the vitamin E-supplemented nor in the vitamin E-deficient rats significant changes in CYP mRNA levels occurred in the liver. Likewise, CYP26A1 and its mRNA expression were not affected in vivo by varying vitamin E supplementation in the liver of laying hens and were not affected in hepatocytes in vitro [117]. Hence, it has been shown in mouse melanoma cell line B16 in vitro that γ-tocotrienol upregulates the expression of CYP1A1-sensitive aryl hydrocarbon receptor [116]. Furthermore, γ-tocopherol induced the expression CYP1A1, NAD(P)H dehydrogenase 1 (NQO1), γGCS, heme oxygenase 1 and PPARγ and decreased the tumour volume and multiplicity in a rat model of oestrogen-induced breast cancer [23]. In a rodent model of mammary carcinogenesis, dietary y-tocopherol regulates the expression of oestrogen receptor α, PPARγ and Nrf2 [94].

PXR has been postulated to play a role in the metabolism of α-tocopherol due to the upregulation of hepatic CYP P450, which has been verified in PXR-humanized, wild-type and Pxr-null mouse models [47]. Gene expression analysis revealed significantly increased expression of CYP3a11, as well as that of several other P450s only in wild-type mice. This study revealed that α-tocopherol is a partial agonist of PXR and that PXR is necessary for CYP3a induction by α-tocopherol.

Overall, targets of transcriptional regulation in response to vitamin E induction comprise antioxidant defence, blood clotting, inflammation and cell adhesion. Furthermore, vitamin E regulates genes encoding proteins centrally involved in lipid and cholesterol uptake, as well as those involved in cholesterol synthesis and steroidogenesis (Fig. 7.4).

Tissue-Specific Gene Expression

Vitamin E also seems to influence the expression of several genes in the rat brain. These tissue-specific gene expressions regulated by vitamin E encode proteins associated with hormones and hormone metabolism, nerve growth, apoptosis, dopaminergic neurotransmission and clearance of amyloid-β and advanced glycated end products [91]. In neuroblastoma cells, α-tocotrienol and tocopherols increased the release of amyloid-β by increased amyloidogenic amyloid precursor protein (APP) processing and decreased the degradation of amyloid-β, leading to the formation of neuritic plaques [38]. However, in rats receiving deficient, marginal or sufficient supplements of vitamin E over 6 months, the mRNA concentrations of genes involved in the formation (APP-binding family member 1, a disintegrin and metalloprotease domain 10, β-site APP-cleaving enzyme 1) or degradation (neprilysin, insulin-degrading enzyme, endothelin-converting enzyme) of amyloid-β in the brain [35] were evaluated. In the

cortex and hippocampus of our rats, dietary vitamin E did not affect the mRNA concentrations of the measured genes; hence, the role of vitamin E in brain function remains controversial.

In the kidney of diabetic rats, tocotrienol-rich fraction supplementation downregulated the expression of TGF-β, fibronectin 1 and collagen (COL) IV [93]. Furthermore, the tocotrienol-rich fraction protects against H_2O_2-induced oxidative stress in human skin fibroblast culture by modulating the expression of COL I and COL III genes with a concomitant increase in the rate of total collagen synthesis [63].

In addition, it has been shown that γ-tocotrienol induces cell cycle arrest and apoptosis via activating the BCL2-associated X protein-mediated mitochondrial and AMPK signalling pathways in adipocytes [113]. In human skin melanocytes, the tocotrienol-rich fraction and tocopherol downregulate the expression of tyrosinase (TYR) and TYR-related protein 1 and 2 genes [64].

Likewise, in a study to clarify the distribution of α-tocopherol-associated gene expression in major tissues and change in expression patterns induced by orally administered α-tocopherol to calves, the mean mRNA expression levels for α-TTP, afamin, ABCA1 and tocopherol-associated protein (TAP) were greatest in the liver, whereas scavenger receptor B1 mRNA was greatest in the adrenal gland [41]. The gene for CYP4F2 was most highly expressed in the liver, testes and adrenal gland. In addition, dietary vitamin E influences the expression of the α-TTP gene in the sheep liver, heart, spleen, lung, kidney, *longissimus dorsi* muscle and gluteus muscle in a tissue-specific way [120].

Taken together, vitamin E exhibits significant effects on gene expression with potential downstream effects.

Table 7.1 provides a brief selection of genes differentially regulated by Vitamin E in cultured cells and in vivo as reported in the literature

Vitamin E and DNA Methylation

Research on the function of essential micronutrients, such as vitamin E, in the epigenetic regulation of gene expression is an area of increasingly recognized importance. In this context, we have recently studied the effect of dietary vitamin E deficiency (6 months) on global and specific DNA methylation patterns in the rat liver [32]. 5α-R1 and γGCS were analysed for promoter methylation in putative CpG island regions. We have chosen these two enzymes for promoter methylation analysis because both enzymes were regulated at the mRNA level by α-tocopherol deficiency (see Chap. 2) in our rat study [6].

Under the conditions investigated, vitamin E deficiency was not associated with different CpG methylations of the analysed promoter regions of 5α-R1 and γGCS, respectively. Importantly, global DNA methylation was not significantly different between vitamin E-deficient and vitamin E-supplemented rats. These results suggest that vitamin E may not regulate hepatic DNA methylation patterns in rats. Further studies are needed to test the hypothesis regarding whether vitamin E deficiency may affect the epigenetic modification of histones by acetylation, methylation, phosphorylation, ubiquitination, sumoylation or isomerization. However, recently, in a global gene expression study, the epigenetic influences of parental diet and vitamin E intake on the embryonic zebrafish transcriptome were analysed [71]. Despite overt morphologic consistency, significant differences in gene expression suggested a perturbed energy metabolism and mitochondrial function. Genes affected were the transcript levels of APP, ApoE, nuclear receptor 4A3, cAMP response element-binding (CREB) protein, PPARγ and PGC1A and 1B. Thus, these findings demonstrate that Vitamin E in the parental zebrafish diet has a direct impact on the embryonic transcriptome.

Furthermore, in prostate tumours from the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse, it was shown that a γ-tocopherol-rich mixture of tocopherols maintains Nrf2 expression via epigenetic inhibition of CpG methylation in the Nrf2 promoter region [43]. The protein expression of DNA methyltransferase 1, 3A and 3B was lower in the prostate of the γ-tocopherol-supplemented group than that in the controls. In addition, TRAMP-C1 cells were treated with

Table 7.1 Selection of genes differentially regulated by Vitamin E in cultured cells and in vivo

Gene name	Gene symbol	Vitamin E form	Tissue/cell	Species	Function of the gene product	Reference
Metabolism						
α-Tocopherol transfer protein	αTTP	α-Tocopherol	Liver	Rat	α-Tocopherol transfer	[30]
Acetyl-CoA acetyltransferase 1	ACAT1	α-Tocopherol	Muscle	Lamp	Energy metabolism	[81]
Transglutaminase 2	TGM2	γ-Tocopherol	Prostate cancer cells	Human	Protein metabolism	[105]
Lipid uptake						
Scavenger receptor CD36	CD36	α-Tocopherol	Liver	Rat	Fatty acid metabolism; receptor for oxidized LDL	[6]
Scavenger receptor class B type 1	SR-BI	α-Tocopherol	Type II pneumocytes	Rat	Receptor for HDL	[52]
Scavenger receptor class A	SR-A I/II	α-Tocopherol	Peritoneal macrophages	Rabbit	Receptor for modified LDL	[103]
Low-density lipoprotein receptor	LDL-R	α-Tocopherol	HepG2 hepatoma cells	Human	Uptake of LDL; key regulator of cholesterol transport	[80]
			Subcutaneous fat	Lamp		[81]
		Tocotrienol	3T3-L1 preadipocytes	Mouse		[18]
Cholesterol, steroid and lipid metabolism						
3-Hydroxy-3-methylglutaryl coenzyme A reductase	HMGCR	α-Tocopherol	HepG2 hepatoma cells	Human	Rate-limiting enzyme for cholesterol biosynthesis	[80]
		γ-Tocopherol	Primary endothelial cells			[81]
3-Hydroxy-3-methylglutaryl coenzyme A synthase	HMGCS1	α-Tocopherol	HepG2 hepatoma cells	Human	De novo cholesterol biosynthetic pathway	[107]
			Subcutaneous fat	Lamp		[57]
Isopentenyl diphosphate delta isomerase	IPPI	α-Tocopherol	HepG2 hepatoma cells	Human	De novo cholesterol biosynthetic pathway	[107]
7-Dehydrocholesterol reductase	DHCR7					
Farnesyl pyrophosphate synthetase	FPPS					
Sterol regulatory element-binding transcription factor 1	SREBF1	α-Tocopherol	Subcutaneous fat	Lamp	De novo cholesterol biosynthetic pathway	[57]
ATP-binding cassette transporter1	ABCA1	Vitamin E	Aortae	Rabbit	Transport of cholesterol	[15]
		α-Tocopherol		ApoE knockout mice		[100]
		α-Tocopherol	Liver	Calves		[41]
Sterol regulatory element-binding protein2	SREBP2	α- and/or γ-tocopherol	Liver	Chicken	Immune response	[53]

Table 7.1 (continued)

Gene name	Gene symbol	Vitamin E form	Tissue/cell	Species	Function of the gene product	Reference
Fas cell surface death receptor	FAS	Tocotrienol	3T3-L1 preadipocytes	Mouse	Lipid biosynthesis	[18]
Stearoyl-CoA desaturase 1	SCD1					
Acetyl-CoA-carboxylase 1	ACC1					
Adiponectin receptor 2	ADIPOR2					
Uncoupling protein 2	UCP2				ß-Oxidation	
Carnitine palmitoyltransferase 1	CPT1					
Carnitine palmitoyltransferase 1A	CPT1A	γ-Tocotrienol	Primary hepatocytes	Rat	ß-Oxidation	[73]
C/EBP homologous protein	CHOP					
5-α-steroid reductase type 1	5α-R1	α-Tocopherol	Liver	Rat	Testosterone metabolism	[6, 87]
Antioxidant defence						
γ-Glutamyl-cysteinyl synthetase (regulatory subunit)	γGCS	α-Tocopherol	Liver	Rat	Glutathione synthesis	[6]
		γ-Tocopherol	Oestrogen-induced breast cancer cells	Rat		[23]
Glutathione peroxidase 3	GPX3	α-Tocopherol	Testes	Sheep	Antioxidative enzyme	[114]
Glutathione S-transferase-α1	GSTα1					
Superoxide dismutase 1 + 2	SOD 1 + 2	Tocotrienol-rich fraction	Diploid fibroblasts	Human	Antioxidative enzyme	[25]
Chopper chaperone for SOD	CCS1					
Catalase	CAT					
Forkhead-box-protein	FOXO3A					
Peroxiredoxin	PRDX6					
Coagulation						
Coagulation factor IX	FIX	α-Tocopherol	Liver	Rat	Intrinsic coagulation pathway	[6]
Thrombomodulin	CD141	γ-Tocopherol	Primary endothelial cells	Human	Receptor for thrombin	[81]
Inflammation, cell adhesion and immune response						
Selectin E	SELE / CD62E	α-Tocopherol	Aortic endothelial cells	Human	Cell adhesion molecule	[112]
Vascular cell adhesion molecule 1	VCAM1	α-Tocopherol	Aortic endothelial cells	Human	Recruitment of monocytes	
Intercellular adhesion molecule-1	ICAM1		Epithelial cells	Mouse		[13]
Integrin, α M	ITGAM	α-Tocopherol	Umbilical vein endothelial cells	Human	Immune response	[102]

(continued)

Table 7.1 (continued)

Gene name	Gene symbol	Vitamin E form	Tissue/cell	Species	Function of the gene product	Reference
Integrin alpha 2b	ITG	α-Tocopherol	Erythroleukaemia cells	Human	Platelet adhesion	[20]
Interleukin 2	IL2	α-Tocopherol	Naïve T cells	Mouse	Proliferation of T and B lymphocytes, immune response	[2]
Interleukin 4	IL4	α-Tocopherol	Peripheral blood T cells	Human	Inflammation and chemotaxis of inflammatory cells	[58]
Interleukin 6	IL6	α-Tocopherol	Spleen	Chicken	Immune response	[49]
5-Lipoxygenase	5-LOX	α-Tocopherol	Activated monocytes	Human	Leukotriene synthesis	[24]
Inducible nitric oxide synthase	iNOS	α-Tocopherol	Gastric mucosa	Rat	Nitric oxide production	[76]
Interferon regulatory factors	IRFs	α- and/or γ-tocopherol	Liver	Chicken	Immune response	[53]
Lymphocyte antigen 96 encoding gene	LY96					
Toll-like receptor 2	TLR2					
Leukocyte ribonuclease A	RSFR				Bactericidal activity	
Cyclooxygenase-2	COX2	α-, β-, γ-, δ-tocopherol	RAW 264.7 macrophages	Mouse	Prostaglandin synthesis	[72]
Interferon-induced transmembrane protein 3	IFITM3	Tocotrienol-rich fraction	MCF-7 breast cancer cells	Human	Immune response	[85]
Interferon-induced transmembrane protein 2	IFITM2	α-Tocopherol, tocotrienol-rich fraction, α-, γ-tocotrienol				
Ferritin heavy polypeptide	FTH1	γ-Tocotrienol				
Cell signalling and cycle regulation						
Cyclin D1	CCND1	α-Tocopherol	LNCaP prostate carcinoma cells	Human	Cell cycle regulation	[40]
Cyclin E1	CCNE1					
Cyclins	CCNs	γ-Tocopherol	Prostate cancer cells			[105]
Fas ligand	CD95L	α-Tocopherol	Peripheral blood cells	Human	Induction of apoptosis	[59]
Peroxisome proliferators-activated receptor γ	PPARγ	α-Tocopherol	SW480 colon cancer cells	Human	Regulator of adipocyte differentiation	[98]
Peroxisome proliferators-activated receptor α	PPARα	α- and/or γ-tocopherol	Liver	Chicken	Immune response	[53]

Table 7.1 (continued)

Gene name	Gene symbol	Vitamin E form	Tissue/cell	Species	Function of the gene product	Reference
Extracellular matrix						
Collagen α1	COL α1	α-Tocopherol	Liver	Mouse	Matrix protein	[21]
Collagen IVα3	COL IVα3	δ-Tocotrienol	MCF-7 breast cancer cells	Human	Matrix protein	[85]
Matrix metallopeptidase 1 (interstitial collagenase)	MMP1	α-Tocopherol	Skin fibroblasts	Human	Breakdown of interstitial collagens, types I, II, III	[86]
Matrix metallopeptidase 19	MMP19	α-Tocopherol	Peripheral blood mononuclear cells	Human	Cellular proliferation, migration and adhesion to type I collagen	[69]
Connective tissue growth factor	CTGF	α-Tocopherol	Aortic vascular smooth muscle cells	Human	Endothelial cell function	[69]
Cytoarchitecture						
ß-Tropomyosin 2	TPM2	α-Tocopherol	Lung	Mouse	Cytoskeleton	[77]
α-Tropomyosin 1	TPM1	α-Tocopherol	Vascular smooth muscle cells	Rat	Actin binding protein; actin-myosin interaction	[4]
Smooth muscle α-actin	ACTA2	α-Tocopherol	Aorta	Rabbit	Cytoskeleton	[48]

γ-tocopherol, which inhibited methylation in the Nrf2 promoter in vitro, and the protein expression levels of Nrf2 and NQO1 were higher than those in untreated controls. Hence, the supplementation of γ-tocopherol leads to higher Nrf2 expression and may contribute to the prevention of prostate tumorigenesis in TRAMP mice.

Vitamin E-Regulated miRNAs

miRNAs are small (22 nucleotides long), noncoding RNAs, which are single-stranded in their mature form. It has been established that miRNA post-transcriptionally influences gene expression by binding at the 3′ untranslated region of mRNA and thereby affecting their translation into proteins [14]. Each miRNA binds many different target mRNAs, allowing the post-transcriptional suppression of many different genes or potentially entire pathways, by a single miRNA [56].

In order to investigate the role of dietary *RRR*-α-tocopherol on miRNA expression in liver, rats were fed with diets deficient or sufficient in vitamin E for 6 months [36]. miRNAs that were earlier presented to participate in processes that were related to vitamin E, in particular lipid metabolism (miRNA-122a) [27], cancer progression and inflammation (miRNA-125b) [78], were used in this study. Vitamin E deficiency significantly lowered the levels of miRNA-122a and miRNA-125b. Besides its role in lipid metabolism, miRNA-122 level was reduced in hepatocellular carcinomas in rodents and humans [54]. As it has been shown that miRNA-125b was downregulated in human prostate cancer tissues [78, 83], lung cancer cell lines [74] and breast cancer [44], a function for miRNA-125b in carcinogenesis has been proposed as well. Results from mouse spleens after whole-body irradiation suggest that γ-tocotrienol pretreatment reverses the expression of several miRNAs that are

involved in postirradiation haematopoiesis. Therefore, in silico cellular pathway analyses have implicated the ERK/P38 mitogen-activated protein kinase pathway as a target signalling pathway [37]. In senescent human diploid fibroblasts, the tocotrienol-rich fraction reduces senescence-associated miR-34a expression and increases miR-20a expression in young human diploid fibroblasts [51]. Furthermore, the tocotrienol-rich fraction increases miR-449a expression in both young and senescent cells. On the other hand, the expression of miR-34a was upregulated in non-small-cell lung cancer cells by δ-tocotrienol, thereby suppressing neurogenic locus notch homologue protein 1 and its downstream targets, including hairy and enhancer of split 1, CCND1, baculoviral inhibitor of apoptosis repeat-containing 5 and BCL2 [45].

A function of miRNA-125b in chronic inflammation is backed up by a study by Tili et al. [104] that described TNFα as a direct target of miRNA-125b. A decrease in miRNA-125b caused increased TNFα generation after LPS stimulation. The authors hypothesized that the decreased miRNA-125b levels, as observed in the liver of vitamin E-deficient rats, may be related to an enhanced inflammatory reaction due to vitamin E deficiency. In the liver of an experimental fish model (*Nile tilapia*), the expression of miRNAs (miR-21, miR-223, miR-146a, miR-125b, miR-181a, miR-16, miR-155 and miR-122) that were reported to be related to oxidative stress was analysed after feeding with a vitamin E-deficient (0 mg/kg) or a vitamin E-excessive (2500 mg/kg) diet [101]. The results showed that vitamin E deficiency decreased the expression of miR-223, miR-146a, miR-16 and miR-122, while excessive supplementation of vitamin E increased the expression levels of all eight miRNAs.

Thus, vitamin E controls cell signalling not only at the mRNA level but also at the miRNA level [36].

Although gene chip and micro-RNA technologies have helped to identify vitamin E-sensitive molecular targets, transcription factors that are specifically regulated by vitamin E have not yet been found [16]. Finally, a receptor that specifically binds to vitamin E is currently unknown and warrants further research.

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Chapter 8
Metabolomic Approaches in Vitamin E Research

John K. Lodge

Keywords Metabolomics · Proteomics · Vitamin E · Metabolites · Human · Animal

Key Points

- Metabolomics aims to characterise changes to the complement of metabolites in a biological sample (metabolome) and is gaining interest in nutrition research as it can define perturbations to metabolism induced by dietary factors.
- In humans, vitamin E supplementation influences phospholipid metabolism and amino acid metabolism, and discriminatory metabolites have included several vitamin E metabolites.
- Metabolomic studies in animal models include studies on zebrafish foetal development, which demonstrated changes to antioxidant status and lipid peroxidation with vitamin E deficiency and rodent models of vitamin E deficiency that showed influences on central metabolism.
- Metabolomics has proven to be a useful research tool to identify novel functions of vitamin E.

Introduction to Metabolomics

Metabolomics can be summarised as a global analysis or characterisation of all metabolites measured in a biological sample (the metabolome). Metabolomic technology aims to provide a global description of all the metabolites present in a biofluid at a given time [1, 2]. The metabolome is a complex mixture of thousands of compounds that arise from endogenous metabolic pathways and exogenous sources such as diet. Foods contain thousands of compounds which give rise to a myriad of different metabolites present in a wide range of concentrations. Several commentary articles in recent years have suggested that metabolomics will have great value for nutritional studies [3–5]. By virtue of its comprehensive and non-selective approach to sample analysis, metabolomic procedures can define metabolic signals indicative of dietary factors not previously considered. Metabolomics has been used recently for several types of investigation relevant to humans including diagnosis of disease [6–8], mode of drug/toxin action [9, 10], characterisation of novel foods [11, 12], characterising novel

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_8

metabolic pathways [13, 14] and identification of biomarkers of dietary exposure [15–18]. There is increasing interest in the use of metabolomics to understand the effects nutritional intervention has on a system. A major challenge is to understand the relationship between nutrition and chronic disease, and a metabolomics-based approach could be used analytically to predict susceptibility to metabolic diseases and elucidate molecular mechanisms that can explain the beneficial effects of dietary interventions, either at the whole diet or single nutrient level.

Methodological Considerations for Metabolomic Studies

Metabolite profiling techniques can be either targeted or nontargeted, depending if the overall aim is hypothesis testing or hypothesis-generating (Fig. 8.1). Targeted approaches are usually hypothesis testing as typically the aim is to measure the change in that specific metabolite or series of metabolites [19]. Nontargeted approaches are sometimes more hypothesis-generating as they will provide a global change to metabolome with limited information of the metabolite species, and from there more targeted approaches can be developed. Metabolomics as a research technique requires technology to generate metabolite profiles from samples. Typically NMR spectroscopy or mass spectrometry (either liquid chromatography LC/MS or gas chromatography GC/MS) is used. These techniques are often complimentary as different chemistries of metabolites require different ionisations, and there is not one technology that can successfully display all metabolites in a sample from a single analysis. A typical workflow is shown in Fig. 8.2 and can be simplified into several steps, each having their own considerations:

(1) *Sample collection*. Blood concentrations of many metabolites are under metabolic regulation, but generally the plasma metabolome is dynamic and responsive to acute changes to diet and metabolism. Metabolite profiles have been shown to be influenced by the blood collection tubes [20],

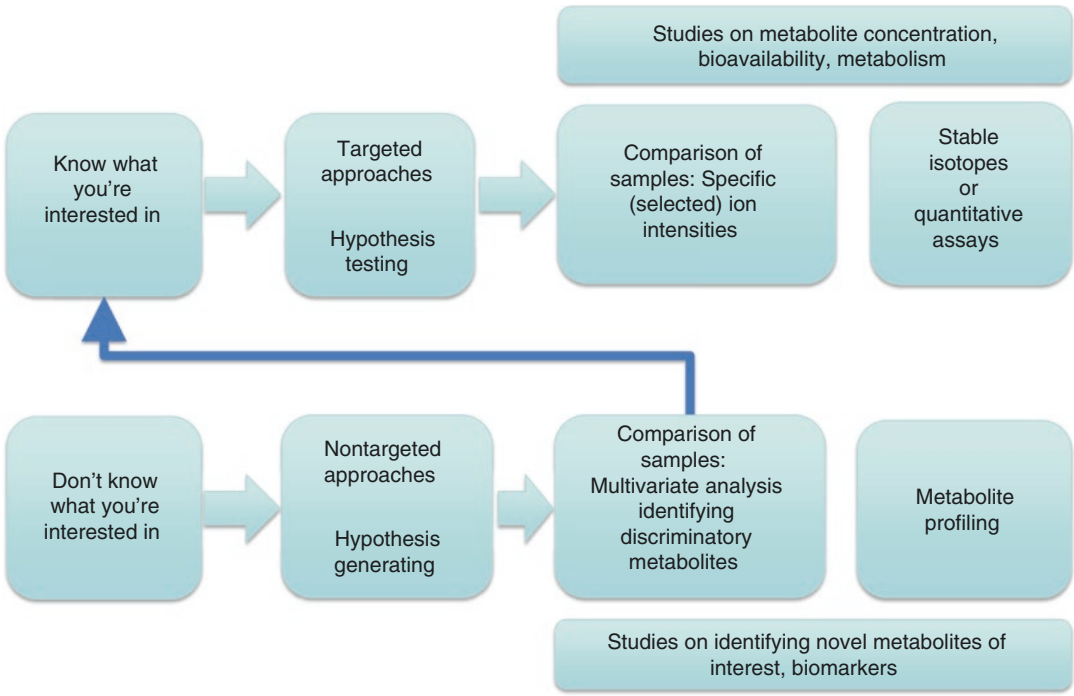

Fig. 8.1 Diagrammatic representation of the different metabolomic approaches for deciding an experimental approach

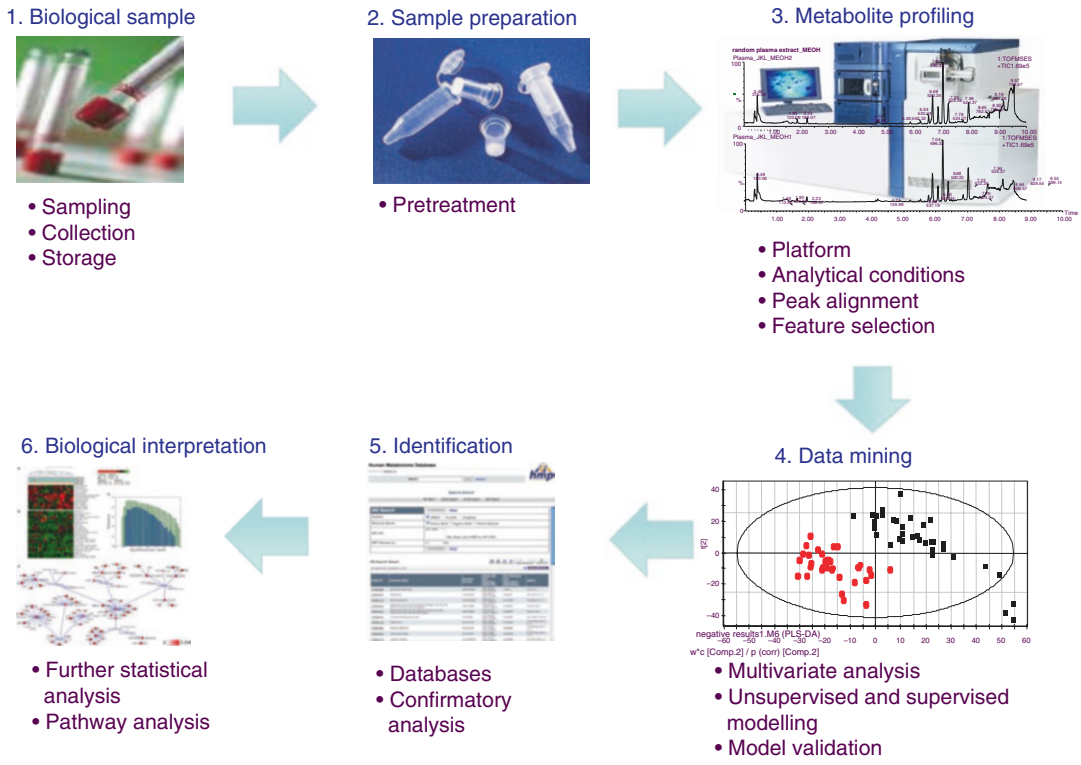

Fig. 8.2 Typical steps in a metabolomic experimental workflow. See test for further details

clotting times and clotting procedure [21]. Urine can be viewed as an 'endpoint' of metabolism and not under any metabolic regulation as for plasma; however, profiles are influenced by diurnal variation [22] and are reflective of acute dietary intake [23]. It has been established that spot urines are quite representative of total urine collections which limit the requirement for a full 24-h collection [24]. It is also important that collection and storage must be consistent between samples as variation can arise from the types of collection tube used, use of preservatives and storage conditions (temperature, time). Sample storage is important; for example, no differences were found in urinary or plasma metabolite profiles when samples were stored for 0 or 24 h at 4°C [25], but for longer storage times, sub-zero conditions (<−20°C) should be used [26]. Freeze-thaw cycles do not appear to influence metabolite profiles using LC/MS [26].

(2) *Sample preparation.* This is an important issue depending on the choice of analytical platform as samples may require a degree of pretreatment which adds considerable analytical variation and also impacts on the level of information obtained, especially as different solvents will result in different metabolite profiles. NMR requires no sample pretreatment, and so the spectrum contains information on small molecular weight compounds, lipids and proteins. Mass spectroscopy procedures require removal of proteins, and various methods for protein precipitation have been compared in terms of the number of features obtained [27, 28] with similar observations finding chilled methanol treatment to be the most optimal for plasma samples. Similarly there have also been attempts to develop and optimise the methodology for LC/MS-based metabolomic studies for urine samples [29, 30]. It is clear that the analytical conditions associated with mass spectroscopy approaches need to be further optimised and standardised as sample pretreatment, run time, type of column, temperature and gradient all influence the profiles obtained.

(3) *Metabolite profiling.* Metabolomics requires a means of sample analysis to produce metabolite profiles or fingerprints. Analytical design and variation in relation to metabolomics have been extensively investigated [21, 22, 28, 31]. As each platform has its own analytical requirements, variation arises from the methodology used in the study and from the instrument itself. NMR and mass spectroscopy are very different techniques, and the choice between them is often down to what is available; however, sensitivity is relatively low with NMR, and in many NMR reports, the same series of metabolites are commonly found as discriminating species. MS is more sensitive and can detect metabolites at orders of magnitude lower than NMR, but sample pretreatment is a major issue. Relevant analytical strategies to assess vitamin E status have been published [32].

(4) *Multivariate data analysis.* The data produced by metabolomic experiments are highly complex, for example, over 5000 features differing in intensity by orders of magnitude are typically represented in LC/MS analysis of plasma or urine. As the technology aims to characterise changes to the metabolome in discreet sets of samples, powerful multivariate statistical analysis techniques are required to process the data for sample classification and clustering/discrimination. These techniques essentially are data compression tools; by reducing the dimensionality of the data, it becomes easier to visualise graphically so that patterns between groups of data (samples) can be revealed. Both unsupervised and supervised techniques are commonly used. Principal component analysis (PCA) is an unsupervised method for sample classification. The principal components represent variance between samples, and so samples that are similar in their metabolite profile will cluster together in a scores plot. An important aspect of this method is that it does not require information about sample classification and as such is a good starting point to visualise the data. However, PCA is often unable to classify similar groups, which is typically found in nutritional studies, and so other techniques are often used. Supervised methods, such as partial least squares-discriminant analysis (PLS-DA), include information on sample classification making it more likely that patterns of change between groups are visualised. However, the downside is that this can lead to overanalysis, for example, PLS-DA will separate groups from random data [33], and as such these methods require appropriate model validation steps and adequate training and predictive sample sets, usually with independent data. Clustering methods as described above only visualise discriminated sample groups demonstrating if metabolite profiles between sample groups are different. They need to be used alongside classification tools that provide a metric quantifying the degree of fit of the model and the discriminating ability. To confirm that the variation between samples is real, standard statistical techniques can be applied to compare the intensity of discriminatory metabolites between samples. It should be noted that data analysis steps require careful consideration and interpretation in order to be confident of obtaining meaningful data.

(5) *Feature identification.* Multivariate analysis will visualise trends between sample groups based on variation, but it is important to be able to identify those discriminatory metabolites that are causing the variation. Metabolite databases (e.g. Human Metabolite Database [34], METLIN [35], KEGG) are useful to putatively identify discriminating metabolites; however, as the complete metabolome remains to be fully understood, there is not one source for all metabolites, and unknowns are still a major problem requiring further analytical work. A full confirmation generally requires comparisons with pure standards analysed under identical conditions as for the samples and further analysis (e.g. MS/MS experiments in mass spectroscopy) need to be performed. Occasionally this could also mean the chemical synthesis of standards if standards are unavailable to purchase.

(6) *Biological interpretation.* There is a complex relationship between genotype, metabolic phenotype, diet, lifestyle and environment and nutritional needs [36]. Tissues and cells respond to the delivery of nutrients by adapting their metabolic processes through the regulation of gene transcription, protein levels and enzyme activity. The endpoint of these processes is that the levels of metabolites change; however, the tools available to investigate downstream effects rely on obtain-

ing quality metabolite data; otherwise incorrect assumptions can be made. Pathway analysis is such a procedure to help with biological interpretations. Such analysis would involve identifying metabolites that are common to a biological pathway; the more metabolites in a pathway, the more confident that the results is real. Such analysis is a guide but can lead to novel hypotheses and further explanatory work.

Metabolomic Studies in Humans Following Vitamin E Supplementation

Vitamin E and its forms are well established for their antioxidant properties that will not be discussed here. The discovery of several α-tocopherol cellular binding proteins appeared to suggest that vitamin E had alternative functions. Metabolomics has the potential to identify novel mechanisms due to its global approach, and this has been the aim of several metabolomic studies. The first study involving vitamin E supplementation [37] gave 12 normolipidaemic male subjects 400 mg/d of *RRR*-α-tocopherol acetate for 4 weeks and compared the plasma metabolome before and after supplementation using LC/MS on a time-of-flight mass spectrometer (LC/TOF-MS) following a methanol-based extraction (4:1 ratio of methanol to sample) and removal of plasma proteins. They found significant changes to the metabolome using supervised classification methods (Fig. 8.3) but not using unsupervised methods highlighting that the changes to the metabolome were relatively minor. The discrimination between the supplementation regimes was found to be due primarily to an increase/decrease in a series of lysophospholipid species that were characterised and confirmed by MS/MS [37]. The study was not well controlled, which does limit the impact of the results, but the authors did use additional methodologies to confirm the metabolite identifications.

A similar study gave ten participants (presumably mixed gender) *all-rac*-α-tocopherol acetate 600 IU (equivalent to 400 mg/d of natural α-tocopherol) for 2 weeks following a 6-day supplementation period of 55 g/d of raw almonds [38] (almonds are relatively high in vitamin E content). Urine and serum samples were taken before and after each supplementation regime. Serum again underwent a methanol extraction (4:1 ratio), and urine samples were diluted 1:1 with an acetonitrile/water solution. Samples were analysed using a LC/TOF-MS approach. Interestingly the authors did not report

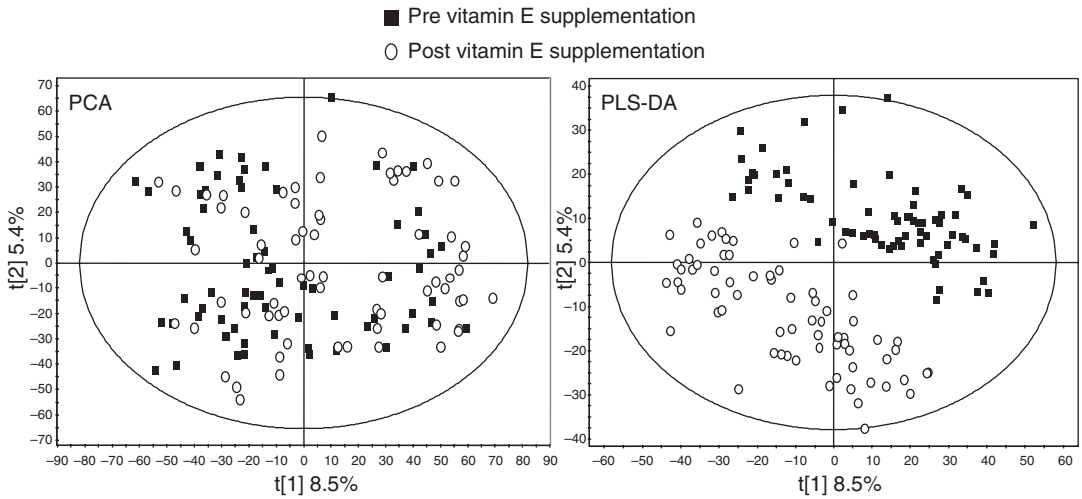

Fig. 8.3 The effect of vitamin E supplementation on the plasma metabolome as represented by score plots from either PCA or PLS-DA. Taken from [37]

any other significant changes to the plasma metabolome apart from three novel vitamin E metabolites [38]. The authors did comment that there was large interindividual variation, and not all the metabolites were produced in all subjects; there was a lack of discrimination with principal component analysis. This was also found by Wong and Lodge [37], and discrimination between vitamin E treatments was only found using supervised modelling, in that case PLS-DA. However, it is interesting that even though both studies used similar levels of vitamin E intake, they had different results.

These studies had relatively small sample sizes ($n = 10$) and small study durations, and these limitations were overcome in a metabolomic investigation of the α-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) trial [39]. In this study, the authors used a random subset of 100 male participants that received 50 mg/d of *all-rac*-α-tocopherol acetate or placebo for 5–8 years. It must be stated that all subjects in this trial were current cigarette smokers, and it is well established that smoking status does impact on vitamin E status and metabolism [40, 41]. They used serum samples and again used methanol to extract metabolites and remove proteins. Analysis was performed using a combination of tandem mass spectroscopy and GC/MS in a more targeted approach that measured 516 compounds (outsourced to Metabolon Inc., Durham, NC, USA). The authors found 24 metabolites that were significantly changed in response to VE supplementation [39]. The most significant discriminatory factors were vitamin E metabolites that were increased following vitamin E supplementation, while β- and γ-tocopherols were decreased. Interestingly, three structurally similar amino acids decreased following supplementation (beta-alanine, ornithine and acetyl-lysine) [39], and the authors suggested that this could be a consequence of the oxidation of the phytyl side chain [39] and so still related to vitamin E metabolism; however, in their discussion the authors do not mention that this could be evidence for alternative roles for vitamin E.

The ATBC study was a large clinical trial to examine the effectiveness of VE supplementation for prostate cancer and all-cause mortality. Vitamin E supplementation has also been used to improve hepatic histology in non-alcoholic steatohepatitis (NASH) [42]. In their study Cheng et al. used metabolomics to profile those patients that either responded or did not respond to vitamin E supplementation in order to identify metabolic predictors of response to vitamin E [42]. The trial itself involved a VE supplementation regime of 800 IU *all-rac*-α-tocopherol acetate (equivalent to approx. 600 mg) per day for 96 weeks versus placebo. Plasma samples and a similar analytical approach to that of above was used in which the targeted metabolomic analysis was outsourced (Metabolon Inc., Durham, NC, USA). Fifteen subjects that either responded or did not respond to VE supplementation (based on a decrease in NAS score) were selected for metabolomic profiling. The authors did not report on metabolomic differences influenced by VE supplementation (comparison of VE vs placebo); nevertheless they did observe a number of metabolites that could be classified as biomarkers of VE response [42]. Interestingly some of these metabolites are involved in the glutathione pathway (γ-glutamyl amino acids), while others (phenyl-propionic acid and indole-propionic acid) were predictors of response at baseline [42]. It is interesting that different metabolites were classified as biomarkers either at baseline or at end of the study (following supplementation).

Proteomics is a complimentary technique to metabolomics, and they share similar analytical methodologies (e.g. they can be both LC/MS driven). Proteomics is the study of the compliment of proteins in a sample (the proteome). The only proteomic studies linked to vitamin E status were associations between plasma VE concentrations and plasma protein concentrations [43, 44]; no supplementation regimes were used. In one such study, a total of >1000 participants within a small age range (20–29 years) provided a fasting blood sample, which was then analysed for VE concentration, and subjects were characterised to tertiles of VE concentration. Tertiles ranged from <24.2 to >32.2 μmol/l, and 57 plasma proteins were quantified; the proteins were selected based on their relationship to chronic disease and so were semi-targeted. The authors found several proteins that were significantly associated to VE concentration including apoCIII, fibrinogens and fibronectin [43]. Although the authors state correctly that investigating plasma proteins could link to biological pathways regulated by VE, this study is an association, and further work is necessary in a supplementation study to assess

VE effects. In a further study on the association between plasma VE concentrations and the plasma proteome, West et al. [44] analysed the plasma proteome of 500 Nepalese children (6–8 years) together with plasma α- and γ-tocopherol concentrations and found 119 proteins out of 982 that correlated with α-tocopherol concentration. Out of these, 61 were positively correlated, and these included several apoproteins such as apoCIII (found previously associated with VE concentration [43]), apoB, apoE and multifunctional proteins involved in vascular lipid, vitamin, mineral and hormonal transport, for example, retinol-binding protein. The study found 58 proteins that were negatively correlated [44], and these included nutrient transporters, proteins involved in homeostasis, cellular and extracellular matrix adhesion, vascular cell adhesion, anticoagulative control, responses to inflammation and redox haemostasis [44]. The study also highlighted 12 proteins that were positively correlated with γ-tocopherol concentration; some of these proteins were also correlated to α-tocopherol concentration, such as apoCIII [44]. This study followed on from a previous analysis of the sample set that was focussed on micronutrient deficiency and status [45], identified an association between α-tocopherol concentration and apoCIII and highlighted that proteomic approaches are useful for determining functional relationships [45].

Metabolomic Studies in Animals

Animal studies provide good insights into the functional role of vitamin E, and there have been several metabolomic studies in model systems aimed at defining either established roles of vitamin E and/or the potential to define new roles.

Studies in Rodents

The effects of VE deficiency on the liver have been investigated using metabolomic approaches. In their study, Moazzami et al. [46] took rats fed either vitamin E-sufficient or vitamin E-deficient diets and used ^{1}H-NMR methodology to profile polar extracts of the livers. They found eight metabolites that discriminated between VE-deficient and VE-sufficient treatments, six of these were significantly higher in intensity in VE-deficient rats, and these were lysine, valine, carnitine, choline, tyrosine and inosine. Two metabolites had reduced intensity in VE-deficient rats, glucose and uridine 5-monophosphate. The authors attempted to relate these changes to effects on central metabolism, although changes in tyrosine metabolism have been found previously [47]. This study appeared to be extended further (by the same first author) comparing more extensive vitamin E diets and comparing VE-deficient, VE-marginal, VE-sufficient and VE-fortified animals but again comparing polar extracts of the liver [48]. In their nontargeted ^{1}H-NMR-based approach, the study found lower glucose content in VE-deficient rats, similar to previous observations [46], and higher content of creatine, phosphocholine and betaine, although the study could not easily differentiate the VE-sufficient diets. The authors also performed targeted analysis of metabolites associated with glucose and lipid metabolism and interestingly performed transcriptional analysis to determine if these changes correlated to their metabolomic analysis [48]. The authors conclude that VE deficiency can alter hepatic energy metabolism in the rat. Central metabolism changes induced by exercise in the rat have also been investigated using a nontargeted GC/MS-based approach [49], in which the authors found that α-tocopherol was a discriminatory feature that was increased in the *plantaris* muscle of exercise-trained rats but decreased in plasma [49]. The authors suggested that this could be an adaptive response with α-tocopherol moving from the plasma to muscle in response to increased oxidative stress and to aid in repair as vitamin E supplementation has been shown to benefit exercise-induced muscle damage [49].

Studies in Zebrafish

Vitamin E has long been known for a role in foetal development and embryogenesis in animals; however, this has yet to be shown in mammals and humans. Metabolomic approaches have been used to study the role of embryonic VE deficiency in zebrafish development. In one study zebrafish from VE-deficient larvae had significant behavioural defects even after dietary VE remediation [50]. Samples were obtained from homogenised larvae using methanol and water (80:20), and targeted metabolomics using LC/TOF-MS methodology revealed a number of metabolites related to cellular antioxidant network that were dysregulated with VE deficiency. For example, the study found lower levels of ascorbic acid and glutathione, elevated levels of NADPH and $NADP^+$ and increased levels of glucose [50]. These changes are consistent with VE deficiency causing oxidative stress and increasing metabolic flux through the pentose phosphate pathway [50]. They also performed targeted analysis on a number of LC-PUFA derivatives and found that VE deficiency causes increased content of oxidised lipids and isoprostanes [50]. Interestingly, the same group went on to use a nontargeted lipidomics approach using triple TOF LC/MS methodology to investigate susceptible lipids in the brains of zebrafish during VE deficiency [51, 52]. They found 1-hexadecanoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine [DHA-PC 38:6, PC 16:0/22:6] to have the most significant and greatest fold differences between VE-deficient and VE-supplemented groups. Furthermore VE-deficient brains were significantly lower in 19 lysophospholipid species but had increased hydroxyl DHA-PC, suggesting that VE deficiency induces phospholipid remodelling; the authors suggested that this was a consequence of increased lipid peroxidation [51, 52], but it is interesting that a metabolomic investigation of VE supplementation in humans also highlighted links to phospholipid metabolism [37].

Vitamin E Metabolism

There have been several targeted and nontargeted studies in humans and in animal models that either aimed to study vitamin E metabolism or revealed metabolites of vitamin E as being important discriminatory metabolites. Vitamin E metabolism will be discussed elsewhere (see also Chaps. 4 and 6), but two studies have used metabolomics to identify novel vitamin E metabolites.

In one study, pregnane X receptor (PXR)-null and wild-type mice were treated with a control or a PXR activator; urine samples (before and after treatments) were collected and diluted 1:4 with acetonitrile, while plasma samples were diluted 1:20 with acetonitrile and profiled by LC/TOF-MS [53]. Discriminatory metabolites that were decreased after treatment (PXR activator or placebo) were found to be metabolites of vitamin E itself, namely, α- and γ-carboxyethyl-hydroxychroman (CEHC) glucuronide and glucoside, the latter being identified in this study [53]. The same authors went on to expand their studies in mice and humans [38]. Mice were fed a vitamin E-deficient or vitamin E-enriched diet, and blood, urine and faeces samples were collected, while humans ($n = 10$) were given 600 IU *all rac*-tocopherol acetate for 2 weeks and blood and 24-h urine samples collected. Plasma underwent a methanol extraction (4:1 ratio), and urine samples were diluted 1:1 with an acetonitrile/water solution. Samples were analysed using LC/TOF-MS. The studies were able to identify three novel urinary metabolites, α-CEHC glycine, a glycine glucuronide and α-CEHC taurine, and these metabolites were fully characterised by preparation of standards and MS/MS experiments. The study did not mention any further discriminatory features apart from vitamin E metabolites; nevertheless it demonstrates the use of metabolomics to identify and characterise metabolites of vitamin E.

Conclusions

Vitamin E is well characterised as an antioxidant agent; however, novel functions for vitamin E have been investigated with the aid of metabolomics. Studies in humans have demonstrated changes to the urinary or plasma metabolome with vitamin E supplementation, while studies in animals have found changes to the metabolome with models of vitamin E deficiency. Studies are consistent in that many have demonstrated vitamin E metabolites as discriminatory species, but with different study designs, there is limited consistency in effects on metabolism. Nevertheless, metabolomics remains a powerful research tool to identify novel functions of vitamin E.

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Chapter 9
The Tocopherol Transfer Protein: Regulator of Vitamin E Status

Jeffrey Atkinson, Varsha Thakur, and Danny Manor

Keywords Tocopherol · Transfer protein · Phosphoinositide · Oxidative stress

Key Points

- TTP is the only known specific regulator of α-tocopherol status in vertebrates.
- Genetic defects in the gene encoding TTP cause vitamin E deficiency in humans.
- Vectorial transport of vitamin across cells by TTP is driven by opposing concentration gradients of tocopherol and PI(4,5)P_2 between organelles.
- Expression of TTP in the CNS protects vulnerable neurons from oxidative stress.
- TTP expression and activity are regulated to maintain vitamin E homeostasis.

Introduction

Many molecules that are essential for the growth and survival of cells and organisms are virtually or poorly insoluble in aqueous solvents. The list of such ligands is long and includes lipophilic molecules that are de novo synthesized within cells (e.g., phospholipids, fatty acids, cholesterol, sex hormones), as well as essential hydrophobic compounds that are derived from the diet (e.g., the fat-soluble vitamins A, D, and E). The poor solubility of such hydrophobic molecules in aqueous solution poses significant thermodynamic and kinetic barriers on their movement in the aqueous milieu of cells and

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_9

tissues. Two general mechanisms evolved to achieve regulated transport of such lipids through the cytosol: (1) transport within the bilayers of lipid vesicles, as seen in the trafficking of newly synthesized phospholipids and sterols from the endoplasmic reticulum to other intracellular organelles [1–4], and (2) by lipid binding and transfer proteins. These proteins bind their lipid ligands (retinoids, some sterols, tocopherol, squalene, etc.) with high affinity and specificity and have been shown to accelerate the movement of those lipids between lipid membranes in vitro. Several distinct structural motifs have evolved to function as specific lipid binding domains and occur in lipid transfer proteins such as the fatty acid binding proteins (FABPs), StAR-related lipid-transfer (START) domain proteins, oxysterol binding proteins (OSBP) and related proteins (ORPs), and phosphatidylinositol transfer proteins (PITPs). Of specific relevance to this chapter is a large family of proteins that contain the CRAL-TRIO lipid binding domain which includes the vitamin E binding protein TTP (SMART-SM00516; PFAM-PF00650). The defining protein of this family is the yeast phospholipid transfer protein Sec14, containing a phospholipid binding cavity with a floor made of four parallel and two antiparallel beta-strands. Several α-helices cover the β-sheet, and the binding site is gated by a flexible hinged helix ("the lid"). The Sec14 domain is found in 6024 proteins across all eukaryotic phyla, with as many as 49 identified and predicted Sec14 domain-containing proteins in humans. In addition to Sec14, the CRAL-TRIO family [5–7] includes the tocopherol transfer protein (discussed in detail below), neurofibromin [8], the yeast Sfh proteins [9], supernatant protein factor [10, 11], the human Sec14-like proteins (e.g., Sec14L4), Dbl-like activators of Rho GTPases [12–14], the tyrosine phosphatase MEG2 [15], and clavesin [16].

Tocopherol Binding Proteins It is generally believed that tocopherol binding proteins serve to "solubilize" the hydrophobic vitamin, thereby facilitating its transport to intracellular sites. Indeed, incubation of crude cytosol fractions with radioactively labeled α-tocopherol followed by chromatographic separation revealed the presence of tocopherol binding proteins in a number of tissues (e.g., [17–21]). Since in cells the hydrophobic tocopherol resides predominantly in membranes, specific binding proteins may also be expected to interact with lipid bilayers. Experimentally, this criterion has been applied in the form of "tocopherol transfer assays" that measure each protein's ability to facilitate the transfer of a labeled tocopherol between lipid vesicles (e.g., [22, 23]).

It is important to note that conclusive identification of a protein's "real" physiological ligand using in vitro binding assays should be interpreted with caution. Unlike water-soluble protein-ligand interactions, many lipophilic ligands will exhibit a measurable affinity to most hydrophobic binding pockets within protein folds (e.g., cholesterol binds to CRAL-TRIO proteins' binding pockets, although no evidence exists that this behavior has any physiological relevance). The threshold for non-specific binding needs to be evaluated directly and carefully [24, 25].

TTP The best characterized tocopherol binding and transfer protein is the α-tocopherol transfer protein (TTP), that belongs to the CRAL-TRIO protein family [26, 27], and is the focus of this chapter. Inoue and colleagues reported the identification, purification, and cloning of a soluble, 33 kDa protein that bound tocopherol with high affinity from rat liver cytosol [22, 23] and, later, its cloning from a human liver cDNA library [28]. The gene encoding TTP is highly conserved among species (94% homology between the rat, mouse, and human proteins). Two in vitro biochemical hallmarks were assigned as "signature" activities of TTP: (1) high affinity binding of tocopherol and (2) facilitation of tocopherol transfer between lipid membranes [20, 29, 30]. The ligand binding preference of this protein is: α-*RRR*-tocopherol>> β- tocopherol >> γ- tocopherol, δ tocopherol, essentially identical to the biological potency of the different tocols in bioassays [30]. Thus, it is generally accepted that TTP is the major physiological mechanism responsible for the selective retention of α-*RRR*- tocopherol in vivo and, therefore, a key mediator of vitamin E action [31]. While expression of TTP is highest in the liver, it is becoming apparent that the protein is also expressed in the brain and placenta and that expression in these tissues is of high physiological importance [32–35].

The role of TTP and tocopherol in human health is underscored by the fact that heritable mutations in the TTP gene are the only known cause for the autosomal recessive disorder ataxia with isolated vitamin E deficiency (AVED; [36]). AVED patients present progressive neurodegeneration and low (or undetectable) serum levels of α-tocopherol. Multiple mutations in TTP were identified in AVED patients that impair the protein's function. We have recently shown that clinical severity of the disease is correlated with defects in TTP function in vitro and in cultured cells [37–40]. The role of TTP in vitamin E status and human health was underscored by observations made in mouse models in which expression of the *ttpA* gene has been disrupted [41–43]. While the homozygous TTP$^{-/-}$ mice are normal in many respects, their levels of circulating tocopherol are extremely low, and they manifest three major pathologies that underscore the critical roles of α-tocopherol in health. In support of epidemiological correlations between vitamin E intake and cardiovascular disease, TTP$^{-/-}$ mice are more susceptible to formation of atherosclerotic lesions [41]. Additionally, female TTP$^{-/-}$ mice are infertile ([43]), in accordance with the original identification of vitamin E as a "fertility factor" (see below). As TTP$^{-/-}$ mice age, they display cerebellar ataxia symptoms, reminiscent of those associated with AVED in human patients ([42]; see below).

Heart-TTP This ~14 kDa polypeptide was demonstrated to bind labeled α-tocopherol in homogenates prepared from rat, rabbit, and bovine hearts [44–46], rat liver [45, 47], and human placenta [48, 49]. The protein was later said to be "present in all tissues" [50], but since the protein was never sequenced, it is impossible to determine whether the biochemical activity demonstrated in different tissues originates from the same biochemical entity. Since thorough biochemical characterization was described only for the form purified from the heart, we focus our discussion on this isoform and refer to it as "heart-TTP." Since its identification >20 years ago, heart-TTP received surprisingly limited experimental attention. Current knowledge of this protein can be summarized as follows: (1) The protein was purified to homogeneity using conventional chromatography approaches, yet has never been sequenced nor cloned. (2) Purified heart-TTP displays rapid and saturable binding to [^{3}H]-α-tocopherol. (3) The purified protein displays pronounced substrate specificity for α-tocopherol, showing no binding for oleic acid, neither to the γ or δ vitamers of tocopherol. (4) Binding of [^{3}H]-α-tocopherol is tight (Kd = 3 nM) and stoichiometric (0.9 mole α-tocopherol per mole protein). (5) The protein is monomeric in solution. (6) Heart-TTP facilitates the in vitro transfer of α-tocopherol between lipid vesicles.

Tocopherol-Associated Protein (TAP)/Supernatant Protein Factor (SPF) Stocker et al. [51] isolated and later cloned [52] a 45 kDa protein from bovine liver that bound radiolabeled α-tocopherol and termed it "tocopherol-associated protein" (TAP). It was later noted that this protein is identical to a squalene binding protein that functions in the de novo cholesterol biosynthetic pathway, previously purified and characterized by Bloch's group [53–55]. Moreover, competitive ligand binding measurements revealed that this protein's affinity for α-tocopherol is at the non-specific range (>600 nM; [24]), shedding doubt on the relevance of this protein to vitamin E biology [24, 56, 57]. Thus, although SPF is not a bona fide tocopherol binding protein, its documented impact on cell proliferation and behavior [58–60] renders it a fascinating subject of study on the roles that sterol binding proteins play in cell biology. It is important to note that recent changes in nomenclature now refer to this protein as Sec14L2.

Three-Dimensional Structure of TTP

Published three-dimensional structures of the human [61, 62] and mouse [63] TTP isoforms reveal a compact protein, with a deeply buried hydrophobic ligand binding pocket, and a charged surface that renders the protein highly soluble (Fig. 9.1a). Comparison of the apo- and holo-structures reveals a

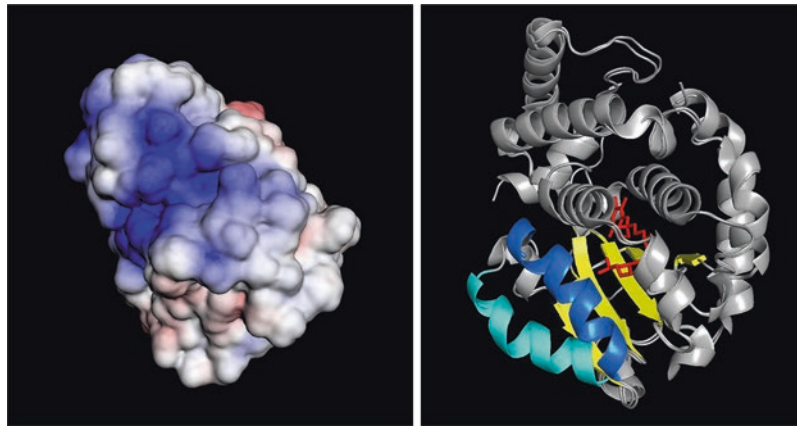

Fig. 9.1 Three-dimensional structure of TTP. **Left panel**: Depiction of the protein's surface electrostatic potential. PyMOL was used for visualization, and the electrostatic surface was calculated using the PyMOL plugin APBS via the PDB2PQR Server (http://nbcr-222.ucsd.edu/pdb2pqr_2.1.1/). The PARSE force field was used, and PROPKA to assign protonation states at pH 7. The solvent accessible surface was visualized using 3DMol and colored from −5 kT/e (dark blue) to +5 kT/e (dark red). The deep blue patch is largely a result of residues R59, R68, K190, R192, K217, and R221. **Right panel**: Backbone aligned x-ray crystal structures of TTP in the open ligand-free form (PDB 1OIZ) and closed ligand-bound form (PDB 1OIP). The mobile helical gate comprising residues 201–213 is colored dark blue in the closed form and aquamarine in the open form. Tocopherol bound to 1OIP is shown in red

significant conformational change that occurs upon ligand binding. The major movement of tertiary structure occurs in a helical "lid" (residues 201–213) that covers the entrance to the binding site, a structural feature conserved among all members of the CRAL-TRIO protein family. The flexibility of the helical lid is illustrated by measurements of the distance between the alpha-carbons of F213 and K177 in TTP which is ~16.0 Å in the ligand-free conformation and shortens to 6.5 Å in the tocopherol-bound conformation [61]. Similar ligand-induced conformational shifts are seen in the yeast phospholipid transfer protein Sec14 [64]. The currently prevailing hypothesis regarding ligand transfer by CRAL-TRIO proteins proposes that the amphipathic lid provides a molecular mediator for membrane insertion and ligand exchange [27, 64, 65]. Such mobile structural units appear to be a common feature of ligand-induced conformational selection in other proteins and enzymes as well [66–68].

The biochemical function of TTP, transferring the lipophilic α-tocopherol between intracellular membranes, necessitates extraction of the ligand from one bilayer and depositing it in another. For this activity the protein must, at least transiently, interact with lipid bilayers. While structural determinations of the protein were based on soluble preparations, and did not include membranes, calculations on the free energy changes on protein insertion into a hydrophobic phase have revealed that indeed, TTP utilizes specific residues to interact with membranes, as shown in Fig. 9.2. Interactions with a membrane occur by insertion of TTP's hydrophobic residues into the outer leaflet of the bilayer. Residues F169, F165, I202, and M209 are particularly important, as they provide the side chains that insert into the bilayer. Accordingly, substitution of either F165 or F169 with aspartic acid all but abolishes membrane binding of TTP and abrogates its activity in facilitating vitamin E transport in cells [65].

Recent findings provide important mechanistic insights into how the lid mediates membrane association and ligand retrieval/release by TTP [63, 69]. The key appears to involve an additional ligand, PI(4,5)P_2, which binds to a patch of basic residues on TTP's surface. Binding of PI(4,5)P_2 to TTP induces a conformational change whereby the lid "opens," allowing for the release of the bound vitamin E and its deposition into the "target" membrane. The interior hydrophobic surface of the lid may actually insert into the bilayer at this step as suggested in calculations of the free energy of insertion of these protein features into a hydrophobic phase [65, 70]. This ligand exchange mechanism appears

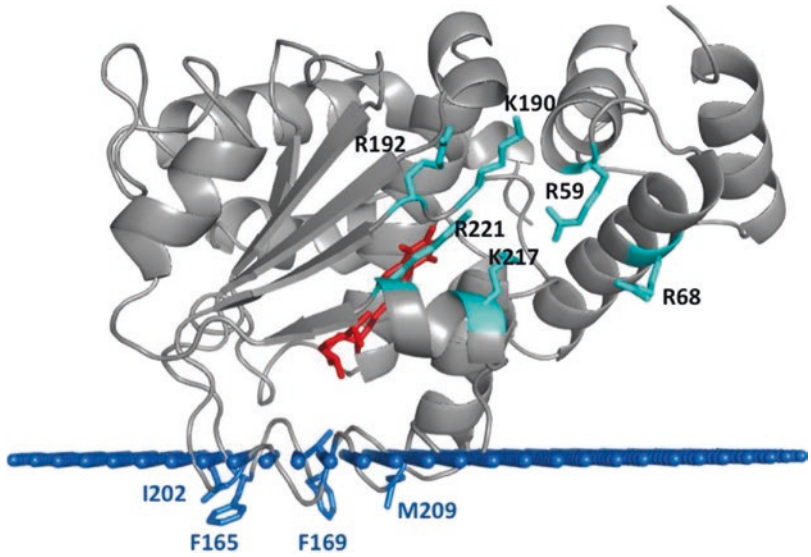

Fig. 9.2 Calculated orientation of human tocopherol transfer protein (PDB 1OIP) with respect to a hydrophobic phase of a phospholipid bilayer. Image is downloaded from the Orientation of Proteins in Membranes database at http://opm.phar.umich.edu. The plane of blue spheres marks the approximate surface of the acyl chain methylene carbons moving away from the glycerol moiety. Residues F165, F169, I202, and M209 in blue are shown deeply inserted into the interior of the bilayer. Tocopherol is shown in red. Residues in aquamarine are key basic amino acids making up the basic patch as seen in Fig. 9.1 (left)

Table 9.1 Ligand and membrane binding affinities of wild-type TTP and K217A

Protein	K_d for PM vesicles (μM)	PM B_{max} (μRIU)	PM + 4% $PI(4,5)P_2$ B_{max} (μRIU)
TTP(wild-type)	1.3 ± 0.26	270 (100%)	470 (175%)
TTP(K217A)	0.43 ± 0.09	570 (100%)	320 (56%)

(i) *PM* plasma membrane lipids (44% DOPC 20% DOPE, 19% cholesterol, 10% sphingomyelin, 5% DOPS, 2% DOPA). When 4% $PI(4,5)P_2$ was included, PC content was reduced by the same amount
(ii) SPR protein injections were made at concentrations of protein near the K_d values for the respective proteins: wtTTP = 1.3 μM; K217A = 0.5 μM. μRIU = micro-refractive index units

to be conserved from yeast to man, as it has been observed/suggested in the homologous CRAL-TRIO proteins Sec14 [27] and CRALBP [71].

A key feature of the dynamic ligand-induced conformational switching in TTP appears to be mediated by Lys217. Substitution of this residue eliminated TTP binding to $PI(4,5)P_2$, abolished TTP-mediated tocopherol secretion in cultured hepatocytes, and disrupted the protein's intracellular localization to a pattern where the protein constitutively resides near the plasma membrane [63]. These findings are supported by in vitro studies in which we determined the affinity of TTP proteins to model plasma membranes (Table 9.1). We found that TTP(K217A) bound NBD-tocopherol with similar affinity as the wild-type protein, indicating that the substitution did not lead to gross structural perturbations [65]. Using surface plasmon resonance (SPR), we determined the ability of the proteins to bind to tethered vesicles mimicking plasma membrane lipid composition. We found that the K217A protein bound to membranes with slightly higher affinity than the wild-type TTP. However, when the experiments included membranes containing $PI(4,5)P_2$, the K217A mutant protein showed profoundly weakened affinity to the membranes, as compared to wild-type TTP. Preliminary SPR studies also suggest that the presence of tocopherol weakens TTP's affinity to $PI(4,5)P_2$-containing membranes.

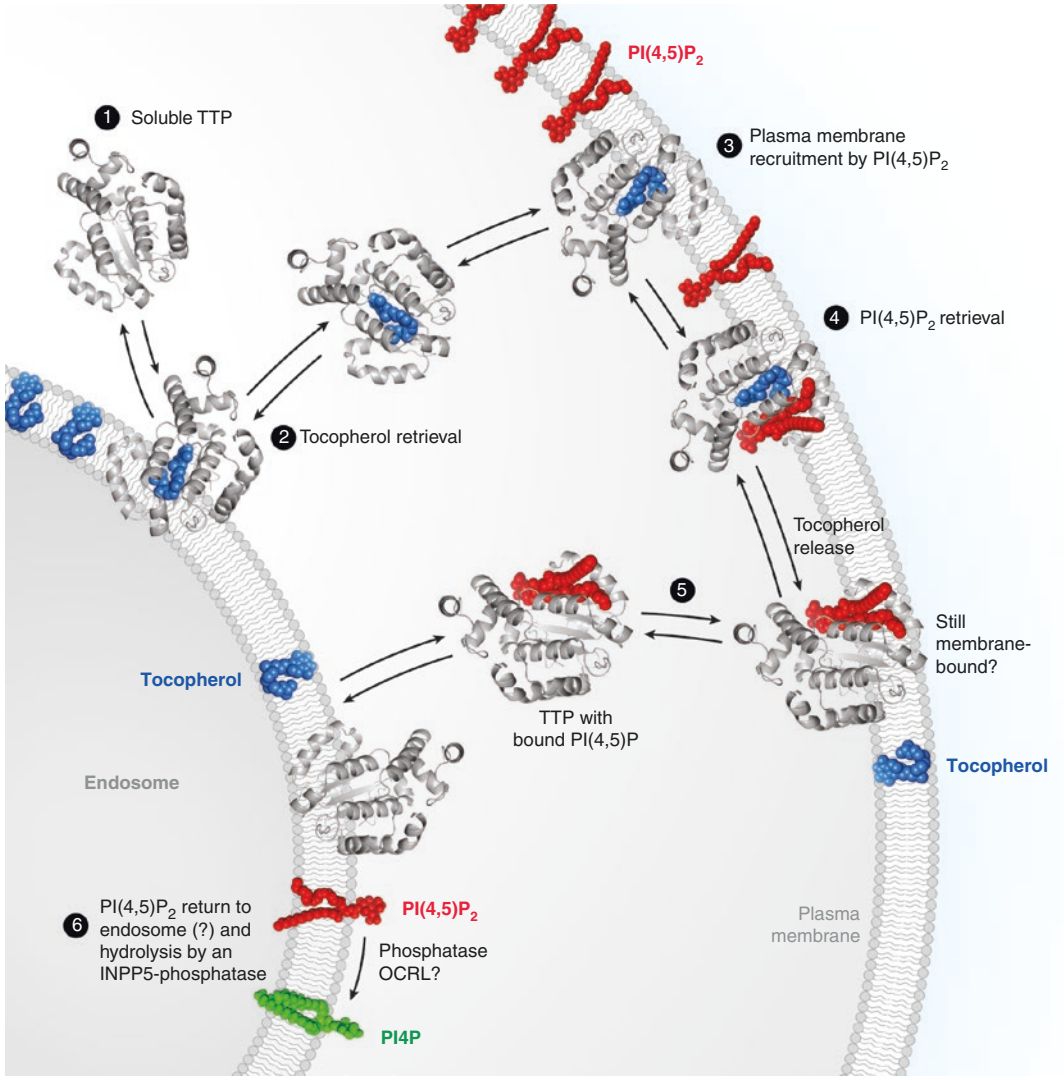

Fig. 9.3 Tocopherol transport across cells. See text for details

These data show that TTP's affinity to lipid bilayers is increased by the presence of $PI(4,5)P_2$ and that Lys217 is critical for this discriminatory binding behavior. Thus, the K217A mutant seems to have lost the ability to complete the ligand exchange reaction; it therefore retains appreciable affinity for tocopherol even when bound to $PI(4,5)P_2$. The mutant protein therefore remains bound to both ligands and "stuck" along the membrane in cells (i.e., cannot complete step 4 in the model shown in Fig. 9.3).

Mechanisms of TTP-Mediated Tocopherol Transfer In Vivo

In cells, the non-vesicular movement of small molecules by transfer proteins requires elements of temporal and spatial control. The protein-ligand complex should form where the two partners are at concentrations that drive favorable thermodynamics of binding. Furthermore, once the protein-ligand

complex is formed, directional transfer of the ligand cargo to a new location requires a favorable binding event elsewhere in the cell. Finally, to achieve net vectoral transport of the ligand, directionality of the process must be maintained. We propose a model for the mechanism by which TTP transports vitamin E across cells that relies on two principle features: the exchange of tocopherol and PI(4,5)P_2 (see above) and concentration gradients of the two ligands between different intracellular locations. This model, depicted in Fig. 9.3, is based on experimental findings [63, 69, 72] and on a similar phenomenon, namely, the transport of sterols and phosphoinositides, by members of the OSBP family [73–76].

According to our model, TTP picks up α-tocopherol from the site of the vitamin's arrival in cells, the endocytic compartment [39]. Interaction between TTP and endosomal vesicles is driven by the high concentrations of tocopherol at this site and possibly by the high curvature of these membranes [77, 78] and is mediated by a hydrophobic face of the protein that offers two key phenylalanine residues for submersion into the hydrophobic membrane (residues F169, F165; see Fig. 9.2 and [65, 79]). The binding of tocopherol shifts the conformation of the helical lid (including residues 198–221) to close on the binding site, thereby weakening the protein's affinity for the bilayer. The protein then dissociates to the cytosol as a soluble TTP-tocopherol complex. The TTP-tocopherol complex then partitions to the plasma membrane (3) where concentrations of PI(4,5)P_2 are high [80]. Although TTP does not contain a known binding motif specific for phosphoinositides, it contains a basic patch on the surface (comprising Arg59, Arg68, Lys190, Arg192, Lys217, Arg221) [63, 72] that offers a favorable environment for electrostatic and H-bonding interactions with the phosphorylated inositol head group of PI(4,5)P_2 [63].

Although the detailed sequence of the ligand exchange event is not yet clear, the end result is the deposition of tocopherol into the plasma membrane bilayer and abstraction of PI(4,5)P_2 by TTP from the membrane (5). The TTP-PI(4,5)P_2 complex then departs to the cytosol and returns to the endosomal compartment (6) where the PI(4,5)P_2 is dropped off and is hydrolyzed to PI(4)P by an endosomal resident phosphatase such as OCRL [81–84]. Alternatively, TTP might specifically promote the hydrolysis of bound PI(4,5)P_2 by presenting it to a plasma membrane-resident phosphatase such as SHIP2 [85, 86]. Directionality of the transport process is governed by the concentration gradients of PI(4,5)P_2 (high in plasma membrane and low in endosomes) and an opposite concentration gradient of tocopherol (high in the endosomal compartment, low in plasma membrane) [72].

TTP and Neurological Function

The primary manifestations of vitamin E deficiency in laboratory animals are neuropathologies, similar to those presented by human AVED patients [36, 87, 88], underscoring the critical roles that α-tocopherol plays in the CNS. We refer the reader to a detailed review on this topic that was recently published elsewhere [89, 90] and only briefly discuss here a few points.

TTP is expressed at significant levels in glia (astrocytes), the cells that provide metabolic support for neurons. In these cells, TTP regulates supply of α-tocopherol to neighboring neurons, in a manner highly reminiscent of the protein function in hepatocytes, as depicted in Fig. 9.4.

Specifically, TTP mediates the trans-cellular transport of the vitamin from vesicles of the endocytic compartment to a transporter at the astrocyte plasma membrane, from which the vitamin is exported by a transporter to an acceptor apolipoprotein for delivery to its final destination. The two tissues utilize slightly different membrane transporters (ABCA1 in hepatocytes vs. ABCG4 in the CNS), different lipoprotein acceptors (ApoA-based in hepatocytes vs. ApoE-based in the CNS), and different destinations (systemic circulation in hepatocytes vs. cerebrospinal fluid in the CNS) yet the overall process seems to share most functional steps and players. Importantly, in both tissues expression of the *TTPA* gene is stimulated under conditions of oxidative stress.

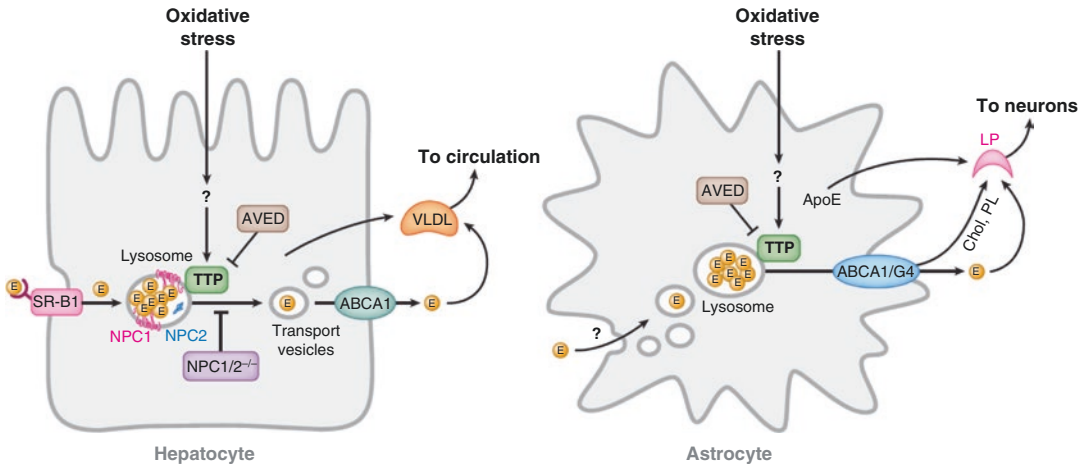

Fig. 9.4 Mechanisms of TTP-facilitated tocopherol transport in hepatocytes and astrocytes. (Drawn after Ulatowski, 2013 [91])

Expression of TTP in the liver ensures supply of diet-derived tocopherol to the circulation and, in turn, to all extra-hepatic tissues. What, then, is the evolutionary need that selected for "redundant" expression of the protein in the CNS? The unique sensitivity of the CNS to oxidative damage might be a key answer to this question. Thus, TTP appears to serve two protective functions in the CNS: to provide and "safeguard" a separate pool of vitamin E that sustains neurological function and to regulate vitamin E transport to neurons in a manner independent from body-wide fluctuations. It is possible, and likely, that similar reasons dictated expression of the protein in the female reproductive system.

TTP and Fertility

Almost a century ago, vitamin E was identified as a plant component that is critical for female fertility in laboratory animals (hence the name tocopherol, *Gr.* to bear offspring). Specifically, vitamin E was shown to be critical to successful pregnancy in rodents; without it, pregnant females would resorb the developing embryo into the uterine wall and pregnancy would be terminated [92]. Considerations of vitamin E actions often rely in part on this biological activity (the gestation-resorption assay) [93, 94]. Since vitamin E deficiency is not common in humans, deeper understanding of this phenomenon came only after the advent of the TTP-null mice. Shortly after generating this genetic model, Arai and colleagues reported that TTP-null crosses were not fertile. They further found that male fertility was not affected, that pregnancies could be rescued by supplementation with α-tocopherol or a synthetic lipophilic antioxidant, and that infertility was an outcome of placental defect [43]. In a later report, the same group demonstrated that transfer of fertilized TTP-null eggs to wild-type females subverted the fertility defect. Taken together, these finding demonstrated that adequate α-tocopherol delivery via TTP is critical for placental function, but not for embryonic development per se [95]. Thus, it appears that the situation in the reproductive system is analogous to that in the CNS: TTP's critical function is in supplying the oxidative stress-susceptible embryo with the antioxidant, and defects in TTP lead to functional failure. A question that begs urgent research is whether these findings have relevance to female fertility in humans. It is worth noting in

this regard that TTP is expressed in human placental trophoblasts [34, 96] and that expression of the protein increases upon induction of oxidative stress in cultured human trophoblasts [97]. These observations strongly suggest that a similar scenario exists. The urgency of research into this question is underscored by the extremely high prevalence of unexplained miscarriages in women [98], the abundance of single nucleotide polymorphisms in the *TTPA* gene [99], and the total inattention at present to vitamin E status in women of child-bearing age.

Vitamin E Homeostasis: Dynamic Regulation of TTP

For a long time, vitamin E status was assumed to be a passive reflection of dietary intake ("we are what we eat"). Recent evidence, however, demonstrates that TTP's function is not merely to transport the vitamin among tissues and compartments, but also to **regulate** tissue levels of vitamin E in response to changing physiological conditions. Regulation of vitamin E status via changes in TTP actions is achieved through a number of mechanisms:

- *Transcriptional regulation of TTP.* Expression of the gene encoding TTP is not constant. Rather, TTP mRNA levels were shown to be regulated by a number of transcriptional modulators [99] including hormones (e.g., TNFα), nuclear receptors (e.g., PPAR), second messengers (cAMP), and hypoxia mediators (DFX) [99]. Although the physiological or pathological conditions under which these stimuli affect TTP expression in vivo are yet to be described, the data clearly demonstrate that TTP expression, and in turn vitamin E levels and distribution, is dynamically regulated. Especially intriguing are the findings showing that TTP expression is markedly enhanced under conditions where vitamin E is in demand, i.e., under elevated oxidative stress or when the need for increased trafficking rises, i.e., upon elevated levels of α-tocopherol.
- *Post-translational regulation of TTP.* A more immediate and transient regulation of TTP happen at the post-translational level. Thus, it was shown that tocopherol increases the lifetime of the TTP protein by protection of the protein from proteasomal degradation [100]. Moreover, our recent findings show that the TTP protein is transiently phosphorylated on tyrosine residue(s) and that this modification has profound effect on the protein's activity. The data show that TTP is phosphorylated on tyrosine residue(s) (Fig. 9.5a), that the phosphorylation is stimulated by serum-derived growth factors (Fig. 9.5b), that substitution of Tyr269 to phenylalanine markedly reduces phosphorylation on TTP (Fig. 9.5c), and that the signature biological activity of TTP, namely, facilitation of tocopherol secretion from cultured hepatocytes, is abolished by the Tyr269Phe mutation as well as by treatment with Iressa, a selective inhibitor of the epidermal growth factor receptor (EGFR; Fig. 9.5d). Taken together, these data strongly support the notion that TTP-mediated tocopherol transport is regulated by classical mitogenic signaling cascades.

Current Gaps in Our Understanding of TTP and Vitamin E Despite a wealth of information derived from decades of vitamin E research, a number of important questions remain unanswered and await detailed focused attention from experimental scientists:

- Is vitamin E status associated with infertility in women, and what is the impact of TTP defects in this phenomenon?
- How is TTP activity regulated in normal and pathological states?
- What is the function of TTP in yet unexplored sites of expression (e.g., lung, kidney)?
- What are the medical consequences of subclinical vitamin E deficiency and polymorphisms in the *TTPA* gene?

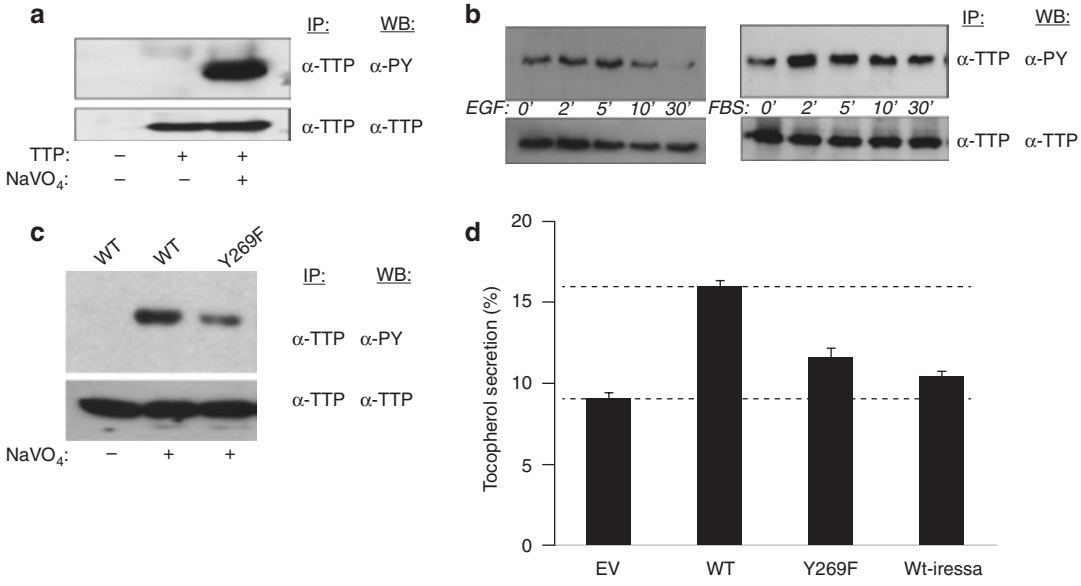

Fig. 9.5 Regulated phosphorylation of tyrosine residue(s) in TTP. All experiments were done in cultured HepG2 hepatocytes. (**a**) **TTP is tyrosine-phosphorylated.** TTP was immunoprecipitated using anti-TTP antibodies in lysates from cells transfected with TTP (or mock)-encoding plasmids. Tyrosine phosphorylation was visualized by immunoblotting with an anti-PY antibody (clone 4G10), in the presence or absence of the phosphatase inhibitor sodium vanadate. (**b**) **Tyrosine phosphorylation of TTP is stimulated by growth factors.** TTP-transfected cells were serum-starved for 20 h and then stimulated for the indicated time with epidermal growth factor (EGF; 50 ng/ml) or bovine serum (FBS; 10%) prior to analysis as in panel A. (**c**) **Tyr269 is a likely phosphorylation site in TTP.** Cells were transfected with the indicated construct prior to analysis as in panel A. (**d**) **Tyrosine phosphorylation of TTP regulates tocopherol secretion in cultured hepatocytes.** Cells were transfected as indicated, and secretion of loaded [^{14}C]-α-tocopherol was measured as in (Qian *Biochemistry*, **2006**, *45*: 8236). Where indicated, the EGFR inhibitor Iressa (Gefitinib; 5 μM) was added to the culture media during the secretion phase of the experiment

Conclusion

Since the earliest discovery of fatty acid [101, 102] and phospholipid [103, 104] binding proteins, function has been deduced from in vitro intermembrane transfer assays. We are now growing to appreciate that the function of lipid transfer proteins is more nuanced than ligand binding and random membrane encounters. How is directional ligand transport achieved and controlled? Many transfer proteins recognize specific membrane lipid compositions, often guided by organelle- and membrane-specific phosphatidylinositol phosphates [105] and by protein-protein interactions at membrane contact sites [2, 74, 75].

In vertebrates, the tocopherol transfer protein is responsible for the selective retention of dietary α-tocopherol during passage of the vitamin through the liver and for its secretion into plasma lipoproteins. Kono et al. [63, 69] have described the possible first steps in TTP-mediated tocopherol transport as specific recognition of plasma membrane PI(4,5)P_2 by the TTP-tocopherol complex that is followed by ligand exchange of phospholipid for the bound tocopherol. Still, aspects of this delivery cycle remain to be described. Is the PI(4,5)P_2 actually extracted from the plasma membrane in vivo? Which phosphatase is responsible for turnover of the extracted PI(4,5)P_2 and where does this hydrolysis occur? Answers to these questions will likely require tools for altering PIP metabolism [106–109], as well as fluorescent and bio-conjugated forms of phosphoinositides [110] to enable tracking and identification of potential protein binding partners.

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Chapter 10
The Role of Lipid Rafts in Mediating the Anticancer Effects of γ-Tocotrienol

Paul W. Sylvester

Keywords γ-Tocotrienol · Lipid rafts · Exosomes · Breast cancer · HER · Heregulin

Key Points

- γ-Tocotrienol displays potent antiproliferative and apoptotic activity against a wide range of cancers, particularly breast cancer.
- A large percentage of human breast cancers display aberrant receptor tyrosine kinase activity, including receptors within the HER family, c-Met, and VEGF among others.
- Heregulin is a potent ligand that activates HER3 and HER4 receptors, and overexpression of this ligand is associated with the development of chemotherapy resistance.
- Exosomes from breast cancer cells treated with γ-tocotrienol contain significantly less heregulin and are significantly less potent in stimulating HER3/HER4 heterodimerization, activation, and mitogenic signaling.
- The anticancer effects of γ-tocotrienol are associated with its accumulation in the lipid raft microdomain, where it appears to interference with the receptor tyrosine kinase dimerization and activation in human breast cancer cells.
- The anticancer effects of γ-tocotrienol result, at least in part, by directly disrupting lipid raft integrity by directly interfering with HER receptor dimerization and signaling within the lipid rafts and indirectly by reducing exosome heregulin content and subsequent autocrine/paracrine mitogenic stimulation.

Vitamin E as an Anticancer Agent

Early studies showed that diets supplemented with α-tocopherol inhibited carcinogen-induced in rodents [1, 2], whereas other studies could not confirm that dietary supplementation with α-tocopherol inhibited carcinogen-induced mammary tumorigenesis when given alone [3, 4] but did display synergistic anticancer activity when given in combination with selenium [5]. Although these studies suggested that dietary supplementation with α-tocopherol may provide a protective effect against the

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_10

development of cancer in humans, epidemiological studies have overwhelmingly shown little or no evidence that α-tocopherol plays a role in the prevention of human cancer [6]. It is now clearly established that most in vivo studies investigating the inhibitory effects of α-tocopherol have shown negative results [7, 8]. Furthermore, highly controlled in vitro studies have shown that α-tocopherol has little or no anticancer activity, even when extremely high concentrations are used [9–11].

The anticancer effects of tocotrienols were indirectly discovered in dietary studies that investigated the role of high fat intake on the development of mammary tumorigenesis in laboratory animals. Experiments showed that while most diets containing various types of animal or vegetable fats were found to stimulate mammary tumorigenesis, high palm oil diets were found to significantly inhibit the development and growth of carcinogen-induced mammary cancer in rats [12]. Since palm oil contains high levels of tocotrienols, it was hypothesized that tocotrienols may be responsible for mediating the anticancer effects of palm oil diets. Furthermore, subsequent studies showed that palm oil diets stripped of tocotrienols no longer displayed a protective anticancer effect [13]. Initial dose-response studies showed that the relative anticancer potency of individual tocotrienol isoforms was characterized as δ-tocotrienol ≥ γ-tocotrienol > α-tocotrienol > δ-tocopherol >> γ- and α-tocopherol [10, 14]. Interestingly, treatment doses of tocotrienol that induced significant antiproliferative and apoptotic effects on breast cancer cells had no effect on normal mammary epithelial cell growth or viability, demonstrating that these treatment effects of tocotrienols show significant selectivity against neoplastic versus normal mammary epithelial cells [10, 14, 15].

In contrast to natural isoforms, synthetic derivatives of tocopherols and tocotrienols display enhanced anticancer activity. For example, both acetate and succinate ether derivatives of α-tocopherol display significantly greater anticancer and apoptotic activity than the natural form of this vitamin E isoform [16, 17]. Similarly, several novel prodrug and polar forms for α-, γ-, and δ-tocotrienol have been synthesized that are absorbed passively through the gut and bypass the transporter mechanisms normally involved in tocotrienol absorption and metabolic breakdown [18]. Once in the blood and tissue, these prodrug and polar forms of tocotrienol are metabolized to the natural parent compound, which can then act to directly inhibit tumor growth and viability. These novel prodrug and polar derivatives of tocotrienol have been shown to display both enhanced bioavailability and higher therapeutic responsiveness than their natural parent forms [18]. In addition, oxazine derivatives and γ- and δ-tocotrienol were found to be more potent in suppressing mammary tumor cell growth in vitro and in in vivo experimental models as compared to γ- and δ-tocotrienol, respectively [19]. These findings suggest that derivatives of tocopherols and tocotrienol may provide significant benefit in the treatment of cancer.

Tocotrienols and Breast Cancer

Breast cancer is the most common type of cancer and second leading cause of cancer-related deaths in women [20]. Traditionally, chemotherapeutic agents used in the treatment of breast cancer are associated with low response rates and development of severe side effects due to their nonselective cytotoxic activity [20]. As a result, there has been increasing interest to develop novel therapeutic agents that specifically target signaling molecules that promote growth, survival, and metastasis of malignant cells while at the same time having little or no toxic effects against normal cells. Approximately 25% of all breast cancers display HER2 overexpression and/or excessive HER2 signaling, and the majority of these cancers are highly metastatic, multidrug resistance and correspond to a poor patient prognosis [21].

During the past few decades, numerous studies have been conducted to determine the intracellular signaling mechanism(s) involved in mediating the anticancer effects of tocotrienols. Experimental results have shown that tocotrienols specifically inhibit several growth and survival signaling pathways associated with the suppression of HER receptor mitogenic signaling, particularly the PI3K/Akt/mTOR [15, 22, 23], JAKs/Stat [24], and MAPK pathways [25]. Since γ-tocotrienol displays a wide range of inhibitory effects on a variety of receptor tyrosine kinases, and lipid raft microdomains

recruit and activate various receptor tyrosine kinases in a wide range of cancer cell types, it was hypothesized that γ-tocotrienol might accumulate in the lipid raft microdomains of cancer cells and interfere with receptor tyrosine kinase dimerization, activation, and signaling. The following review will discuss the inhibitory effects of γ-tocotrienol on lipid raft integrity and corresponding suppression of HER2 receptor activation and signaling, as well as γ-tocotrienol suppression of exosome-dependent proliferation of human breast cancer cells.

Lipid rafts play an important role in normal signal transduction in relation to the recruitment and activation of various types of receptor tyrosine kinases, such as HER family members, c-Met, VEGF, and others [26, 27]. Lipid raft microdomains within the plasma membrane are enriched with sphingolipids and cholesterol and are very stable and rigid structures as compared to the surrounding more fluid plasma membrane [28, 29]. Ligand binding to its receptor results in the translocation and dimerization of the receptor within the lipid raft microdomain. Following dimerization, the cytoplasmic domain of the receptor undergoes tyrosine phosphorylation [27, 30, 31]. These tyrosine autophosphorylation sites are required for the interaction and activation of downstream substrates involved in mediating intracellular second messenger signal transduction. In addition, lipid rafts play a critical role in modulating the production, content, and secretion of cup-shaped exosomes that can be found in high concentration in media obtained from tumor cells grown in culture, as well as fluid collected from malignant pleural effusion [32]. Exosomes also play a role in promoting tumor cell growth, T-cell apoptosis, and chemoresistance [33]. Exosomes released into the extracellular environment transport various oncogenic mitogens including EGF, amphiregulin, heregulin, HGF, and VEGF, which can then locally stimulate autocrine-/paracrine-mediated cancer cell growth and progression [34–36].

Since γ-tocotrienol displays a wide range of inhibitory effects on a variety of receptor tyrosine kinases, and lipid raft microdomains recruit and activate various receptor tyrosine kinases in a wide range of cancer cell types, it was hypothesized that γ-tocotrienol might accumulate in the lipid raft microdomains of cancer cells and interfere with receptor tyrosine kinase dimerization, activation, and signaling. The following review will discuss the inhibitory effects of γ-tocotrienol on lipid raft integrity and corresponding suppression of HER2 receptor activation and signaling, as well as γ-tocotrienol suppression of exosome-dependent proliferation of human breast cancer cells.

HER Receptor Tyrosine Kinases

The HER family consists of four transmembrane receptors (HER1, HER2, HER3, and HER4) [37]. All HER receptors have a glycosylated extracellular ligand-binding domain, a transmembrane domain, and an intracellular catalytic tyrosine kinase domain [38]. Although a large number of ligands have been identified that activate HER1, HER3, and HER4, no known ligand has been identified that binds directly to HER2 [38]. In contrast, HER3 lacks the tyrosine kinase domain [38]. However, both HER2 and HER3 can participate in signal transduction by acting as co-receptors in the formation of heterodimers [38]. Dimerization is an important step in the activation of HER receptors, and the intensity of activation of the downstream signaling depends on the type of dimer/heterodimer formed. Ligands to HER receptors induce conformation changes in the receptor that exposes the dimerization loop [39]. HER2 differs from the other HER family members in that even in the absence of ligand binding, HER2 is constitutively in favorable dimerization conformation and is a preferred dimerization partner for all other HER receptors [40]. Although HER3 lacks intrinsic tyrosine kinase activity, ligand binding and dimerization with other HER receptors result in the cross phosphorylation of the intracellular domain of HER3, which can lead to the activation of various substrates, particularly the PI3K/Akt signaling pathway [41]. HER3 serves as a critical co-receptor of HER2, and its expression is a rate-limiting factor for HER2-induced cancer cell survival and proliferation. In HER2-positive breast cancer tissues, HER2 displays preferential dimerization and cross phosphorylation with HER3, but not HER1 [42].

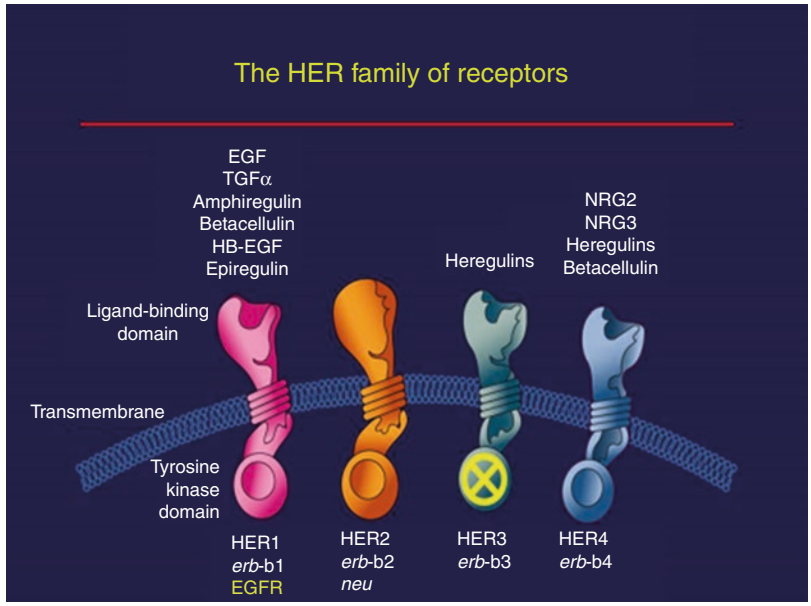

Fig. 10.1 Structural characteristics and ligand binding of HER receptor family member

Unregulated and/or excessive HER receptor activation and signaling is associated with the promotion of cancer cell proliferation, survival, and metastasis and can result from the overexpression of HER ligands or receptors and mutations in receptor domains that can result in constitutive activation or dysregulation of receptor signaling [38, 42]. The general mechanism for the activation of HER family receptors involves a specific ligand binding to the extracellular domain and initiate activation, dimerization, and autophosphorylation. The juxtaposed cytoplasmic kinase domains catalyze the phosphorylation of tyrosine residues, usually in the activation segment, that leads to protein kinase activation. The kinase domains also catalyze the phosphorylation of additional tyrosine residues that create docking sites for adaptor proteins or enzymes involved in downstream signaling [38]. The docking proteins contain modular Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains (or both) that recognize phosphotyrosine in sequence-specific contexts. This phosphorylation is accomplished in transactivation when the first member of the dimer mediates the phosphorylation of the second, and the second member mediates phosphorylation of the first [43]. The HER signaling networks consist of several modules that are interconnected and overlapping [44]. These include PI3K/Akt pathway, Ras/Raf/MEK/ERK1/2 pathway, and PLCγ pathway. The PI3K/Akt pathway plays an important role in mediating cell survival, and the Ras/ERK1/2 and PLCγ pathways participate in cell proliferation [45]. These and other HER signaling modules participate in cell adhesion, cell motility, development, and angiogenesis [46]. Figure 10.1 illustrates the general structure and ligand binding characteristics of the difference members in the HER family of receptors.

Lipid Rafts

The fluid mosaic model of plasma membranes has undergone revision in recent years to incorporate the concept that membranes exhibit detergent-resistant subdomains or lipid rafts with distinctive protein and lipid compositions [47]. The composition of lipid rafts differs from the surrounding membrane in that it is enriched with sphingolipids and cholesterol [47]. These lipids preferentially incorporated into membrane microdomains, which results in a highly stable and rigid structure. Lipid

rafts are divided into two subtypes identified as planar or non-caveolar lipid rafts and caveolae lipid rafts [47]. Planar rafts are defined as non-invaginated microdomains lacking specific morphological features, whereas caveolae lipid rafts are tube-like invaginations of the plasma membrane that contain specific scaffolding proteins called caveolins [47].

Specific proteins are constitutive components of lipid rafts and are essential for normal lipid raft formation and function. Caveolin-1 is such a protein that acts as a scaffolding to promote caveolar raft formation caveolin-1 [48, 49]. Caveolin-1 is a hairpin protein that plays a role in caveolae-mediated signaling, endocytosis, and transport. In addition, flotillin proteins are required for non-caveolar lipid raft formation and microdomains. Flotillin-1 and flotillin-2 function to promote co-assembly signaling and anchoring proteins in plasma membrane microdomains, which allow a concerted interaction between signaling molecules, such as Src family kinases and the small GTPases [48, 49]. Flotillins can also interact with caveolin-1 and/or serve as a functional substitute in caveolin-1-deficient MCF-7 breast cancer cells [32, 50].

In summary, lipid raft microdomains within the plasma membrane organize signaling molecules into functional complexes, and the central organizing proteins are those that provide a scaffolding called the caveolin-scaffolding domain (CSD). Caveolin-1 in caveolar rafts serves this function and interacts through its CSD with G proteins, endothelial nitric oxide synthase (eNOS), adenylate cyclase isoforms, and a series of cytoplasmic kinases (Src family members, MAPK, protein kinase A, and protein kinase C) [51, 52]. The "caveolin signaling hypothesis" proposes that the binding of signaling proteins with its CSD regulates signal transduction by activating the signaling partners within the complex milieu [51, 52]. A switch between raft and non-raft localization of signaling components appears to function as an important regulatory mechanism. Receptor tyrosine kinases such as HER2 are signaling proteins that upon activation are found in high concentrations within the lipid raft microdomains. Studies have shown that caveolin-1 is responsible for maintaining Akt in an activated state by inhibiting protein phosphatases 1 and 2A through CSD entrapment, whereas flotillins interact with Src family cytoplasmic tyrosine kinases and small Rho-family GTPases through the CSD [53]. Figure 10.2 illustrates the general structure and scaffolding of signaling within a lipid raft microdomain.

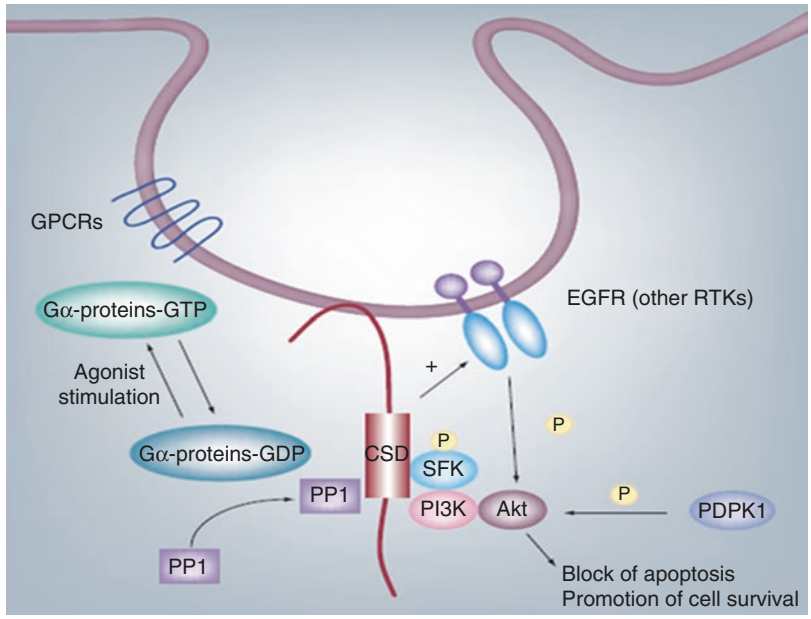

Fig. 10.2 Scaffolding of signaling pathways associated with lipid raft microdomains. (Adapted from Staubach and Hanisch [32], reference #9)

Exosomes

Aggressive, drug-resistant cancers maintain a robust network of biological interactions involving gene-gene, gene-microRNA, protein-protein, as well as the parallel signaling of intracellular and intercellular influences [54, 55]. The fluidity of such biological interactions is maintained by constant influx and efflux of materials across nuclear and plasma membrane. Exosomes represent a vesicular transport system by which cells communicate with their surrounding environment [56]. Exosomes are between 20 and 100 nm in diameter and secreted by a wide range of mammalian cell types [57]. They are composed of a lipid bilayer membrane surrounding a small volume of cytoplasm that lacks cellular organelles. Exosomes contain various molecular constituents of their cellular origin, including proteins, RNAs, and miRNAs [58, 59]. Exosomes derived from the intracellular endosomal compartment are formed by a ceramide-triggered process of inward budding of the lipid raft microdomain [60]. This process encapsulates cytoplasmic RNA molecules and functional proteins into exosomes. Afterward, these multivesicular bodies fuse with the cellular membrane to release exosomes into the extracellular space [61]. Figure 10.3 shows the biogenesis and release of exosomes from the plasma membrane.

Tumor cells exchange oncogenic proteins by way of exosome-mediated transfer. Exosomes carry different sets of tumor mitogens, such as HER receptor tyrosine kinase ligands, that stimulate cancer cell growth and expression of anti-apoptotic genes and promote anchorage-independent growth [58, 62]. Furthermore, cancer cells, expressing a mutant K-RAS gene, are capable of releasing exosomes with mutant K-Ras proteins. The exosome cargo internalized by cells expressing the wild-type K-Ras can be induced to express malignant phenotypic behavior [63].

γ-Tocotrienol and Cancer

The vitamin E family of compounds is composed of eight naturally occurring isoforms that are further divided into two subgroups called tocopherols and tocotrienols. Tocopherols are much more abundant in nature and can be found in nuts, whole grains, dark green vegetables, egg yolk, and various vegetable oils, whereas tocotrienols are relatively rare and found only in limited sources such as palm oil, rice bran oil, and annatto bean oil [64]. Both tocopherols and tocotrienols are characterized by a chromanol ring structure methylated to various degrees at the 5, 7, and 8 positions to form specific

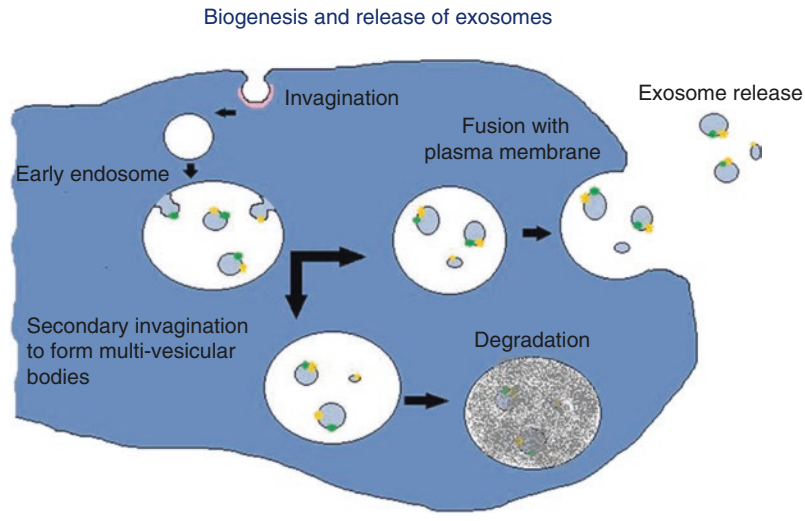

Fig. 10.3 Exosome biogenesis originating from the invagination of plasma membrane to ultimately form multivesicular bodies that will then fuse with either lysosomes for degradation or the plasma membrane to release exosomes. (Adapted from Raposo and Stoorvogel [61], reference #44)

isoforms classified as α, β, γ, and δ-tocopherols or tocotrienols. Studies have shown that relative anticancer potency of tocotrienol isoforms was determined to be δ-tocotrienol ≥ γ-tocotrienol > α-tocotrienol [10, 14]. In addition, tocotrienols have been shown to have selective antiproliferative and apoptotic effects on mammary cancer cells at doses that have little or no effect on the viability of normal mouse and human mammary epithelial cells [10, 14, 15, 65].

Tocotrienol suppression of downstream signaling pathways is directly related to a suppression in membrane receptor activation. Early studies showed that the antiproliferative effects of γ-tocotrienol on mammary cancer cells were associated with suppression of HER3 activation [66]. Subsequent studies showed that tocotrienols are able to inhibit a wide range of receptors such as VEGFR [67], HER2-4 [24, 68, 69], MET [70, 71], and Frizzled-7 (FZD7) [72]. Furthermore, combined treatment of tocotrienols with other phytochemicals and chemotherapeutic agents shows synergistic anticancer activity [24, 25, 70, 73–75]. In addition to suppressing mitogen-dependent mitogenesis, treatment with very high doses of γ-tocotrienol is cytotoxic and initiates apoptosis, necrosis, and autophagy in cancer cells [15, 76, 77].

The antiproliferative effects of tocotrienols in cancer cells are associated with a suppression in mitogen-dependent signaling [78]. Specifically, γ-tocotrienol treatment inhibits PI3K/Akt activation in a dose- and time-dependent manner, and these effects were not found to be associated with an increased in either PTEN or PP2A phosphatase activity [76]. Furthermore, tocotrienol suppression of Akt corresponds with a decrease in the activity of the transcription factor, NFκB, by suppressing the activation of IKK-α/β [22]. In addition to the PI3K/Akt signaling, γ-tocotrienol also inhibits PKCα activation [78]. Other investigations have shown that tocotrienol inhibition of EGF-dependent mitogenesis in preneoplastic CL-S1 mouse mammary epithelial cells resulted from an inhibition of G-protein-mediated activation of adenylyl cyclase and cAMP production as well as reduction in phosphorylated (activated) ERK1 and ERK 2 [79]. Tocotrienol suppression of mitogen-dependent cancer cell growth is also associated with blockade of cell cycle progression [80], the mevalonate pathway [81], cancer cell adipogenesis [82], glycolysis [83], angiogenesis [64], and epithelial-mesenchymal transition [70, 72].

Although studies have also shown that the anticancer effects of γ-tocotrienol are associated with the suppression in HER2 mitogenic signaling, the exact cellular/molecular target involved in mediating these inhibitory effects of γ-tocotrienol is presently unknown. Since γ-tocotrienol displays a wide range of inhibitory effects on a variety of receptor tyrosine kinases, it was hypothesized that γ-tocotrienol accumulation and disruption within the lipid raft microdomains act to attenuate receptor tyrosine kinase dimerization, activation, and signaling as well as significantly reduced exosome receptor ligand content and mitogenic biopotency.

Antiproliferative Effects of γ-Tocotrienol Are Associated with Disruption in Lipid Raft Integrity

Recent studies have provided evidence that demonstrates that the antiproliferative effects of γ-tocotrienol on HER2 receptor activation and signaling in HER2-positive SKBR3 and BT474 human breast cancer cells are associated with a disruption in lipid raft integrity and function [84]. Treatment with 4 μM γ-tocotrienol (IC_{50} dose) significantly inhibited EGF-dependent SKBR3 and BT474 breast cancer cell growth and Ki-67 positive staining after a 5-day culture period, as compared to cells in their corresponding vehicle-treated control groups [84]. Positive staining for the nuclear protein Ki-67 is an indicator of active cell proliferation and occurs in all active phases of the cell cycle including G_1, S, G_2, and M phases, but is not found during the resting G_0 phase [85]. Furthermore, these growth-inhibitory effects of γ-tocotrienol occur in the absence of caspase-3 activation, indicating that its antiproliferative effects did not result from the induction of apoptosis. Figure 10.4 shows that treatment of

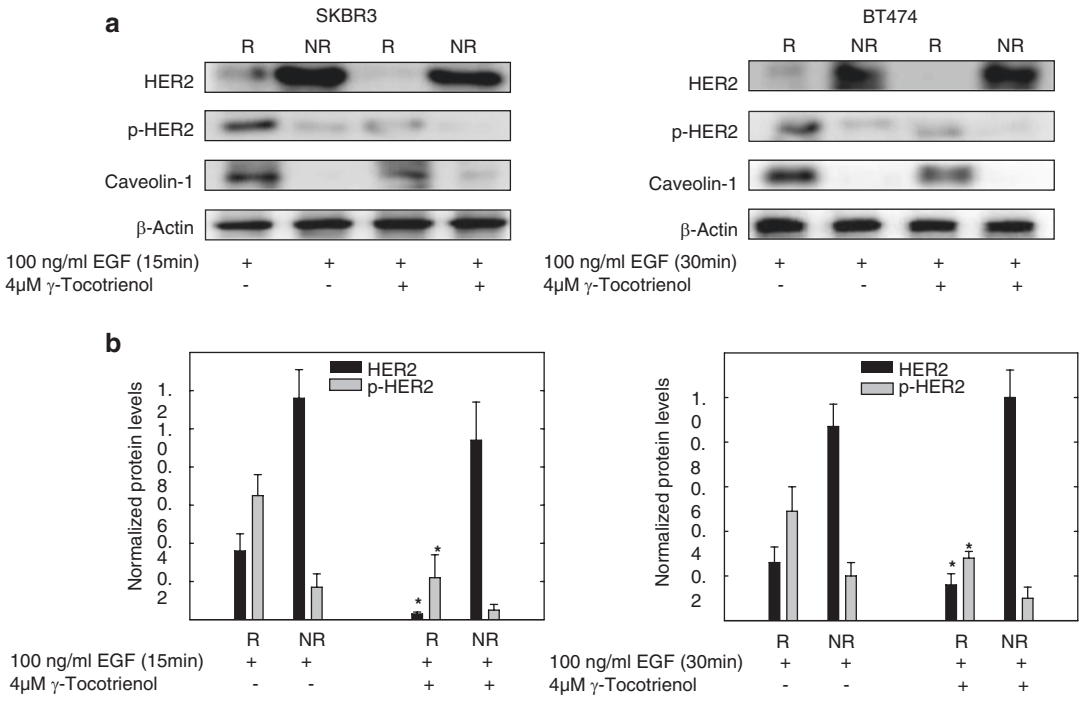

Fig. 10.4 Effects of γ-tocotrienol on total HER2, phosphorylated HER2, and caveolin-1 levels in lipid raft and non-lipid raft microdomains of the plasma membrane of HER2-positive SKBR3 and BT474 human breast cancer cells. (**a**) Lipid raft [R] and non-lipid raft [NR] fractions were isolated from cells in each treatment group and subjected to Western blotting analysis. (**b**) Scanning densitometric analysis was performed on Western blots, and integrated optical density for each band was normalized with their corresponding β-actin levels. $^*P < 0.05$ as compared with their respective vehicle-treated control groups. (Adapted from Alawin et al. [84], reference #69)

mitogen-starved SKBR3 and BT474 cells with 100 ng/ml EGF resulted in a transient increase in HER2 phosphorylation (activation) that peaked between 15 and 30 min after EGF exposure in vehicle-treated control cells [84]. In contrast, cells treated with γ-tocotrienol showed a significant reduction in EGF-induced HER2 phosphorylation (Fig. 10.4). Additional studies showed that γ-tocotrienol treatment also induced a decrease in phosphorylated HER2 monomer and dimer levels in these HER2-positive breast cancer cells, as compared to cells in their corresponding vehicle-treated control groups.

Because EGF-induced dimerization and phosphorylation of HER2 occur in lipid raft microdomains [86, 87], additional studies were conducted to determine if the inhibitory effects of γ-tocotrienol result from an interference with HER2 functional localization within lipid raft microdomains. Caveolin-1 is an established cellular marker for lipid raft microdomains [86]. Figure 10.5 provides the analysis of plasma membrane subcellular fractions and shows the presence of caveolin-1 in the lipid raft fraction and an absence or very low presence in the non-lipid raft fraction of SKBR3 and BT474 control breast cancer cells [84]. In addition, subcellular fractions obtained from mitogen-starved cells treated with or without 4 μM γ-tocotrienol and 100 ng/ml EGF showed that total HER2 levels were low in the lipid raft microdomain and high in the non-lipid raft microdomain in all treatment groups. However, phosphorylated HER2 levels were relatively high in the lipid raft subcellular fraction and relatively low in the non-lipid raft subcellular fraction of both SKBR3 and BT474 vehicle-treated control groups. Furthermore, γ-tocotrienol treatment caused a large reduction in phosphorylated HER2 levels following EGF exposure in both breast cancer cell types, as compared to cells in their corresponding vehicle-treated control groups [84]. These findings strongly suggest that γ-tocotrienol directly inhibits EGF-dependent HER2 phosphorylation and dimerization within the lipid raft microdomains.

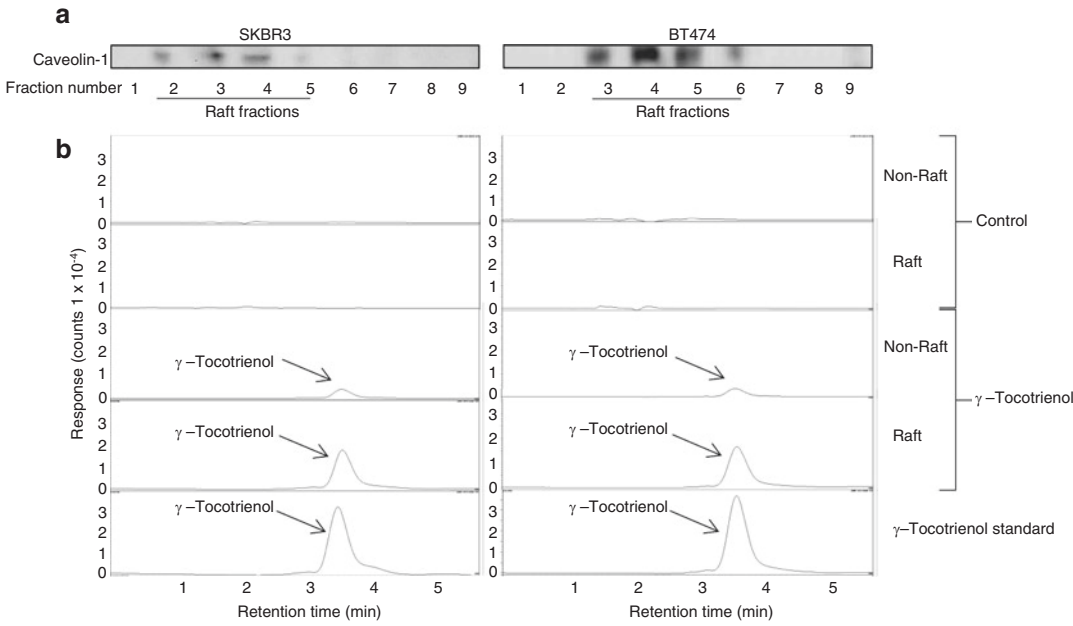

Fig. 10.5 HPLC analysis of γ-tocotrienol levels in lipid raft and non-lipid raft isolated fractions of SKBR3 and BT474 human breast cancer cells. (**a**) Lipid raft and non-lipid raft fractions were isolated from cells in each treatment group and subjected to Western blotting analysis. Caveolin-1-rich fractions were designated as lipid raft fractions. (**b**) Lipid raft and non-lipid raft fractions were then pooled, and γ-tocotrienol levels in these fractions were determined by HPLC analysis as compared to the γ-tocotrienol standard. (Adapted from Alawin et al. [84], reference #69)

Isolated subcellular fractions of lipid raft and non-lipid raft microdomains showed that γ-tocotrienol was not detected in either the lipid raft or non-lipid raft fractions obtained from vehicle-treated SKBR3 and BT474 breast cancer cells [84]. However, subcellular fractions obtained from γ-tocotrienol-treated breast cancer cells showed that γ-tocotrienol levels were highest in the lipid raft fraction as compared to the non-lipid raft subcellular fraction. Dialkylcarbocyanines (DilC16) are a lipophilic molecular probe that preferentially bind to intact lipid raft microdomains [88]. Positive DilC16 labeling showed very high intensity in the lipid raft fractions extracted from vehicle-treated SKBR3 and BT474 breast cancer cells but greatly reduced in the lipid raft fraction extracted from γ-tocotrienol-treated cells [84]. Additional studies showed that disruption of lipid raft integrity with 2-hydroxypro pyl-beta-cyclodextrin (HPβCD) significantly reduced γ-tocotrienol accumulation within the lipid raft microdomain and caused a corresponding decrease in the antiproliferative effects of γ-tocotrienol in SKBR3 and BT474 breast cancer cells [84]. Results from these studies demonstrate that the anticancer effects of γ-tocotrienol result from its accumulation within the lipid raft microdomain of the plasma membrane, where it appears to disrupt with HER2 dimerization, activation, and mitogenic signaling in HER2-positive SKBR3 and BT474 human breast cancer cells.

γ-Tocotrienol Disruption of Lipid Raft Integrity Results in a Reduction in Exosome Heregulin Content and Mitogenic Potency

Lipid rafts play a critical role in modulating the production and content of exosomes secreted into interstitial fluid surrounding cancer cells [32]. Exosomes released into the extracellular environment transport various oncogenic mitogens including EGF, amphiregulin, heregulin, HGF, and VEGF,

which can then stimulate local autocrine-/paracrine-mediated cancer cell growth and progression [34–36]. Previous studies showed that γ-tocotrienol selectively localizes within the lipid raft microdomains and prevents HER2 dimerization, activation, and mitogenic signaling in human breast cancer cells [84]. Since lipid rafts play an important role in exosome production, studies were conducted to investigate γ-tocotrienol effects on exosome-dependent proliferation of HER3-positive human T47D breast cancer cells [89].

Exosomes were isolated from human T47D breast cancer cells following a 24-h culture period in the presence of RPMI 1640 media supplemented with pre-ultracentrifuged fetal bovine serum (exosome-free serum), as previously described [90]. T47D cells were then grown in culture and maintained in serum-free defined media containing 0–100 μg/ml exosomes [89]. Treatment with 20–40 μg/ml exosomes significantly increased growth of T47D breast cancer cells in a dose-responsive manner, as compared to cells maintained in exosome-free control media. In other studies, T47D breast cancer cells were starved with serum-/exosome-free defined media for a 24 h period and afterward treated with 100 μg/ml exosomes. Exosome treatment caused a gradual decrease in total HER3 and HER4 levels and a corresponding large increase in tyrosine-phosphorylated (activated) HER3 and HER4 levels, with maximal phosphorylation observed within 15 min following exosomes exposure.

Characterization studies showed that isolated exosomes contained significantly high levels of the lipid raft proteins flotillin-1 and caveolin-1 and were devoid of the cellular markers Rab6 and cytochrome c. In addition, T47D-derived exosomes displayed intense positive staining for DilC16, which is a lipophilic molecular probe that preferentially binds to intact lipid raft microdomains [88]. Scanning electron micrographs also showed that these particles display a cup-shaped morphology, the hallmark structure of exosomes with particle size ranging from 20 to 100 nm and a mean diameter of 37.3 nm [88].

Subsequent studies showed that treatment with 0–10 μM γ-tocotrienol significantly inhibited exosomes-dependent T47D breast cancer cell growth in a dose-responsive manner as compared to cells in the vehicle-treated control group [89]. Co-immunoprecipitation of HER3/HER4 heterodimers showed that following a 24 h period in serum-/exosome-free defined media, T47D cells displayed relatively low heterodimer levels. However, following an acute exposure to 100 μg/ml exosomes, HER3/HER4 heterodimer levels significantly increased, and this effect was blocked by co-treatment with 5 μM γ-tocotrienol. Additional studies confirmed these findings using immunocytochemical fluorescent staining to visualizing exosome-induced phosphorylation (activation) of HER3 and HER4 in vehicle-treated controls, whereas this effect was blocked in cells receiving combined treatment with γ-tocotrienol [89]. In addition, T47D cells cultured in serum-/exosome-free defined media displayed relatively low levels of phosphorylated FAK, caveolin-1, Akt, and mTOR, total PI3K and Akt, and conversely high levels of total FAK and caveolin-1 [89]. However, following a 15 min exposure to 100 μg/ml exosomes, cells showed a large increase in phosphorylated FAK, caveolin-1, Akt and mTOR, as well as elevations in total PI3K and Akt. Combined treatment with 3–7 μM γ-tocotrienol completely blocked exosome-induced elevations in phosphorylated FAK, caveolin-1, Akt and mTOR, as well as the exosome-induced increase in total PI3K, but not total Akt levels.

γ-Tocotrienol-induced suppression of exosomes-induced HER3/HER4 heterodimerization and autophosphorylation suggests that γ-tocotrienol may be acting specifically within the lipid raft microdomains to bring about these inhibitory effects. Assay of γ-tocotrienol with isolated subcellular fractions of lipid raft and non-lipid raft fractions, as determined by caveolin-1 levels, showed that γ-tocotrienol was not detected in either lipid raft or non-lipid raft fractions obtained from vehicle-treated T47D breast cancer cells. However, subcellular fractions obtained from T47D cells treated with γ-tocotrienol showed that γ-tocotrienol levels were highest in the lipid raft fraction as compared to the non-lipid raft subcellular fraction [89].

Because exosomes originate from lipid raft microdomains [91], γ-tocotrienol-induced disruption of lipid raft integrity may also result in a disruption in exosome production, content, and release. Figure 10.6a shows that exosomes isolated from culture media of T47D breast cancer cells treated with 5 μM γ-tocotrienol were found to have little or no growth stimulatory effect on T47D cells cul-

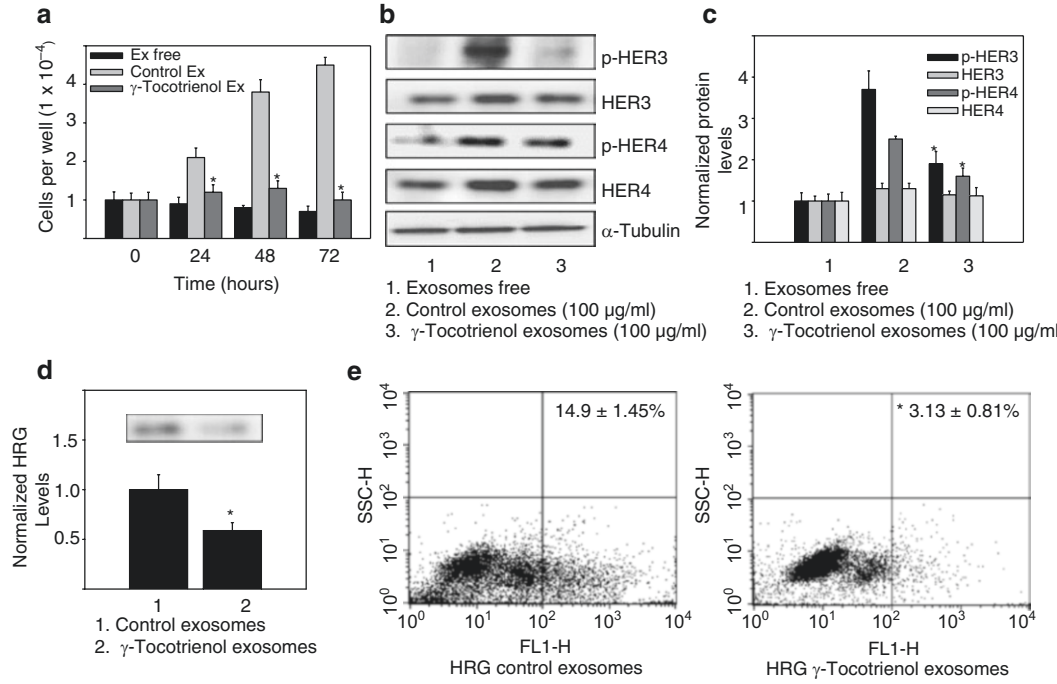

Fig. 10.6 Effects of exosomes derived from γ-tocotrienol-treated T47D cells on T47D breast cancer cell growth. (**a**) T47D cells were divided into different groups and treated with serum-free defined media containing 0 μg/ml exosomes (Ex), 20 μg/ml exosomes (Ex) isolated from control T47D cells, or 20 μg/ml exosomes (Ex) isolated from γ-tocotrienol-treated T47D cells for a 3-day culture period. Vertical bars indicate mean cell number ± SEM in each treatment group. *$P < 0.05$ compared with cells treated in media containing exosomes obtained from the vehicle-treated control group. (**b**) Western blot analysis of exosomes derived from γ-tocotrienol-treated T47D cells on HER3 and HER4 activation. Following exosomes stimulation, cells were isolated, and whole cell lysates were prepared for Western blot analysis for total and phosphorylated HER3 (p-HER3) and HER4 (p-HER4). (**c**) Scanning densitometric analysis was performed on all blots shown in B. Vertical bars indicate the fold change in protein levels in various treatment groups ± SEM as compared with their respective 100 μg/ml exosome-treated control group. *$P < 0.05$ compared to their respective exosome-treated vehicle-treated control group. (**d**) Western blot and scanning densitometric analysis of HPβCD and γ-tocotrienol effects on the relative levels of heregulin in exosomes obtained from the media of T47D mammary cancer cells. Lysates were prepared from exosomes in each treatment group and subjected to Western blot analysis heregulin. Vertical bars indicate the fold change in protein levels in various treatment groups ± SEM as compared with exosomes obtained from the media of T47D cells grown in control media. *$P < 0.05$ compared to exosome from the vehicle-treated control group. (**e**) γ-Tocotrienol effects on the relative levels of heregulin in exosomes from T47D mammary cancer cells as determined by flow cytometry. Exosomes isolated from either vehicle- or γ-tocotrienol-treated cells were adsorbed on magnetic beads, incubated with anti-heregulin, and then subjected to flow cytometry analysis. Exosomes displaying positive heregulin level appear in the lower right quadrant of each dot plot. The numbers appearing in the upper right quadrant of each dot plot represent the mean percentage ± SEM of exosomes with heregulin in their respective treatment group. *$P < 0.05$ as compared with exosomes obtained from cells grown in the vehicle-treated control group

tured in serum-free defined media, as compared to exosomes obtained from the culture media of T47D cells maintained in vehicle-treated control media [89]. Furthermore, a 15 min exposure to 100 μg/ml exosomes derived from γ-tocotrienol-treated T47D breast cancer cells showed a significant reduction in phosphorylated (activated) HER3 and HER4 levels, but had no effect on total levels of these receptor tyrosine kinases, as compared to T47D cells treated with exosomes isolated from the media of vehicle-treated T47D cells (Fig. 10.6b). Additional studies showed that exosomes isolated from γ-tocotrienol-treated T47D cells contained significantly lower heregulin levels (Fig. 10.6d, e), a potent ligand for HER3 and HER4 tyrosine kinase receptors, as compared to exosomes isolated from the media of vehicle-treated T47D cells [89].

These findings demonstrate that exosomes isolated from the media of cultured T47D human breast cancer cells are capable of stimulating T47D cell growth in serum-free defined culture media containing only exosomes as a mitogenic agent. Furthermore, these exosomes contain high levels of the heregulin, an endogenous ligand that activates and induces heterodimerization of HER3/HER4 receptors located in the lipid raft microdomains of T47D cells. Additional studies showed exosomes isolated from the media of cultured T47D cells treated with a growth-inhibitory dose (5 μM) of γ-tocotrienol contained significantly lower levels of heregulin and were significantly less potent in stimulating HER3/HER4 activation and heterodimerization, as compared to exosomes obtained from T47D cells maintained in control media. Taken together, these results indicate that exosomes produced by T47D cells provide a potent autocrine/paracrine mechanism for stimulating breast cancer cell proliferation and exposure to γ-tocotrienol results in a significant reduction in exosome mitogenic biopotency.

Conclusion

Because a significant percentage of human breast cancers display aberrant receptor tyrosine kinase signaling, a variety of chemotherapeutic agents have been developed that target these receptors. Various agents currently approved for clinical use include monoclonal antibodies and tyrosine kinase inhibitors. Unfortunately, the clinical effectiveness of these agents has been found to be limited due to the cooperation between different types of receptor tyrosine kinases to form heterodimers within the lipid raft microdomain [21, 26, 86]. Heterodimer cooperativity allows cancer cells to escape the inhibitory effects of these chemotherapeutic agents [21, 92–94]. However, results have also shown that combined treatment of γ-tocotrienol with receptor tyrosine kinase inhibitors results in a synergistic anticancer response [21, 92–94]. Recent publications have provided further evidence demonstrating that use of chemotherapeutic agents that target receptor tyrosine kinases as well as lipid raft integrity provides significant benefits in the treatment of human breast cancer and the prevention in the development of acquired drug resistance.

γ-Tocotrienol-induced disruption of lipid raft integrity also explains the mechanism(s) mediating the ubiquitous inhibitory effects of γ-tocotrienol on a wide variety of receptor tyrosine kinases and their downstream signaling cascades and is consistent with the current understanding of the intimate relationship between plasma membrane lipid raft microdomains and extracellular exosomes. Experimental findings demonstrate that a primary mechanism by which γ-tocotrienol is able to mediate such a wide range of inhibitory effects on receptor tyrosine kinase activation and dimerization is mediated by its preferential accumulation and disruption of the lipid raft microdomain. Mitogenic exosomes are derived from lipid rafts, and γ-tocotrienol-induced disruption of breast cancer cell lipid raft microdomains produces exosomes that contain relatively low levels of heregulin and display a corresponding low level of mitogenic biopotency. Since heregulin stimulates HER3/HER4 activation, heterodimerization, and mitogenic signaling, the antiproliferative effects of γ-tocotrienol appear to result both from a suppression in both receptor tyrosine kinase activation and from a reduction in exosome-mediated autocrine/paracrine mitogenic activity. These findings are of particular interest because aberrant HER3/HER4 signaling is associated with poor patient prognosis in breast and many other forms of cancer [95] and suggest that γ-tocotrienol treatment may provide benefit in the treatment of breast cancer characterized by aberrant receptor tyrosine kinase signaling, particularly cancers that display excessive HER3/HER4 expression and signaling.

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Chapter 11
Interaction Between Vitamin E and Polyunsaturated Fatty Acids

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Keywords Signal transduction · Gene expression · PUFA · Vitamin E · Omega-3 · Fish oil · Membrane microdomains · Lipid rafts · Fatty acids · Transport · Lipids · Atherosclerosis · NASH · Neurodegeneration

Key Points

- Vitamin E and PUFA are insoluble in water and share similar and/or overlapping pathways for their uptake, distribution, metabolism, and molecular action as structural membrane components and active lipid mediators.
- At sufficient levels, vitamin E inhibits free radical attack on PUFA and preserves it against peroxidation, nitration, and depletion, whereas excess PUFA may lead to depletion of vitamin E.
- Vitamin E influences the production of PUFA-derived active lipid mediators by directly binding to enzymes involved in their biosynthesis and thereby has indirect regulatory actions on associated signal transduction and gene expression.
- Vitamin E and PUFA form an interdependent chemical and biological pair of cellular lipid mediators that interact and influence each other with regulatory consequences on cell physiology and pathophysiology.

Cholesterol, phospholipids (PL) containing fatty acids (FA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and vitamin E are principal components of cell membranes that influence each other and determine membrane properties and function. Their relative levels in cell membranes influence the physical properties of the plasma membrane and the structure and composition of membrane microdomains such as lipid rafts/caveolae and non-rafts important for the localization and activity of enzymes, receptors, and transporters involved in signal transduction, gene expression, secretion, endocytosis, and intercellular cell-to-cell communication and in maintaining vital cellular processes. Moreover, both PUFA and vitamin E can be metabolized to a number of active

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_11

lipid mediators that are important for physiological and pathophysiological cellular processes. Vitamin E protects PUFA from oxidation and nitration indicating that the balance between vitamin E and PUFA is an important determinant for maintaining their regulatory activities in signal transduction and gene expression relevant for immune, inflammatory, developmental, and metabolic pathways. In this chapter, the interactions of vitamin E and PUFA are reviewed by comparing their mechanisms of uptake, transport, distribution, metabolism, interaction, and regulatory roles in signal transduction and gene expression and in maintaining normal cell membrane physiology.

Dietary Sources of Vitamin E and PUFA

Cholesterol, fatty acids (FA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and vitamin E are the main active components of the plasma membrane of almost all higher organisms. Vitamin E is an essential fat-soluble vitamin which is comprised of eight naturally occurring analogues, four as tocopherols (αT, βT, γT, δT) with saturated phytyl side chains and four as tocotrienols (αTT, βTT, γTT, δTT) with unsaturated isoprenoid side chains. The most widely accepted biological function of vitamin E is related to its antioxidant activity and in particular to the prevention of peroxidation of PUFA in membranes. However, in recent years several biological non-antioxidant activities of vitamin E and vitamin E metabolites (e.g., α-tocopheryl phosphate (αTP), long-chain carboxyethylhydroxychromans (LC-CEHC), and CEHC) have been described as well [1–3]. Among the tocopherols, RRR-α-tocopherol (αT) has the highest biological activity and is the most widely available form in foods. The antioxidant activity of the other analogues is within the same range, but since only αT is preferentially enriched in the body, it is considered to be the analogue with the essential vitamin E activity [4]. The most commonly used synthetic form of vitamin E comprises approximately equal amounts of each of the eight side-chain stereoisomers as α-tocopheryl acetate (αTA) in which the chromanol hydroxy group is esterified to increase its stability by protecting it from oxidation and to increase its water solubility in supplements. One mg of synthetic all racemic αTA is regarded as 1 international unit (1 U), and the potency of natural form of αT is set to be equal to 1.49 IU. The biological activity of γT is about 1/10th of that of αT.

Vegetable oils are the richest dietary sources of vitamin E (200–1000 mg/kg) [5, 6]. Vegetables seed oils such as corn oil, soybean oil, safflower oil, palm oil, nuts, whole grain, and wheat germ are rich sources of tocopherols. Sunflower seed oil is considered an excellent source of αT, and corn oil is the second best. However, corn oil and soybean oil contain the highest amounts of γT which is the major form of vitamin E in US diet [6, 7]. Among the brands, sunflower oil, canola oil, and corn oil are classified as a good food source of tocopherols [8], whereas animal products are generally a relatively poor source of vitamin E.

The n-3 and n-6 polyunsaturated fatty acids (omega-3 or omega-6 PUFA, respectively) are nutritionally essential since they cannot be synthesized de novo from two-carbon fragments in vertebrates. Three major categories of PUFA are n-3, n-6, and n-9 PUFA depending on the first unsaturated double bond of the carbon chain from the terminal methyl end. Important PUFA are the long-chain PUFA (LC-PUFA) arachidonic acid (AA, 20:4n-6), docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and docosapentaenoic acid (DPA, 22:5n-6) and their nutritionally essential precursors linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) (reviewed in [9, 10]). Similar to vitamin E, PUFA and also monounsaturated fatty acids (MUFA) are abundant in vegetable oils (10–70%), with a n-6/n-3 ratio between 80 and 10, whereas peanuts and almonds contain mostly n-6 PUFA [6].

LA and ALA are abundant in nature and can be converted mostly by the liver to n-3 and n-6 LC-PUFA, respectively. LA occurs similar to vitamin E in seeds of most plants (soybean, canola, corn, sunflower, safflower, cottonseed, but not in coconut, cocoa, and palm), whereas AA mostly occurs in meats. ALA, the precursor of n-3 PUFA, is found mostly in green leafy vegetables, walnuts,

and seeds of flax, rape, and chia. The major source of EPA and DHA is seafood such as coldwater fish and products from animals fed a diet high in n-3 PUFA such as eggs and beef from range-fed cattle. Dietary sourced DHA mainly determines the amounts in tissues having a low level of DHA in the sn-2 position of phospholipids, whereas tissues containing already large amounts of DHA in the sn-1 and sn-2 position such as the brain/neurons, rod outer segment, and sperm are less affected by the diet [11].

AA is essential since it is used for eicosanoids biosynthesis and as a constituent of phospholipids in membranes important for signaling. EPA and in particular DHA are essential since they are important for reproduction, embryonic and neuronal development, and visual acuity and as membrane phospholipids such as the ethanolamine and serine phospholipids important for membrane structure [11–13]. In the body, the n-3 and n-6 PUFA have often opposite effects so that the balance between them is relevant for their effects in physiology and pathophysiology [14]. N-3 and n-6 PUFA are differentially metabolized into a number of lipid mediators (prostaglandins, thromboxanes, leukotrienes, eicosanoids, resolvins, lipoxins, neuroprotectins, and others), and since the enzymes involved (desaturases, elongases, P450 epoxygenases, lipoxygenases, cyclooxygenases) recognize both n-3 and- n-6 PUFA, there is competition between them and in some cases also with vitamin E [6, 14]. At the molecular level, PUFA and PUFA metabolites act as active lipid mediators and influence cell signaling and gene expression [15, 16]. PUFA and in particular DHA are essential for the normal functional development of the retina and brain and modulate energy production and storage, immune and inflammatory events, ion channels, and calcium conductance and when in phospholipids in the membrane influence physical properties of membrane microdomains (lipid rafts/caveolae and non-rafts) important for signaling and gene expression with relevance for cancer, insulin resistance, and cardiovascular disease (reviewed in [9, 10, 17–20]).

Since vitamin E as the most important lipid-soluble antioxidant protects PUFA from reactive oxygen and nitrogen species (ROS and RNS, respectively), the requirements of vitamin E are dependent on the levels of PUFA in the diet. It has been recently estimated that with a typical dietary PUFA intake, the vitamin E requirements range between 12 and 20 mg [21, 22], what is within the range of the recommended daily allowance (RDA) of 15 mg vitamin E as determined by the Institute of Medicine [23]. The relative level of vitamin E appears to be particularly important when PUFA are increased in the diet by supplementation because of their well-established health benefits against diseases such as atherosclerosis, inflammation, chronic heart failure, rheumatoid arthritis, allergies, and depression [21, 22].

Uptake and Transport of Vitamin E

Since vitamin E is a fat-soluble vitamin, its absorption is linked to the digestion, micellar emulsification, and absorption of fat [24]. The mechanisms and proteins involved in the transport of vitamin E across the plasma membrane of cells in the intestinal epithelium [25–27], the intracellular transport [28], export, and incorporation into lipoproteins (chylomicrons, HDL, LDL, VLDL) have been recently reviewed [29]. At high concentration vitamin E can be absorbed by passive diffusion mechanisms and bulk endocytosis; however, at least in intestinal epithelial cells, a certain degree of competition between hydrophobic vitamins suggests common mechanisms of uptake involving specific transporters [30–32]. In particular at the lower doses found in the diet and the body, vitamin E transport and distribution is selective and regulated involving specific recognition by carrier proteins, transporters, and receptors in a similar manner as it occurs with FA [33]. Part of this regulation occurs by selective and regulated uptake and transport in cells, plasma, and tissue, specific binding to and activation by enzymes and structural proteins and transcription factors, selective metabolism, and selective secretion/excretion.

When in the circulation, vitamin E is mostly located within lipoproteins (VLDL, LDL, HDL, chylomicrons) and albumin/afamin and delivered to tissues during hydrolysis of triacylglycerides (TG) by lipoprotein lipase and at physiological levels of vitamin E occurs via selective transport by specific transporters such as the LDL receptor (LDLr), the scavenger receptors/fatty acid transporters (CD36, SR-BI), phospholipid transfer proteins (PLTP), the Niemann-Pick C1/like 1 (NPC1L1), and the three tocopherol-associated proteins (TAP1/2/3 or SEC14L2/3/4, respectively) [34–39]. Whereas the transport of all eight major natural analogues of vitamin E (α-, β-, γ-, δ-tocopherol and tocotrienols) across the intestinal epithelium occurs with equal efficiency, the subsequent steps are highly sophisticated and selective, in part to prevent accumulation but mostly because it is geared toward retaining mainly one of the eight natural vitamin E analogues, *RRR*-alpha-tocopherol (αT), by means of selective enrichment and by preventing its metabolism occurring with the other seven analogues of vitamin E [29, 40, 41]. αT is enriched mostly as the consequence of selective retention, protection, and incorporation into very low-density lipoproteins (VLDL) by the liver α-tocopherol transfer protein (αTTP), secretion by ATP-binding cassette transporters A1 and G1 (ABCA1, ABCG1), and enhanced metabolic degradation of the other tocopherols (β-, γ-, and δ-), of excess αT, and of all tocotrienols (TT) by cytochrome P450 enzymes (CYP3A, CYP4F2) and their subsequent elimination as water-soluble vitamin E metabolites (Fig. 11.1) (reviewed in [40, 42]).

Polymorphisms in the genes involved in vitamin E uptake and distribution have been implicated in the bioavailability and bioactivity of vitamin E (uptake, distribution, oxidation, metabolism, molecular action, signaling, and gene expression) (reviewed in [36, 43–45]). Some of these gene polymorphisms (e.g., in αTTP, hTAPs, CD36, SR-BI, NPC1L1, ABCA1, ABCG1, haptoglobin, apoE, cytochrome P450) [45–58] may influence vitamin E levels and function in plasma and tissues [59–64]. The presence of gene polymorphisms in vitamin E regulatory genes may ultimately translate into an altered risk for diseases such as atherosclerosis [65, 66], metabolic syndrome [67], inflammation [54, 68], cancer [69], and degenerative diseases [51], but they also may explain the difficulty to reach clear evidence for beneficial effects in vitamin E supplementation studies [49]. It remains to be investigated whether these polymorphisms influence the level and action of PUFA and whether similar polymorphisms in genes involved in PUFA uptake, tissue distribution, metabolism to bioactive lipid mediators, and molecular action play a role in the individual risk for these diseases.

Uptake and Transport of PUFA

Triacylglycerides (TG) are the quantitatively most important lipids in the human diet, reaching about 100 g per day or more. Depending on the food source, TG contain fatty acyl groups of various length and saturation that are converted to non-esterified fatty acids (NEFA) upon digestion by lipases during gastrointestinal passage. NEFA are bound, transported, and distributed by a number of tissue-specific fatty acid-binding proteins (FAPB) with limited ability to discriminate between the types of NEFA [70]. The route of uptake of PUFA has been recently reviewed [9, 71] and involves oral detection of dietary PUFA by CD36 and GPR120; digestion of TG-PUFA by lingual, gastric, and pancreatic lipases; emulsification; absorption of PUFA by the intestinal epithelial cells involving both passive and facilitated transport by CD36/FAT, plasma membrane fatty acid-binding protein (FABPpm), and six fatty acid transport proteins (FATP1–FATP6) [72]; transport across the cytosol by one of nine tissue-specific cytoplasmic fatty acid-binding proteins (FABPc) [73]; and subsequent incorporation and secretion within chylomicrons [9, 74–76]. At least for DHA, the uptake from the diet can be increased by a mixture of α-tocopheryl phosphate and di-tocopheryl phosphate (TPM) leading to an almost twofold higher plasma concentration [77].

In the periphery, PUFA transport occurs mainly within TG in lipoproteins (VLDL, LDL, HDL) and albumin/afamin. In the liver, PUFA serve as precursors for the synthesis of long-chain PUFA

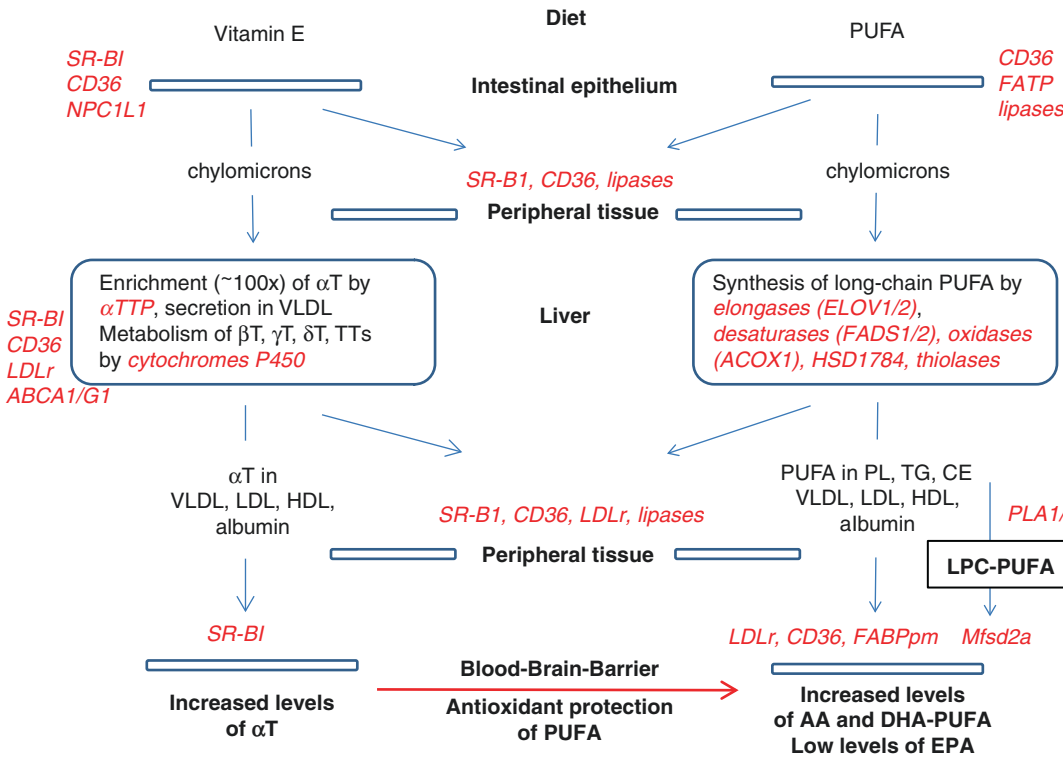

Fig. 11.1 Uptake and transport of vitamin E and PUFA. (**Left**) Vitamin E is taken up across the intestinal epithelium mediated by SR-BI, CD36, and NPC1L1, and then becomes incorporated into chylomicrons and distributed to peripheral tissues and the liver. In the liver, αT is selectively recognized by αTTP and enriched up to 100-fold, whereas βT, γT, δT and αTT, βTT, γTT, δTT and excess αT are metabolized and secreted. From the liver, αT is incorporated into VLDL and distributed to the peripheral tissues within lipoproteins (VLDL, LDL, HDL, albumin, afamin); transport across the blood-brain barrier (BBB) occurs mainly from HDL via SR-BI. (**Right**) After digestion of triacylglycerols (TG) by several lipases, free fatty acids (FA) and PUFA are taken up from the diet via CD36, FATP, and FATPpm and then become incorporated into chylomicrons and distributed to peripheral tissues and the liver. In the liver, long-chain PUFA are synthesized by several enzymes (elongases, desaturases, and P450 epoxygenases) and distributed within phospholipids (PL), triacylglycerides (TG), cholesterol esters (CE), and lipoproteins (VLDL, LDL, HDL, albumin/afamin) to peripheral tissues; transport across the blood-brain barrier (BBB) occurs as lysophosphatidylcholine-PUFA (LPC-PUFA) involving LDLr, CD36, FABPpm, FAPBc, and in particular Mfsd2a. Vitamin E enriched in plasma and brain can protect PUFA from oxidation leading to less oxidative depletion in particular of the essential DHA-PUFA in the brain

(LC-PUFA) by several desaturases (FADS1, FADS2), elongases (ELOV1, ELOV2), and oxidases (ACOX1) in the endoplasmic reticulum and peroxisomes and are then secreted into the circulation within phospholipids (PL), triacylglycerols (TG), and cholesterol esters. CD36 can bind PUFA with nanomolar affinity, and it transports the n-6 PUFAs linoleic acid (LA) and arachidonic acid (AA) (Fig. 11.1) [74, 75], but CD36 ablation only affected MUFA and not PUFA concentrations in the brain [78]. CD36 expression is induced by fish oil PUFA what has been linked to beneficial effects of fish oil administration on the metabolic syndrome [79]. In contrast, CD36 expression and cell surface exposition is inhibited by vitamin E and more so by vitamin E phosphate leading to reduced response to other CD36 ligands such as bacteria, virus, parasites, fatty acids, oxLDL, and possibly cholesterol crystals, and it remains to be investigated whether the uptake of PUFA is also affected [36, 38].

Because vitamin E plays an important role in protecting PUFA from peroxidation and nitration in the body and in particular in the brain, the relative levels of vitamin E and PUFA in the circulation and the transport into the brain are important determinants for a number of diseases [21, 22]. Neither

vitamin E nor essential PUFA such as eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) can be synthesized in the brain. Therefore, they are expected to be transported across the blood-brain barrier (BBB) to maintain long-chain n-3 PUFA and vitamin E at adequate levels to keep the brain function normal. Severe neurological disorders are associated with low plasma levels of vitamin E and PUFA which are primarily manifested by defects in embryonic and in particular brain development and associated increased risk for neurodegenerative diseases (e.g., ataxia, Alzheimer's disease, Parkinson's disease) [12, 13, 18], suggesting existence of transporting systems for vitamin E and PUFA across the BBB system.

Blood-Brain Barrier (BBB) Transport of Vitamin E and n-3 PUFA

Neither vitamin E nor essential PUFA can be synthesized in the brain, and severe diseases associated with low plasma levels of vitamin E and PUFA associate mainly with defects in brain function [12, 13], suggesting that the transport across the blood-brain barrier (BBB) and the protection of PUFA by vitamin E in the body and in particular in the brain play an important role (Fig. 11.1). Interestingly, also severe vitamin E deficiency with clinical symptoms as a result of mutation of the liver αTTP gene involved in vitamin E enrichment in plasma leads to a specific neurological syndrome, ataxia with vitamin E deficiency (AVED), linked to degeneration of Purkinje cells [29]. To cross the BBB, vitamin E is released mainly from HDL and transported via the scavenger receptor BI (SR-BI) which delivers vitamin E to the brain capillary endothelial cells [26, 80]. SR-BI-deficient mice show an up to 70% reduction of brain αT content [81].

DHA, and other omega-3 PUFA important for brain growth which cannot be synthesized in the brain, is highly enriched in the brain suggesting mechanisms for transport across the BBB. Recently, Mfsd2a, a member of the major facilitator superfamily of proteins, has been identified to be expressed in the endothelium of the BBB microvessels [82]. Interestingly, Mfsd2a transports DHA in the form of lysophosphatidylcholine (LPC), but not as unesterified fatty acids, in a sodium-dependent manner suggesting that it is a sodium-dependent LPC symporter. LPC-oleate and LPC-palmitate, two common plasma LPC, are transported by Mfsd2a but not LPC with shorter than 14-carbon acyl chains. Knockout mice for Mfsd2a have markedly reduced uptake of LPC-DHA and other LPC from plasma to the brain [82]. Mfsd2a mediates also the transport of LPC-DHA in the eye relevant for the development of photoreceptor discs [83]. Mutations that inactivate Mfsd2a in zebrafish and that were also detected in humans were associated with a microcephaly syndrome, thus linking inadequate uptake of LPC by Mfsda2a with human brain growth and function [84]. The low aqueous solubility of DHA limits direct cytosolic transport across the endothelial cells of the BBB. In mice, the intracellular carrier protein fatty acid-binding protein 5 (FABP5) is expressed at the BBB, binds to DHA, and is involved in the brain endothelial cell uptake and subsequent BBB transport of DHA, confirming its importance for CNS exposure of this PUFA essential for neuronal function [85, 86].

A protective antioxidant effect of vitamin E on PUFA in the brain has been demonstrated in 1-year-old zebrafish (*Danio rerio*), in which vitamin E deficiency with limiting dietary long-chain PUFA has been linked to a 1/3 lower level of a specific PUFA, 1-hexadecanoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (DHA-PC 38:6, PC 16:0/22:6), most likely as a result of increased peroxidation as suggested from increased level of its hydroxyl-DHA-PC form [12, 13, 87]. However, the different disease symptoms associated with PUFA and vitamin E deficiency suggest that the chemical antioxidant effects of vitamin E in protecting PUFA in the brain may only in part explain the essential function of vitamin E. Accordingly, of 155 lipids surveyed by lipidomics in brain extracts, only four phospholipids (PL) were significantly different [87]. Moreover, since the levels of both saturated and unsaturated LPC were decreased in the brain during vitamin E deficiency, the observed depletion by nearly 60% of 19 different LPC (combined) could also be due to an overall lower level of PUFA in the body,

their lower uptake, synthesis or higher metabolism, and/or their lower transport across the BBB, e.g., by regulatory effects of vitamin E on gene expression of enzymes and transporters relevant for PUFA uptake, transport, synthesis, and metabolism [77, 87]. In fact, in a metabolomics study, vitamin E supplementation (400 mg/d RRR-α-tocopheryl acetate, for 4 weeks) in humans showed increased plasma levels of a number of lysophosphatidylcholine (LPC) species (16:0, 18:0, 18:1, 18:2, 20:3, 22:6) possibly as a result of induction of phospholipase A2 (PLA2) [88]. Some of these LPC species are able to modulate signal transduction and gene expression (e.g., via Ca^{2+} and protein kinase C (PKC)) [89], and some of them may be selectively transported across the BBB by Mfsd2a [82, 84] and furthermore interconverted into DHA [10, 18]. Likewise, in the retina of αTTP-knockout mice deficient in vitamin E, the age-related decrease of n-3 PUFA in the retina was more pronounced when compared to controls, whereas other classes of fatty acids were unchanged or increased, suggesting that a severe deficiency of vitamin E may reduce PUFA uptake and/or enhance their peroxidation, and accelerate retinal degenerative damage with age [90]. In cell culture, vitamin E preserved and protected the cytoplasmic membranes from oxidative injuries and promotes plasma membrane repair [91, 92]. These molecular mechanisms of protective and repair characteristics of vitamin E are not known and need to be investigated further; they may involve its antioxidant activity by protecting cells from free radicals leading to physicochemical stability; they may directly enhance membrane repair by virtue of the biophysical alterations induced in the membrane, e.g., by forming a complex with LPC [88, 93]; or they may be the result of induction of signal transduction and the expression of membrane repair proteins.

Regulatory Effects of Vitamin E and PUFA

The mechanisms by which vitamin E and vitamin E metabolites affect signal transduction and gene expression have been recently reviewed and are only shortly addressed here (Table 11.1) [1, 36, 58, 94–97]. By preventing the oxidation and depletion of PUFA and by influencing the generation of

Table 11.1 Molecular mechanisms of regulatory effects of vitamin E and vitamin E analogues on signal transduction and gene expression (see text for references) (reviewed in [36])

Mechanisms of vitamin E action	Proposed targets involved in signaling and gene expression (examples)
Antioxidant protection against ROS, RNS, and lipid peroxides	Lipids, proteins, micronutrients and other vitamins, PUFA, and DNA/RNA
Alterations of membrane properties	Fluidity, curvature and stability of membranes and membrane microdomains (lipid rafts/caveolae, non-rafts), modulation of Ca^{2+} influx and Ca^{2+}-mediated signaling, membrane repair
Modulation of membrane-protein interactions	PKC, Akt/PKB, PI3K, PHLPP2, PP2A, PLA2, NADPH oxidase
Modulation of membrane receptors	TLR2, TLR4, TLR6
Modulation of gene expression via transcription factors and nuclear receptors	PXR, PPARγ, NRF2, NFκB, RORα, Hif1α, ERβ
Modulation of signal transduction and gene expression by vitamin E and vitamin E phosphate	CD36, PI3Kα, PI3Kγ, Akt/PKB, PLA2, TAP1/SEC14L2, TAP2/SEC14L3, TAP3/SEC14L4
Modulation of receptor cell surface exposition	CD36
Direct interaction with specific enzymes, in part enhanced after conversion to vitamin E metabolites, and modulation of the formation of PUFA-derived lipid mediators	PKCα, PLA2, 5-, 12-, 15- LOX, COX-1, COX-2
Protective effects against PUFA oxidation and modulation of PUFA-metabolic enzymes	Indirect by influencing level and action of PUFA and of PUFA-derived active lipid mediators

PUFA-derived lipid mediators, vitamin E may overall increase the cellular levels of PUFA and thus affect a multitude of regulatory events. Therefore, as described in the following, the regulatory effects of vitamin E on PUFA levels and action may be an additional albeit indirect way by which vitamin E can influence signal transduction and gene expression.

Regulatory Effects and Interaction of Vitamin E and PUFA in Membranes

When inserted into membranes, vitamin E has stabilizing characteristics, and it was originally assumed that this is required in particular to stabilize cholesterol- and sphingomyelin-rich membrane microdomains (lipid rafts) [98, 99]. In contrast, at least in model membranes, vitamin E appears to accumulate more in DHA-rich membrane microdomains having increased fluidity due to the flexible structure of rapidly isomerizing unsaturated fatty acids leading to their stabilization and prevention of lipid peroxidation [98]. DHA-containing phospholipids have an aversion for sterols leading to the lateral segregation of DHA-containing phospholipids into liquid-disordered (l(d)) non-raft microdomains that are depleted in cholesterol, as opposed to the liquid-ordered (l(o)) raft/caveolae microdomains that are enriched in predominantly saturated sphingolipids and cholesterol. Vitamin E and PUFA can modulate the composition, properties, and organization of cholesterol- and sphingolipid-rich lipid rafts (10–200 nm) and the flask-like invaginations called caveolae that are enriched with the structural protein caveolin-1. Caveolae harbor several enzymes and receptors involved in signal transduction, and changes in their structure and function can influence a variety of cellular signaling functions (reviewed in [100]).

Vitamin E in the plasma membrane may influence the local concentration of structural and messenger lipids, their spatial clustering in membrane microdomains (e.g., in lipid rafts/caveolae and non-rafts), or their transbilayer asymmetry and thus change the ability of several enzymes to interact with these membrane structures and to become activated (reviewed in [101]). Conformational changes induced in enzymes when they move between rafts and non-raft microdomains may account for some of the regulatory effects of vitamin E and PUFA on cell function [11]. Vitamin E (αT but not γT) can prevent the apoptotic effects of cholesterol oxides, in particular 7-ketocholesterol, by changing the presence of 7-ketocholesterol in sphingolipid-/cholesterol-enriched lipid raft microdomains [99, 102]. Interestingly, apoptosis induced by 7-ketocholesterol was mediated by dephosphorylation and inactivation of PKB, and αT but not γT prevented the PKB dephosphorylation. Vitamin E also influences the transbilayer asymmetry of phospholipids in the plasma membrane, e.g., by inhibiting the externalization of phosphatidylserine (PS) in erythrocytes with consequent reduced procoagulant properties [103]. Similar to that, vitamin E inhibits hemolysis induced by hemin by increasing the stability of erythrocytes membranes in a non-antioxidant manner [104]. Of interest is that the two main phospholipids that are enriched with DHA, phosphatidylethanolamine (PE) and phosphatidylserine (PS), reside preferentially in the inner leaflet of the plasma membrane [11], and it remains to be shown whether the inhibition of externalization of these phospholipids by vitamin E and the prevention of apoptosis are influenced by the presence of DHA.

PUFA alter basic properties of cell membranes, including stability, fluidity, phase behavior, elastic compressibility, curvature, elasticity, fusion, ion permeability, flip-flop, and the activity of membrane-resident enzymes and proteins (reviewed in [100]). These changes in membrane properties alter the molecular architecture of the plasma membrane and in particular the organization into sphingolipid-cholesterol-enriched lipid rafts and PUFA-enriched non-rafts and in sphingolipid-rich cholesterol-free microdomain and cardiolipin-protein scaffolds in the inner mitochondrial membrane [105–107]. In T-cells, dietary n-3 PUFA-mediated changes in membrane microdomains affect the translocation of activated PKCΘ to the plasma membrane possibly leading to the suppression of the activity of the NFκB and AP-1 transcription factors, of IL-2 secretion, of lymphoproliferation, and of immune-synapse formation [100].

Taken together, vitamin E and PUFA modulate similar cellular processes that can be explained in part by either affecting overlapping molecular targets in the plasma membrane or by influencing the cellular concentrations of each other.

Regulatory Effects of Vitamin E on Metabolism of PUFA to Active Lipid Mediators

The metabolism of PUFA leads to a variety of active lipid mediators (e.g., omega-3 (n-3)-derived families of resolvins, protectins, maresins, prostaglandins, thromboxanes, leukotrienes, and eicosanoids; omega-6 (n-6) – arachidonic acid-derived lipoxins, prostaglandins, thromboxanes, leukotrienes). These lipid mediators have a variety of important cell and immunoregulatory functions during inflammation, tissue regeneration and repair, wound healing, and infection with consequences for diseases such as cardiovascular disease, neurodegenerative disease, obesity, asthma, arthritis, and periodontal disease (reviewed in [14, 108, 109]). Moreover, these lipid mediators play an important role for reproduction by influencing implantation and embryonic development in particular of the brain [12, 13]. Vitamin E may influence the production of PUFA-derived active lipid mediators by directly binding to enzymes involved in their biosynthesis, such as phospholipase A2 (PLA2) [110, 111]; 5-, 12-, and 15-lipoxygenases (5-, 12-, and 15-LOX, respectively) [112–115]; and cyclooxygenase-2 (COX-2) [116]. Vitamin E modulates these enzymes in a vitamin E analogue-specific manner, and some vitamin E metabolites have a higher activity, suggesting that each tocopherol and tocotrienol analogue and metabolite can bind to them with different affinity (reviewed in [6]). The direct binding of αT to 5-LOX inhibits not only its enzymatic activity, but affects also membrane translocation via the inhibition of tyrosine phosphorylation [117]. Similarly, the phospholipase D1 (PLD1) is excluded from lipid rafts upon treatment with DHA leading to disruption of rafts-associated signal transduction possibly accounting for some of its immunoregulatory effects [11]. Thus, in addition to modulating the enzymatic activity of these enzymes by binding to their active site, vitamin E may interfere with the enzyme activation/membrane translocation process or act as a competitive inhibitor and/or affect the action of PUFA-derived lipid mediators [6, 14].

Competition of vitamin E with the binding of several phospholipids occurs with the alpha-tocopherol transfer protein (αTTP) [118, 119] and with the tocopherol-associated proteins (TAP1, TAP2, and TAP3, also known as SEC14L2, SEC14L3, SEC14L4, respectively) affecting PI3K activity relevant for the expression of the vascular endothelial growth factor (VEGF) involved in angiogenesis [37, 120]. In addition to vitamin E, the human TAP proteins (hTAP) bind in vitro several other ligands, such as squalene, phosphatidylinositol, phosphatidylinositol-3,4,5-phosphate, phosphatidylcholine (PC), and phosphatidylserine (PS), and it is unknown whether the degree of unsaturation of the FA influences the binding and exchange with vitamin E (reviewed in [121]). These proteins are involved in transport and PI3K-mediated signal transduction and become activated by vitamin E-mediated lipid exchange and/or dissociation of the inactive complex [37, 120, 122–125]. Moreover, the zebrafish/human SEC14L3/SEC14L2 mediates Wnt/Ca^{2+} signaling by activating phospholipase δ4a (PLδ4a) and subsequent phosphatidylinositol-4,5-biphosphate and Ca^{2+} signaling important for a number of developmental events via its intrinsic GTPase activity, and it remains to be shown whether vitamin E binding can modulate it [126, 127].

Regulatory Effects and Interaction of Vitamin E and PUFA on Signal Transduction and Gene Expression

According to the pecking order of antioxidants, vitamin E has a lower one-electron reduction potential than PUFA and is therefore capable of reducing peroxidized PUFA, thus interfering with the chain reaction of lipid peroxidation together with phospholipid hydroperoxide glutathione peroxidase

(GPx4) [128]. As a consequence of donating a hydrogen, vitamin E forms a resonance-stabilized vitamin E hydroperoxide that can be regenerated by vitamin C (L-ascorbic acid) and glutathione or, if not, convert to tocopheryl quinone/hydroquinone. Dihydroascorbate formed in this reaction is regenerated by dihydroascorbate reductase. Accordingly, the formation of nitrated fatty acids from nitric oxide (·NO)-derived species can be prevented by gamma-tocopherol (γT) able to trap electrophiles including reactive nitrogen species (RNS) [129–132]. By protecting the levels of PUFA from oxidation, nitration, and depletion and by affecting PUFA-metabolic enzymes, vitamin E may thus play a regulatory role within similar signal transduction and gene expression pathways as PUFA and PUFA-derived lipid mediators (Fig. 11.2 and Table 11.2), although such indirect effects of vitamin E are more difficult to assess than direct ones and involve an entire antioxidant network of redox active molecules and enzymes.

PUFA and PUFA-derived lipid mediators function not only by altering membrane lipid composition and properties but also modulate a number of lipid-metabolizing enzymes, membrane receptors (toll-like receptors (TLR 2, 3)), G-coupled receptors, membrane transporters such as CD36/FAT, signal transduction enzymes, and transcription factors including peroxisome proliferator-activated receptors alpha and gamma (PPARα, PPARγ), retinoid X receptors (RXR), liver X receptors alpha and beta (LXRα, LXRβ), hepatic nuclear factor-4 alpha (HNRα), nuclear factor erythroid-derived 2-like 2 (NRF2), and sterol regulatory binding proteins 1 and 2 SREBP-1/SREBP-2 leading to the regulation of genes playing a major role in carbohydrates, fatty acids, triacylglycerides (TG), and cholesterol transport and metabolism (reviewed in [14, 70, 132–136]).

PUFA reduce expression of genes involved in FA and cholesterol synthesis by binding and inactivating UBXD8 leading to inhibition of proteolytic processing of SREBP-1 [137]. PUFA also reduce the expression of L-type pyruvate kinase involved in glycolysis most likely by inhibiting nuclear translocation of MAX-like protein X (MLX)-carbohydrate-responsive element-binding protein (ChREBP) (reviewed in [133]). PUFA but not saturated and monounsaturated FA suppress sterol regulatory element-binding protein 1c (SREBP-1c) expression via activation of farnesoid X receptor (FXR) and suppression of two liver X receptor (LXR) response elements in its promoter leading to inhibition of lipogenesis [138]. On the other hand, PUFA increase peroxisome proliferator-activated receptor alpha (PPARα) crucial for lipid degradation and catabolism [138]. Interestingly, docosahexaenoic acid (DHA) has been reported to be a ligand of the retinoid X receptor (RXR) which regulates gene expression in concert with PPARs [133]. Testicular orphan nuclear receptor 4 (TL4) induces CD36 mRNA and protein expression via the same regulatory site as PPARs in the CD36 promoter, what can be enhanced by PUFA and their metabolites [139]. The same PPAR response element in the CD36 promoter mediates activation in response to oxidized low-density lipoproteins (oxLDL) leading to atherosclerotic foam cell formation, what can be prevented by vitamin E by antagonizing the oxLDL/CD36/PKB/PPARγ signaling pathway [140–142]. In human monocytic THP-1 cells, PUFA (LA, ALA, and DHA) but not of palmitic acid (PA) have anti-inflammatory effects in response to lipopolysaccharides (LPS) by stimulating PPARγ and by inhibiting NF-κB, thus regulating genes such as IL-6, IL-1β, and TNFα [143]. In the skeletal muscle, PUFA are known to improve insulin-mediated glucose metabolism in the insulin-resistant state possibly by stimulating the Akt/PKB-mTOR-S6 K1 pathway (reviewed in [10, 144]). In similar manner, the phosphorylated form of vitamin E (αTP) may alleviate insulin resistance by activating the CD36/PI3K/Akt-signaling pathway via hTAP/SEC14L2-mediated lipid exchange [37, 38, 120].

In human HepG2 cells, cooperative inhibition of UDP-glucuronosyltransferase (UGT1A1) mRNA was only observed upon combined treatment with DHA with vitamin E, whereas SREBP-1 and stearoyl-CoA desaturase (SCD) were inhibited by DHA alone but not by vitamin E [145]. In a study with chicken fed with high n-3 PUFA (linseed oil), administration of either αT, γT, or an αT/γT mixture differently affected gene expression, with γT affecting genes involved in inflammatory processes and the immune response and αT affecting genes involved in lipid and cholesterol metabolism, whereas the mixture of αT/γT affecting genes relevant for fatty acid oxidation and glucose sparing [146].

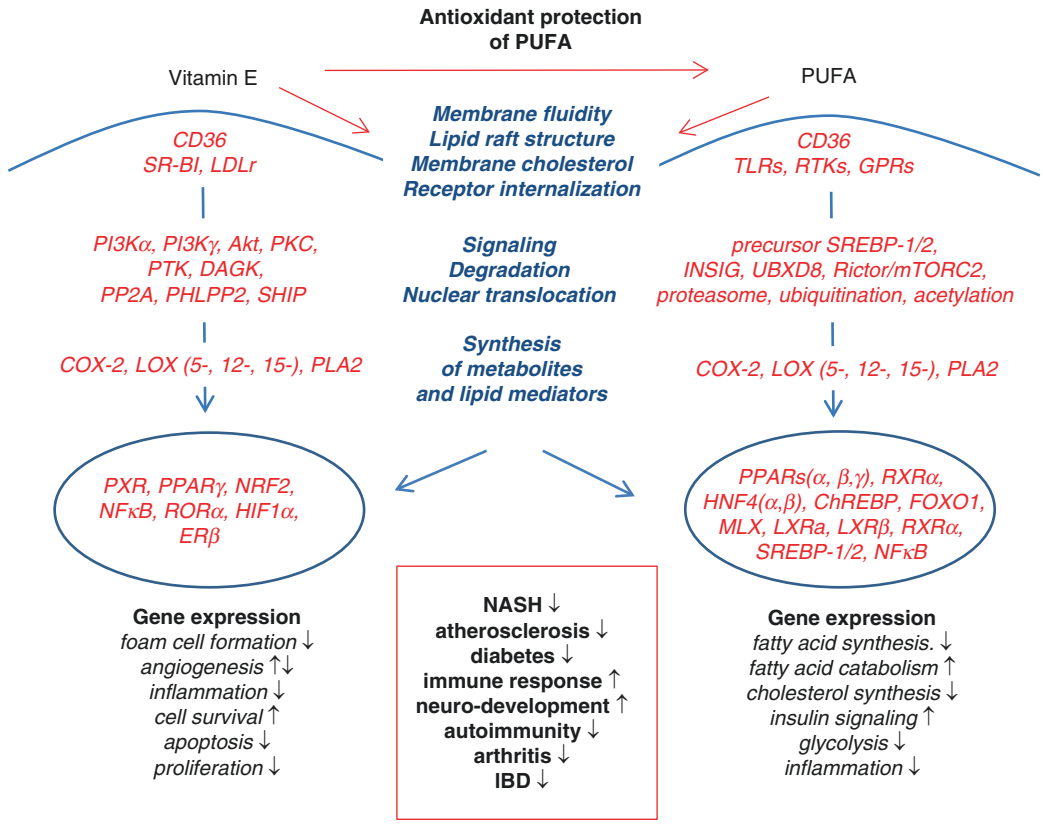

Fig. 11.2 Modulation of signal transduction and gene expression by vitamin E and PUFA**.** (**Left**) Vitamin E is taken up into cells via scavenger receptors CD36, SR-BI, and LDLr and changes the fluidity and structure of the plasma membrane and of microdomains (lipid rafts/caveolae and non-rafts), or it is converted to vitamin E metabolites able to act as active lipid mediators (e.g., α-tocopheryl phosphate, long-chain carboxyethylhydroxychromans (LC-CEHC), and CEHC). Vitamin E modulates the activity of several receptors (CD36) and signal transduction enzymes, including phosphatidylinositol 3-kinase alpha and gamma (PI3Kα and PI3Kγ); Akt/PKB; protein tyrosine kinases (PTKs); diacylglycerol kinase (DAGK); protein phosphatase 2a (PP2A); pleckstrin homology (PH) domain leucine-rich repeat protein phosphatase, isoform 1 (PHLPP1); 5′- and 3′-inositol polyphosphatase (SHIP); cyclooxygenase-2 (COX-2); 5-, 12-, and 15-lipoxygenases (5-, 12-, 15-LOX, respectively); and phospholipase 2 (PLA2). These enzymes and vitamin E modulate gene expression via pregnane X receptor (PXR), peroxisome proliferator-activated receptors alpha and gamma (PPARα, PPARγ), nuclear factor erythroid-derived 2-like 2 (NRF2), nuclear factor kappa B (NFκB), RAR-related orphan receptor alpha (RORα), hypoxia-inducible factor 1 alpha (Hif1α), and estrogen receptor alpha (ERα). Overall, vitamin E has been implicated in regulating genes involved in the prevention of atherosclerotic foam cell formation, lipid homeostasis, immune response, angiogenesis, inflammation, apoptosis/cell survival, and proliferation. (**Right**) PUFA are taken up into cells involving LDLr, CD36, FABPpm, FATP, and in particular as lysophosphatidylcholine-PUFA (LPC-PUFA) by Mfsd2A and change the fluidity and structure of the plasma membrane (lipid rafts/caveolae and non-rafts). PUFA can be converted by a number of enzymes (desaturases, elongases, P450 epoxygenases, cyclooxygenases, lipoxygenases) to active lipid mediators (e.g., arachidonic acid (AA), linoleic acid (LA), alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA), and their derivatives such as leukotrienes, prostaglandins, thromboxanes, lipoxins, resolvins, protectins, and others). These events lead to the modulation of signaling receptors such as toll-like receptors (TLRs 2, 3) and G-coupled receptors (GPR40, GPR41, GPR43, GPR83, GPR119, GPR120) and of a number of enzymes involved in signal transduction (e.g., Src-family kinases (Fyn, c-Yes), PKA, PKC, PLC, PI3K, COX-1, COX-2, Akt/PKB-mTOR-S6 K1, adenylate cyclases), leading to changes in downstream signaling such as by NFκB and Jun N-terminal kinase (JNK). When compared to saturated fatty acids (SFA) and n-6 PUFA, signaling by n-3 PUFA occurs often in the opposite direction. PUFA modulate gene expression either by influencing phosphorylation, ubiquitination, acetylation, and nuclear translocation of specific transcription factors (SREBP-1/SREBP-2, NFκB, ChREBP, max-like protein X (MLX), FOXO1) or by directly interacting with them (PPARα, PPARβ, PPARγ, RXRα, HNF4α, HNFβ, farnesoid X receptor (FXR), and liver X receptors alpha and beta (LXRα, LXRβ)). Vitamin E analogues and metabolites can modulate the synthesis of PUFA-derived lipid mediators and therefore indirectly influence signal transduction and gene expression. Overall, n-3 PUFA have been implicated in lowering fatty acids (FA), triacylglycerides (TG) and cholesterol synthesis, increasing fatty acid catabolism, glycolysis, inflammation, and modulation of embryogenesis and disease development

Table 11.2 PUFA and vitamin E modulate similar transcription factors (examples) (see text for references)

Transcription factors	PUFA and metabolites	Vitamin E and metabolites	Main regulatory pathways affected
SREBP-1c, SREBP-2	Inhibition/activation	Inhibition	Lipid metabolism
ChREBP	Inhibition		Carbohydrate metabolism
LXRα, LXRβ	Inhibition		Lipid metabolism
PPARα	Direct activation	Activation	Lipid metabolism
PPARγ	Direct activation	Activation	Inflammation
TL4	Activation		Lipid metabolism
RXR	Direct activation		Via heterodimer with PPARs, RARs, and LXRs
NFκB	Direct inhibition	Inhibition	Inflammation
Estrogen receptor (ER)	Inhibition	Direct activation	Lipid metabolism, development
HNF-4α	Inhibition		Lipid metabolism
PXR	Activation	Direct activation	Metabolism
NRF2	Activation	Activation	Stress response

In epidemiological studies n-3 PUFA had benefits on cardiovascular health, and intervention studies confirmed primary and secondary prevention of cardiovascular disease [6, 80]. At the mechanistic level, the cardioprotective effects of n-3 PUFA involved anti-inflammation, pro-resolving lipid mediators, modulation of cardiac ion channels, reduction of triglycerides (TG), influence on membrane microdomains and downstream cell signaling pathways, and antithrombotic and antiarrhythmic effects. At the molecular level, PUFA inhibit inflammatory signaling pathways (NFκB) and downregulate fatty acid (FA) synthesis gene expression (SREBP-1c) and upregulate gene expression involved in FA oxidation (PPARα) [147] and of FA transport (CD36) [74, 75, 79]. Interestingly, similar regulatory effects on transcription factors and lipid homeostasis during inflammation and atherosclerosis have been observed for vitamin E [148–150], and they are more pronounced for vitamin E phosphate in models of hypercholesterolemic rabbits and in apoE$^{-/-}$ knockout mice, most likely as a result of reduction of CD36 scavenger receptor overexpression and of downregulation of several pro-inflammatory cytokines (IL-1β, IL-6, IL-8, PAI1, TNFα, and CRP) [151–153], and it is largely unknown whether and to what degree protective effects of vitamin E on PUFA are involved or whether they are possibly due to higher PUFA uptake in the presence of vitamin E phosphate [77] or to generation of higher levels of LPC-PUFA [88].

In preclinical disease models, in observational studies, and in clinical intervention studies, vitamin E has anti-inflammatory and immune-modulatory activities with preventive effects against cardiovascular disease, nonalcoholic steatohepatitis (NASH), asthma, allergies and airway inflammation, neurodegenerative disease, and cancer (reviewed in [6, 80]). It appears possible that the regulatory effects of vitamin E supplementation on disease prevention become more evident when excess PUFA consumption leads to vitamin E depletion, and it remains to be investigated whether in situations of vitamin E insufficiency, excess PUFA supplementation has negative health effects [21, 22]. Pregnant rats obtaining fish oil had lower levels of vitamin E in the fetal brains [154]. Accordingly, in a randomized double-blind human study, a smaller increase of plasma vitamin E concentrations in the presence of concomitantly consumed fish oil was observed that possibly led to the dampened immunoenhancing effect of vitamin E on T-cell function in the elderly [155]. In contrast, in the transgenic fat-1 mouse model, which expressed a *Caenorhabditis elegans* fatty acid desaturase that converts n-6 into n-3 fatty acids and therefore has sufficient n-3 PUFA tissue levels, the induction of nonalcoholic steatohepatitis (NASH), upon feeding a methionine- and choline-deficient diet, was accelerated most likely as a result of increased lipid peroxidation, although other regulatory effects cannot be excluded

at present time [156]. It remains to be investigated whether vitamin E has been depleted in this study and whether supplementation of vitamin E can prevent or reduce oxidative damage, inflammation, aberrant signal transduction and gene expression, and the development of NASH as suggested from animal and human studies [157–160].

Conclusion

Vitamin E and PUFA are essential molecules for humans since they cannot be synthesized from precursors and need to be taken up from the diet. Since both molecules are insoluble in water, they share similar and/or partially overlapping pathways for their uptake, distribution, metabolism, and molecular action as structural membrane components and active lipid mediators. When there is sufficient vitamin E in the diet, the protective function of vitamin E against free radicals in the lipid phase protects PUFA against peroxidation and depletion, whereas excess PUFA in the diet may lead to depletion of vitamin E. Thus, PUFA and vitamin E form an interdependent chemical and biological pair of cellular lipid mediators that interact and influence each other with regulatory consequences on physiology and pathophysiology of a number of diseases. Defining the molecular interactions of PUFA and vitamin E and the consequent regulatory cellular effects in signal transduction and gene expression are emerging topics for future research with importance not only for disease prevention and the design of dietary supplementation studies but also for the determination of adequate dietary reference values.

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Part II
A Global View on Vitamin E Intakes and Status

Chapter 12
The Challenge of Defining Daily Intake Recommendations: Vitamin E and Polyunsaturated Fatty Acids

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Keywords Vitamin E recommendations, PUFA, Omega-3 LC-PUFA, α-tocopherol, Requirement

Key Points

- Dietary intake recommendations for the essential micronutrient vitamin E established in many countries vary, and it appears to be still a challenge to define these despite the wealth of data published.
- It is noteworthy that the reports from the leading agencies like the European Food Safety Authority, the Nordic Nutrition Recommendations, and the Institute of Medicine, following comprehensive reviews, arrive at quite varying vitamin E intake recommendations as they use different approaches and (bio)markers.
- However, it is of interest that they actually all appreciate the fundamental role of the lipid-soluble essential micronutrient vitamin E in protecting PUFA from oxidation.
- Given the proposed health benefits of omega-3 PUFA and the recommendations to increase in particular the long-chain PUFA, the evidence on the interaction of vitamin E and the amount needed to protect PUFA from being oxidized is reviewed.

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_12

Scope

For the essential micronutrient vitamin E, dietary intake recommendations are established in many countries; however, the daily intake recommendations vary (Table 12.1) [1]. The most recent reports on vitamin E intake recommendations are the report on Scientific Opinion on Dietary Reference Values for vitamin E as a-tocopherol from the European Food Safety Authority (EFSA) [2] and the Nordic Nutrition Recommendations (NNR) [3] and the report on Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium and Carotenoids from the Institute of Medicine (IOM) [4]. These reports all provide comprehensive reviews on the literature relevant to define vitamin E recommendations, and for detailed information we refer to these as it would go beyond the scope of this chapter to critically appreciate the wealth of these data. The body of evidence on the biological role of vitamin E in humans is huge, though also rather heterogeneous in design and outcomes, so it may not come as a surprise that the committees involved to establish dietary intake recommendations for vitamin E differ in their approach how to apply these data. The purposes of this chapter are to (i) give a brief summary on the current approaches used to define vitamin E intake recommendations and the resulting recommendations and (ii) examine the possible value of evidence related to the fundamental biological role of vitamin E in humans to define intake requirements which is as the key lipid-soluble antioxidant to protect polyunsaturated fatty acids (PUFAs) in cell membranes from being oxidized in all tissues.

Table 12.1 Overview of dietary reference values for vitamin E for adults

	NCM (2014)[a,b]	D-A-CH (2013)[c]	WHO/FAO (2004)[d]	Afssa (2001)	IOM (2000)[a,b]	SCF (1993)[e,f]	NL (1992)[b,f]	UK (1991)[g]
Age (years)	≥18	19–<25	≥19	20–74	≥19–50	≥18	≥18	>18
Men (mg/day)	10	15	10	12	15	0.4	0.67	>4
Women (mg/day)	8	12	7.5	12	15	0.4	0.67	>3
Age (years)		25–<51						
Men (mg/day)		14						
Women (mg/day)		12						
Age (years)		51–<65						
Men (mg/day)		13						
Women (mg/day)		12						
Age (years)		≥65		≥75				
Men (mg/day)		12		20–50				
Women (mg/day)		11		20–50				

DRVs in α-tocopherol equivalents except for IOM.
NL Netherlands Food and Nutrition Council, *NCM* Nordic Council of Ministers
[a]Applicable to RRR-, RSR-, RRS- and RSS-isomers of α-tocopherol only
[b]PRI
[c]Adequate Intake
[d]Data were insufficient to set PRIs; the indicated figures represent the 'best estimates of requirements' (WHO/FAO, 2004)
[e]'vitamin E requirement'
[f]mg α-TE/g PUFA
[g]'Safe' intakes

What Is Vitamin E?

The term vitamin E used to include eight compounds found in nature (α-, β-, γ-, δ-tocopherol and α-, β-, γ-, δ-tocotrienol) all of which possess antioxidative activity though at different degrees. New scientific insights on the metabolic fate of the different vitamin E forms led the IOM [4] in their report in the year 2000 to limit the bioactive form that is recognized to meet human vitamin E requirements to natural RRR-α-tocopherol. Blood α-tocopherol concentrations are maintained by the preferential binding of α-tocopherol to the α-tocopherol transfer protein (α-TTP) compared to the other tocopherols or tocotrienols. The natural RRR-α-tocopherol has three chiral centers which allows potentially for eight stereoisomers (see Chap. 4 and 9). According to this concept, the synthetically produced all-rac-α-tocopherol which contains the eight stereoisomers of α-tocopherol in equal amounts has only 50% relative activity of RRR-α-tocopherol, because only the 2R-α-tocopherol isomers are relevant to meet vitamin E requirements while the 2S-α-tocopherol isomers are considered to have no relevant biological vitamin E activity due to the lower affinity to the α-tocopherol transport protein (α-TTP). This new definition of the physiologically active vitamin E form as proposed by IOM in the year 2000 has meanwhile been adopted by several other agencies (i.e., EFSA [2], NNR [3], Japan, WHO/FAO [1], though it is worthwhile to mention that, for instance, Australia and New Zealand have not committed to that concept yet [1].

Which Marker to Choose to Set Vitamin E Recommendations?

There is a general agreement that vitamin E is a powerful, chain-breaking antioxidant, which is localized due to its lipophilic nature in lipid compartments such as cell membranes. There it prevents the peroxidation of lipids [5] and thus preserves the cellular membrane integrity. Vitamin E also plays a crucial role in the stability of erythrocytes and the conductivity of central and peripheral nerves [6, 7]. It prevents hemolytic anemia and neurological symptoms of vitamin E deficiency (e. g., ataxia, peripheral neuropathy, myopathy, pigmented retinopathy). This essential role of vitamin E as an antioxidant in the human body has recently been underscored by the approval of a 13.1 European Food Safety Authority (EFSA) health claim [8]. So it appears logical when evaluating the evidence on which to base dietary intake recommendations for vitamin E that the antioxidant property in particular in lipophilic compartments should be leading. In addition, the reports of IOM [4], EFSA [2], and NNR [3] comprehensively reviewed a number of other options on the biological role of vitamin E as possible biomarkers like epidemiological outcomes or clinical endpoints for CVD, mental diseases, diabetes, cataract, etc. and discussed the value of these data as marker to establish dietary intake recommendations. The approach chosen and the rationale used to define those recommendations of three leading agencies, the IOM, EFSA, and NNR, are briefly summarized below.

Institute of Medicine (IOM)

The IOM, which published their report in 2000, built their recommendation on the antioxidative property of vitamin E and chose as the respective biomarker hydrogen peroxide-induced hemolysis.

In their report they conclude "it is recognized that there are great uncertainties in the data utilized to set the α-tocopherol requirements. However, in the absence of other scientifically sound data, hydrogen peroxide-induced hemolysis is the best marker at the present time." This value is derived from the amount needed to prevent peroxide-induced hemolysis in vitamin E-deficient subjects, which was determined

from a limited number of studies performed and published in the 1950s and 1960s to occur at 12 μmol/L serum α-tocopherol [4]: In 4 subjects fed with a vitamin E-deficient diet for more than 6 years, who had plasma tocopherol levels around 6 umol/L, peroxide-induced hemolysis was at about 80%, whereas in 6 healthy controls, the subject with the lowest plasma tocopherol level at which hemolysis was below 12% (which is considered normal) was 12 umol/L. Using the limit of 12 umol/L plasma tocopherol as the criterion for the estimated vitamin E requirement, and assuming that intakes of vitamin E in that low range are reasonably linearly related to plasma tocopherol levels, an intake of 12 mg vitamin E was chosen as the estimated average requirement (EAR) and became the basis to calculate the RDA.

Based on these data, dating back more than 50 years in a group of 10 subjects (four vitamin E depleted, six healthy controls on a regular diet) using the peroxide-induced hemolysis assay as a biomarker, the RDA was calculated as 15 mg a-tocopherol for adult men and women in the USA.

European Food Safety Authority (EFSA)

The EFSA Panel, which published their report in 2015, arrived at the conclusion that the published data which were at the time in the public domain available were not sufficient to derive neither "average requirement," which is equivalent to the EAR, nor to define "population reference intake (PRI)," which is termed RDA in the IOM publication.

The argumentation of the committee as taken from the abstract of their publication goes

> The Panel considers that there is, at present, insufficient data on markers of α-tocopherol intake/status/function (e.g., plasma/serum α-tocopherol concentration, hydrogen peroxide-induced heamolysis, urinary α-CEHC excretion, markers of oxidative damage) to derive the requirement for α-tocopherol. The Panel notes the lack of convergence of the values that would be derived from the use of data on markers of α-tocopherol intake/status or on α-tocopherol kinetics and body pools. The Panel considers that available data on markers of α-tocopherol intake/status/function, on α-tocopherol kinetics and body pools, on the relationship between PUFA intake and α-tocopherol intake/requirement can be used neither on their own nor in combination to derive the requirement for α-tocopherol in adults. The Panel considers that data on the relationship between vitamin E (unspecified form) or α-tocopherol intake and health consequences are inconsistent or limited and cannot be used to derive the requirement for α-tocopherol. … The Panel considers that Average Requirements (ARs) and Population Reference Intakes (PRIs) cannot be set for α-tocopherol. Therefore, the EFSA Panel proposes to set Adequate Intakes (AI) for a-tocopherol.

The assessment of the AI, which is the current intakes in apparently healthy populations, included food consumption data from 13 dietary surveys from 9 countries (Finland, France, Germany, Ireland, Italy, Latvia, the Netherlands, Sweden, and the UK). In adults (≥18 years), the average α-tocopherol intakes ranged between 7.8 and 12.5 mg/day in women and between 8.2 and 16 mg/day in men. The EFSA Panel proposes an AI of 13 mg α-tocopherol per day for men and 11 mg α-tocopherol per day for women, respectively.

Nordic Nutrition Recommendations (NNR)

The NNR [3] concludes that in the absence of signs of vitamin E inadequacy in the general Nordic population, and because no new strong evidence supporting changes since the NNR report published in 2004 have emerged, the recommended intakes (RI) of vitamin E remain unchanged, which is for adults 8 a-TE/d for women and 10 a-TE for men. The markers which were discussed and considered while defining the RI for vitamin E include a-tocopherol kinetic studies, vitamin E plasma concentrations, and role of vitamin E to prevent oxidation of tissue PUFA. Based on a 0.4 a-TE/g PUFA ratio, it was concluded that the estimated average requirement (AR) is 5 and 6 mg a-tocopherol for women and men, respectively.

The reports of the IOM, EFSA, and NNR in one way or another all do appreciate the fundamental role of the lipid-soluble essential micronutrient vitamin E in protecting PUFA from oxidation. Actually, the NNR report defined the AR (equivalent to the EAR in IOM terminology) on this function of vitamin E. However, they also raise a number of questions on how to use these data, and the IOM and the EFSA did not value the evidence on the vitamin E/PUFA ratio to be appropriate to be considered for dietary intake recommendations. However, given the proposed health benefits of omega-3 PUFA and the recommendations to increase in particular the long-chain PUFA, we think it is important to revisit the evidence on the interaction of vitamin E and the amount needed to protect PUFA from being oxidized.

Role of Vitamin E in Relation to PUFA Function

Vitamin E (α-tocopherol) is considered as the major essential lipophilic antioxidant in humans. The antioxidant function of vitamin E is critical for protecting lipoproteins, polyunsaturated fatty acids (PUFAs), and cellular and intracellular membranes from damage. Recently the role of vitamin E as an antioxidant has been evaluated by the European Food Safety Authority expert panel, which concluded that the scientific evidence indicates that "Vitamin E contributes to the protection of cell constituents from oxidative damage" [8]. In vivo and in vitro studies show that vitamin E functions as a chain-breaking antioxidant acting to protect unsaturated lipids from peroxidation by scavenging peroxyl radicals [9]. RRR-α-tocopherol is the isoform which is preferentially absorbed and maintained in the human body [10, 11]. The antioxidant defense system keeps levels of oxidants and antioxidants in balance protecting the body from the effects of oxidative stress [12]. The endogenous antioxidant defense system is not sufficiently efficient on its own to protect human tissues from oxidative damage and must be supported by small molecule antioxidants derived from the diet. Alpha-tocopherol as the major essential lipid-soluble antioxidant in human supports the body's antioxidant defense system. The antioxidant function of vitamin E is especially critical for protecting tissue PUFA from oxidative damage. Animal experiments have shown that increasing the degree of fatty acid unsaturation in tissues increases the peroxidability of the lipids and reduces the time required to develop symptoms of vitamin E deficiency. Thus, the vitamin E requirement will increase with an increase in PUFA consumption and with the degree of unsaturation of the PUFA in the diet [13–15].

Polyunsaturated fatty acids (PUFAs) are classified into two major families the omega-3 and omega-6 fatty acids. Linoleic and alpha-linoleic acids are the parent fatty acids of, respectively, the omega-6 series and the omega-3 series and are both considered as essential as they cannot be synthesized de novo by humans. The omega-6 long-chain polyunsaturated fatty acid (LC-PUFA), arachidonic acid (AA), is synthesized from linoleic acid, and the omega-3 LC-PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are synthesized from alpha-linolenic acid. However, the conversion rates are low, especially for DHA. Thus, the LC-PUFAs are considered as conditionally essential if the endogenous production from the parent fatty acids is inefficient and the limited endogenous production is insufficient to meet nutritional requirements. The omega-6 (AA) and omega-3 (DHA, EPA) long-chain PUFAs (LC-PUFAs) are important structural components of cell membranes, affecting the physicochemical properties of the membranes (fluidity, permeability) and the activity of membrane-bound enzymes, and play an important role in cell signaling, cell division, gene expression, and lipid mediator production [16–18]. LC-PUFA plays important roles in human physiology notably in child development (brain, visual, and immune function) and in cardiovascular, mental, and physical health throughout the life course. Population studies still consistently show that a low n-3 PUFA (EPA and DHA) status is associated with an increased risk of a cardiovascular diseases and cardiac death [19–23]. Moreover, adequate LC-PUFA intake during pregnancy is also important for fetal development and long-term development of children [24].

However, the intake of omega-3 LC-PUFA is low and largely below recommended dietary intake. A worldwide review of nutritional surveys showed that only 45 (representing only 18.9% of the world population) of 266 countries achieved a recommended intake of ≥250 mg/d [25]. Moreover, the current vitamin E intakes are below-recommended intakes in more than 90% of North American as well as in some European Countries [26], and at the same time, people are being encouraged to increase intake of PUFAs, especially those with high degrees of unsaturation, because of their reported health benefits. Thus, the ratio of vitamin E to PUFA in the human diet appears to become more critical and requires a deeper examination.

Impact of Dietary PUFA on Vitamin E Levels

Preclinical Data

In tissues α-tocopherol is incorporated into cell membranes and may affect the membrane's stability and fluidity. Studies in model membranes indicate that vitamin E accumulates in DHA-rich, rather unstructured domains and such stabilize the membrane and protect DHA from oxidative damage [27]. Biophysical experiments indicate that in view of the localization of α-tocopherol in the membrane, the α-tocopherol antioxidant activity occurs at the membrane surface [28]. Lipophilicity and membrane localization of vitamin E explain its antioxidant activity.

The presence of vitamin E is of key importance in cellular membranes rich in highly unsaturated fatty acids such as docosahexaenoic acid (DHA) and arachidonic acid (AA) which are found in high concentrations in the brain, the retina, and some other locations. For example, in α-TTP null mice fed a vitamin E-deficient diet, retinal structure is altered, and lipid peroxidation is enhanced, while the concentration of DHA, the most abundant PUFA in the retina, is decreased [29]. In zebra fish, a model organism for lipid metabolism, vitamin E deficiency led to a decrease in the content of highly unsaturated fatty acids (DHA, AA) as they are consumed by peroxidative processes and impaired cognitive function [30–34]. Those studies support the role of vitamin E in protecting PUFAs from damage to maintain optimal cellular function.

In several animal experiments, diets with various vitamin E/PUFA ratios induced vitamin E deficiency or relieved it [35]. The review of those animal studies concluded that a vitamin E/PUFA ratio of 0.6 mg RRR-α-tocopherol for each g of PUFA is necessary to protect against vitamin E deficiency [35]. A study in rhesus monkey estimated that the minimum requirement was slightly more than 0.36 mg RRR-α-tocopherol per g of linoleic acid consumed and that 0.72 mg RRR-α-tocopherol per g of linoleic acid was nutritionally adequate [36]. Moreover, the relation between vitamin E requirements and the degree of unsaturation of PUFAs was evaluated in young rats, fed diets containing PUFA with different degrees of unsaturation, but having a constant total unsaturation in their dietary fat [14]. The study showed that the relative amounts of α-tocopherol required to protect one mole of mono-, di-, tri-, tetra-, penta-, and hexaenoic fatty acids were approximately in the ratios of 0.3:2:3:4:5:6, respectively, which is consistent with the in vitro susceptibility of unsaturated fatty acids to oxidative damage [14].

Human Evidence

Several human studies also have made attempts to relate the vitamin E requirement to the amount of dietary PUFA mainly based on dietary intake of vitamin E and PUFA. Murphy et al. [37] used the data from the Second National Health and Nutrition Examination Survey (NHANES II) and concluded

that the dietary vitamin E/PUFA ratio decreased with increased PUFA intake from 0.94 for individuals with diets low in PUFA (<5 g/day) to 0.44 for individuals with PUFA intakes above 25 g/day. In previous studies a ratio of 0.43 based on the analysis of composite meals [38] and of 0.52 based on an experimental diet was suggested providing an adequate intake of vitamin E [39]. In growing children, a ratio of 0.4 has been found sufficient to maintain plasma vitamin E levels [40]. A ratio of approximately 0.4 mg RRR-α-tocopherol per g of linoleic acid was considered as adequate in a group of young women consuming repetitively a diet rich in linoleic acid over a period of about 9 months [40]. Finally, the Elgin project, conducted between 1953 and 1967, is still considered as the critical set of data to evaluate the vitamin E requirement of humans through a long-term dietary study [41–44]. In this project, the adequacy of vitamin E intake was evaluated by following the plasma α-tocopherol levels over time and measuring the susceptibility of erythrocytes to in vitro peroxide-induced hemolysis as a sign of vitamin E deficiency in subjects consuming diets with various amounts of PUFA. The data showed that a daily intake of 3–4 mg of α-tocopherol was inadequate [41] and a minimal intake of 4–5 mg/day of α-tocopherol was needed in the basal state, even in the absence of dietary PUFAs. Moreover, the study suggested that individuals ingesting large amounts of linoleic acid (>30 g/day) require more than 30 mg/day of α-tocopherol, while 10 mg/day of α-tocopherol may be at the border of inadequacy in individuals ingesting about 4–7 g/day of linoleic acid [43].

Therefore, Horwitt et al. proposed to quantify the vitamin E requirement in humans by adding to the basal minimum α-tocopherol requirement (4–5 mg/day), needed for normal cellular synthesis and retention of PUFA in membranes, a factor that depended on the dietary PUFA intake [43, 44]. The data from the Elgin project suggest that the minimum vitamin E/PUFA ratio to avoid the development of vitamin E deficiency symptoms is between 0.5 and 0.8 mg α-tocopherol per g of PUFA [35, 45]. Thus, taking into consideration those factors, the α-tocopherol requirements in humans range from 10 mg/day to 30 mg/day depending on the amount of PUFAs in the diet and the tissues [43].

Vitamin E Recommendations and PUFA Intake

The physiological requirement of vitamin E as a function of PUFA intake has been reviewed and discussed in several publications [15, 38, 45–48]. Collectively, the preclinical data, the human evidence, and the reviews of the data show that to quantify the vitamin E requirement, two factors should be taken into account: (1) the minimum requirement to allow for basal metabolism, cellular synthesis, and PUFA retention, even in low-PUFA diets, and (2) the additional vitamin E required to protect and metabolize dietary PUFA. Even so various ratios of vitamin E to PUFA have been proposed to calculate the additional vitamin E requirement as a function of the amount of PUFA in the diet, the estimated optimal vitamin E/PUFA ratio seems to be relatively consistent across studies approximatively ranging from 0.4 to 0.6 mg RRR-α-tocopherol/g of PUFA in the diet when considering a diet with a typical content of PUFAs in which the major PUFA is linoleic acid. Thus, considering a minimum basal requirement of at least 4 mg RRR-α-tocopherol as suggested by the human data from the Elgin project and a ratio of 0.5 mg RRR-α-tocopherol per g of PUFA in the middle of the estimated range (0.4–0.6 mg RRR-α-tocopherol/ g of PUFA), the following formula may reasonably be used to calculate the vitamin E requirement: vitamin E requirement = 4 + (0.5 × amount of PUFA in the diet in g). This formula was developed using data from studies in which linoleic acid was the major PUFA in the diet, and it doesn't take into account the fact that the vitamin E requirement also depends on the degree of unsaturation of the dietary PUFAs [14]. Therefore, it was proposed to estimate the dietary vitamin E requirement by taking the relative vitamin E requirement for individual PUFAs into account [15]. Table 12.2 shows the vitamin E requirement for individual PUFAs using a vitamin E/linoleic acid ratio of 0.5 mg α-TE/g of linoleic acid in the diet and extrapolating the relative vitamin E requirement for the PUFAs with different degrees of unsaturation by using the relative ratios of 0.3, 2, 3, 4,

Table 12.2 Vitamin E requirements for different unsaturated fatty acids found in human diets

Number of double bonds	Fatty acid	Vitamin E requirement (mg/g fatty acid)
1	Oleic acid	0.075
2	Linoleic acid	0.5
3	α-Linolenic acid	0.75
4	Arachidonic acid	1.0
5	Eicosapentaenoic acid (EPA)	1.25
6	Docosahexaenoic acid (DHA)	1.5

Table 12.3 Estimated vitamin E requirement for typical ranges of unsaturated fatty acid intake in western diets

Unsaturated fatty acid	Typical intake range (g/day)	Vitamin E requirement (mg/g fatty acid)
Oleic acid	20–30	1.5–2.3
Linoleic acid	12–21	6.0–10.5
α-Linolenic acid	1.1–2.6	0.8–2.0
Arachidonic acid	0.1–0.3	0.1–0.3
EPA + DHA	0.1–0.5	0.1–0.7
Vitamin E requirement related to unsaturated fatty acid intake	8.5–15.7 mg/day	
Total vitamin E requirement	12.5–20 mg/day[a]	

[a]The estimation is based on the vitamin E requirement related to unsaturated fatty acid intake plus a basal requirement of 4 mg of RRR-α-tocopherol per day

5, and 6 for, respectively, mono-, di-, tri-, tetra-, penta-, and hexaenoic fatty acids. The vitamin E requirement is then calculated using the following formula, in which the minimal basal requirement is 4 mg of RRR-α-tocopherol and Mn is the amount of dietary PUFA with n double bond in grams:

$$\text{Vitamin E requirement}\left(\text{mg TE}\right) = 4 + 0.075\text{M1} + 0.5\text{M2} + 0.75\text{M3} + 1\text{M4} + 1.25\text{M5} + 1.5\text{M6}$$

(M1–M6 are expressed in gram of the respective unsaturated fatty acids).

Table 12.3 shows the calculated vitamin E requirements for typical intake range of the various dietary PUFAs. The unsaturated fatty acid intake ranges indicated in Table 12.3 were estimated from reviews providing a global perspective on dietary fatty acid intake [25, 49, 50]. In a western diet, linoleic acid ranges from about 12 to about 21 g/day when considering an energy intake ranging from 1800 to 3250 Kcal/day, and it is the major PUFA in the diet and the principal determinant of the vitamin E requirement. The calculated total vitamin E requirement using the above formula ranges from 12 to 20 mg of RRR-α-tocopherol/day.

The Institute of Medicine (IOM) and the DACH countries (Germany, Austria, Switzerland) have recognized the role of vitamin E in protecting PUFAs from being oxidized and indicated that the amount of vitamin E needed to keep PUFAs functional in cell membranes is related to the intake of PUFAs [11, 51]. However, even so there are only small differences between the IOM and the DACH vitamin E reference values, the two expert groups used two different methodological approaches to propose recommendations for the dietary vitamin E intake (Table 12.4).The IOM based their vitamin E recommendation on the prevention of deficiency symptoms, using the sensitivity of erythrocytes to hemolysis as a sign of deficiency. They estimated that individuals with a plasma concentration of at least 12 μmol/L of α-tocopherol have a low percent of erythrocyte hemolysis and that a plasma α-tocopherol level of 12 μmol/L corresponds to an intake of about 12 mg/day. Therefore, they concluded that the estimated average requirement (EAR) for vitamin E is 12 mg/day of α-tocopherol to

Table 12.4 Estimated daily vitamin E intake (mg) as reported in the IOM and DACH recommendations

Age range	Estimated daily intake based on EAR according to IOM	Estimated daily according to DACH[a]
0–3 months	–	3
0–6 months	4[b]	–
4–12 months	–	4
7–12 months	5[b]	–
1–3 years	5	Male: 6, Female: 5
4–6 years	–	8
4–8 years	6	–
7–9 years	–	Male: 10, Female: 9
9–13 years	9	–
10–12 years	–	Male: 13, Female: 11
13–14 years	–	Male: 14, Female: 12
14–18 years	12	–
15–18 years	–	Male: 15, Female: 12
>19 years	12	–
19–24 years	–	Male: 15, Female: 12
25–50 years	–	Male: 14, Female: 12
51–64 years	–	Male: 13, Female: 12
>65 years	–	Male: 12, Female: 11
Pregnant women	12	13
Lactating women	16	17

EAR estimated average requirement, *IOM* Institute of Medicine
[a]Estimated intake based on adequate intake in a diet principally consisting of human milk
[b]Equivalent to EAR

prevent erythrocyte hemolysis and a recommended dietary allowance (RDA) of 15 mg/day of α-tocopherol for both men and women was set from the EAR plus twice the coefficient of variation (10%) to take into account the individual needs. The DACH recommendations estimated the vitamin E requirement by taking into account a basal vitamin E requirement plus an additional requirement based on the dietary intake of PUFAs using the above described methodology. In the formula, a basal vitamin E requirement of 4 mg/day and a ratio of 0.4 mg of α-tocopherol per g of dietary linoleic acid were used to determine the ratios between vitamin E and dietary PUFAs with different degrees of unsaturation as proposed by Witting and Horwitt [14, 40, 43]. They concluded that based on a typical dietary intake of PUFAs which differs between women and men due to difference in energy intake, the dietary vitamin E requirements for adult women and men were set to 12 and 15 mg/day, respectively. Thus, despite of using two different methods, the IOM and the DACH proposed vitamin E reference values are very similar.

In summary, a number of studies showed that the vitamin E requirement is related to the dietary intake of PUFAs and that to quantify the vitamin E requirement, a basal vitamin E requirement plus an additional vitamin E requirement for dietary PUFAs might be considered. The preclinical and human evidence indicates that a minimal basal requirement of 4–5 mg/day of RRR-α-tocopherol is required even when the diet is very low in PUFAs. There has been no consensus on the exact vitamin E/PUFA ratio to determine the vitamin requirement since a precise vitamin E/PUFA ratio may not be applicable to all types of diet and health status. However, the vitamin E requirement increases with an increase in PUFA consumption and with the degree of unsaturation of the PUFA in the diet. Thus, the published human data indicate that the additional vitamin E requirement ranges from 0.4 to 0.6 mg RRR-α-tocopherol/g of PUFA in the diet, for a diet with an average content of PUFAs in which linoleic acid is the main dietary PUFA. Moreover, animal studies show that for fatty acids with a higher degree of unsaturation, the vitamin E requirement increases almost linearly with the degree of unsatu-

ration of the PUFA in the relative ratios of 0.3, 2, 3, 4, 5, and 6 for, respectively, mono-, di-, tri-, tetra-, penta-, and hexaenoic fatty acids. All together the animal and the human data show that assuming a typical intake of dietary PUFA, the estimated requirement for vitamin E ranges from 12 to 20 mg/day.

Conclusions

When reviewing and comparing the approaches used by different agencies in different continents to define dietary intake recommendations for vitamin E, it becomes obvious that despite all the scientific progress, new insights on a molecular level, and numerous human studies which generated a wealth of data, it does remain a challenge to settle on dietary intake recommendations for this essential micronutrient.

The reports of the agencies we have chosen to look at (IOM, EFSA, NNR) for the purpose of this matter did a great effort in reviewing in depth the tremendous amount of literature available as can be looked up in their detailed reports (2,3,4). However, following a very stringent process, the critical scientific scrutiny led the scientist involved to different judgments of the relevance of the evidence, and respectively, their recommendations differ. The IOM was in favor of using the results of the peroxide-induced hemolysis assay to define an EAR and calculate a RDA thereof, the NNR defined an AR based on the vitamin E/PUFA ratio to provide a basic protection of PUFA calculated on linoleic acid intake, and the EFSA could not commit to any biomarker and based their recommendations on the current AI. Accordingly, the resulting daily intake recommendations for vitamin E differ quite a bit from 15 mg for adults by the IOM to 8 mg for women and 10 mg for men as proposed by the NNR, and EFSA is in-between with 11mg for women and 13 mg for men, respectively.

A key element to define dietary intake recommendations for vitamin E is obviously the (bio)marker chosen, and all these agencies mentioned struggle to agree on the appropriate marker. This may not only be because of very heterogeneous designs and outcomes of the respective studies or high doses applied in human studies looking at clinical endpoints or inconsistent outcomes – it also may reflect the fact that most of the studies done are not set up to define dietary intake requirements and even if they are they provide only a snapshot information. For instance, if we look at kinetic studies, technically speaking they provide very sound information, but to apply these data it depends quite a bit how the fat absorption in a given individual at a given time point actually is. In this respect, it is clear the challenge will continue; however, answers are needed for public health decisions.

In this chapter we revisited the data which are related to the fundamental role of vitamin E which again is to work as a chain-breaking lipid-soluble antioxidant and by doing so serves as a main element to protect PUFAs in all the different tissues in the human body. It is interesting that the IOM, EFSA, and NNR all do actually appreciate this role of vitamin E. Now what appears to be important is not only to consider the vitamin E/PUFA ratio to provide a basic protection related to linoleic acid intake, which is the main PUFA source in our diet. In addition, animal and human data support the fact that the degree of unsaturation of PUFA, the number of double bonds in other words, should be considered as well in particular as there is now recommendations to increase the intake of long-chain PUFA, because of their reported health benefits. As our understanding of the role of the long-chain PUFAs in human health has increased over the last years and continues to do so, it may be beneficial to the process of setting RDAs to revisit this approach again, and if there are gaps, define them and try to get the respective scientific answers.

In conclusion, in view of the importance of vitamin E and PUFA in human health, it is critical to ensure an adequate intake of vitamin E in humans, particularly when the dietary PUFA intake is increased. Therefore, further research is needed to better clarify the relation and interactions between vitamin E intake and the consumption of highly unsaturated fatty acids, especially omega-3 LC-PUFAs (DHA; EPA).

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Chapter 13
Vitamin E Intake and Serum Levels in the General Population: A Global Perspective

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Keywords α-tocopherol · Intake · Serum · Status · Global · Vitamin E reference values

Key Points

- Dietary intake recommendations for vitamin E vary considerably by different countries and organizations.
- Vitamin E intake values worldwide are often below the recommended levels.
- Rather than relying only on dietary intake reports, serum concentration of α-tocopherol is considered as a promising biomarker to define vitamin E status.
- Measuring vitamin E serum concentration could provide a reference ranging from deficiency to suboptimal and desired levels.
- A substantial part of the global population does not reach the functional deficiency threshold serum level for α-tocopherol.
- Certain health benefits and even some therapeutic effects may be attributed to higher vitamin E serum concentrations.
- Vitamin E supplementation above the RDA may be targeted to selected individuals or high-risk groups, for improving, e.g., immune function, cardiovascular and liver health, and cognitive function.

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_13

Intake Recommendations for Vitamin E

Dietary intake recommendations for vitamin E are set in many countries (Table 13.1); however there is an ongoing need to review, establish, and harmonize dietary vitamin E requirements and daily allowances across populations. The current adult Dietary Reference Values (DRVs) as used in Europe vary considerably, ranging from 3 mg/day (UK) to 15 mg/day (German-speaking countries) for adults, and refer to vitamin E's antioxidant role in preserving the integrity of the cell membrane. The French Food Safety Agency (AFSSA) derived a separate reference value of 20–50 mg/day for adults aged 75 years and over, noting possible benefits of this range in preventing age-related disorders such as cardiovascular disease (CVD) and cancer [1]. Peroxide-induced lysis of erythrocytes in vitamin E-deficient subjects has been shown to be preventable by vitamin E supplementation: Compared to subjects experimentally fed for more than 6 years a diet containing <3 mg/day vitamin E, subjects given an additional 12 mg α-tocopherol per day (a total of 15 mg/day) were sufficient to achieve a serum α-tocopherol concentration of 12 μmol/L, or higher [4, 5], a threshold which prevents red blood cells from hemolysis under high-stress conditions and which the US Institute of Medicine (IOM) used

Table 13.1 Adult reference intake values for vitamin E in α-tocopherol equivalents (except for IOM) by different countries/organizations worldwide

	D-A-CH 2015 (a)	NCM 2014 (b) (c)	WHO/ FAO 2004 (d)	AFSSA 2001	IOM 2000 (b) (c)	SCF 1993 (e)(f)	EFSA 2015 (a) (g)	NL 1992 (c)(f)	DH 1991 (h)
Age (years)	19–<25	≥18	≥19	20–74	≥ 19–50	≥18	≥10	≥18	>18
Men (mg/day)	15	10	10	12	15	0.4	13	0.67	>4
Women (mg/day)	12	8	7.5	12	15	0.4	11	0.67	>3
Age (years)	25–<51								
Men (mg/day)	14								
Women (mg/day)	12								
Age (years)	51–<65								
Men (mg/day)	13								
Women (mg/day)	12								
Age (years)	≥65			≥75					
Men (mg/day)	12			20–50					
Women (mg/day)	11			20–50					

D-A-CH Deutschland (Germany)-Austria-Confoederatio Helvetica (Switzerland), *NCM* Nordic Council of Ministers, *WHO/FAO* World Health Organization/Food and Agriculture Organization of the United Nations, *AFSSA* Agence Française de Sécurité Sanitaire des Aliments, *IOM* US Institute of Medicine of the National Academy of Sciences, *SCF* Scientific Committee on Food, *EFSA* European Food Safety Authority, *NL* Netherlands Food and Nutrition Council, *DH* UK Department of Health [1]. One α-tocopherol equivalent is defined by the biological activity of 1 mg natural α-tocopherol in the resorption-gestation test

as a marker to define vitamin E recommendations for the generally healthy population [6]. The above intake level of 12 mg/day, therefore, has been defined as the Estimated Average Requirement (EAR), the amount needed to satisfy the needs of 50% of healthy people, and became the basis to calculate the RDA, a value that is expected to meet the requirements of 97.5% of healthy individuals. In the USA, the RDA for vitamin E is 15 mg/day α-tocopherol in both men and women 14 years of age and older [6]. When an RDA cannot be determined for a group, a recommended intake value (Adequate Intake, AI) is derived based on observed or experimentally determined approximations of nutrient intake by a group of healthy people that are assumed to be adequate in status. In Europe, on the other hand, the European Food Safety Authority (EFSA) recently concluded that the RDA range for α-tocopherol cannot be derived for adults, infants, and children; therefore it should be replaced by a newly defined AI, depending on age, as follows: men, 13 mg/day; women, 11 mg/day; and infants/children, 5–13 mg/day. However, the Panel also noted uncertainties in the available food composition and consumption data, since most EU food composition databases contain values for vitamin E as α-tocopherol equivalents, as well as the contribution of average α-tocopherol intakes to average α-tocopherol equivalent intakes in these countries [1].

Deriving from its role as a potent lipid-soluble antioxidant, vitamin E has an important role to play in the cell membrane to protect polyunsaturated fatty acids (PUFA) from being oxidized. So, it is conceivable that vitamin E requirements can also be defined in relation to dietary PUFA intake: Based on a recommended intake of 0.4–0.6 mg of α-tocopherol per gram of linoleic acid intake, human vitamin E requirements vary from 15 to 25 mg/day [7, 8]. Therefore, PUFA-dependent vitamin E needs should be consistently reflected in the RDAs (see chapter Raederstorff et al.). The probabilistic framework, and dependency of estimates on best available test indicator data, allows for the Dietary Reference Intakes (DRIs) to be periodically reviewed and updated as new information becomes available. The question which will be addressed in this chapter through a recently conducted systematic review is how the current vitamin E status (as measured by vitamin E intakes and serum levels) of populations in various countries differs.

Methodology of a Global Systematic Literature Review

In order to better understand the current α-tocopherol provision worldwide, a recent paper systematically reviewed existing and reported data on vitamin E intakes and serum concentrations in the general population globally, focusing on age groups and gender in different countries [3]. By using a systematic approach, this review was the first to provide a detailed description about the existing data on vitamin E status worldwide, including developed and developing countries. The main characteristics of the study are summarized in the below text box:

- PubMed/MEDLINE database search for original articles on vitamin E status in the general population
- Articles published between January 1, 2000 and July 30, 2012 were considered
- *Outcome of interest*: Vitamin E status assessed by nutritional intake level and/or by plasma or serum concentration of all vitamin E forms, with particular focus on α-tocopherol
- Subgroups by age, gender, and geographic region, as well as by ethnicity, diet type, smoking, and physical activity status
- *Excluded*: studies on patients, clinical settings, and athletes; clinical trials, case-control studies, case reports, case series, and reviews
- RDA = 15 mg/day and EAR = 12 mg/day applied
  Descriptive statistics presented using frequencies and percentages

Table 13.2 Number of studies on vitamin E status by world region, as assessed by intake levels and serum concentrations

World region	n (studies)	% (studies)
Europe (including Russia, Turkey, and Israel)	61	47.7
North America	31	24.2
Western Pacific Region	19	14.8
Africa	6	4.7
Eastern Mediterranean Region	6	4.7
Latin America (including Mexico)	3	2.3
Southeast Asia Region	2	1.6

Altogether 176 articles referring to 132 single studies were included in this review, with a total of 249,637 participants from 46 countries [9–78]. The 176 articles reported 1419 discrete values on vitamin E intake levels or serum concentrations. They ranged from overall values for the total study population to values for specific subgroups, which were defined as subentries. The sample size of individual studies ranged from 10 to 48,776 participants, with a median of $n = 374$. While the majority of studies included data on males and females, 5 studies (3.8%) restricted their focus to males, and 13 studies (9.9%) contained data on females only. The overall proportion of males and females was 36.0% and 64.0%, respectively. On average, participants had a mean age of 49.8 years, ranging from newborns to 106 years. The age group of older adults (aged 50+) was considered in 27.3% of the studies, followed by middle-aged adults (aged 35–49; 20.7%) and adults in general, including younger adults (aged 18+; 15.7%). Most of the studies were conducted in Europe (47.7%), followed by North America (24.2%) and the Western Pacific Region (14.8%) (Table 13.2).

The highest number of publications derived from the periodically repeated NHANES ($n = 13$). Of the 132 studies, about one fourth included participants taking vitamin supplements that could potentially influence the reported serum concentrations. However, supplement use was not sufficiently reported in half of the studies. Fifty-six studies (42.4%) provided nutritional intake data, with 81.3% of the corresponding subentries referring to vitamin E as such. Food frequency questionnaires were used in 36.1% of these studies, followed by 24-h recalls (31.9%) and dietary records (20.8%). A method used to a lesser extent was dietary history (6.9%). In 63.4% of intake values, no adjustment had been performed, 27.1% of intake values were energy adjusted, 8.0% represented a tocopherol-energy ratio, and 0.6% represented a tocopherol-PUFA ratio. Fifty-nine studies (44.7%) provided data on vitamin E status measured in blood, with most of them reporting α-tocopherol concentrations (58.2% of the corresponding subentries). In 97.3% of these studies, high-performance liquid chromatography (HPLC) was used for the measurements. In 60.4% of blood values, no adjustment for blood lipids had been performed; 29.0% of blood values represented a tocopherol-lipid ratio, and 10.6% of blood values had been adjusted for lipids. Seventeen studies (12.9%) included both intake data and status measured in blood.

Vitamin E Intake by Region and Country

Nutritional intake was analyzed for unadjusted α- and γ-tocopherol and all eight isomers together. The median intake was 6.2 mg per day for α-tocopherol and 1 mg per day for γ-tocopherol. For intake of all 8 isomers together, the median was 10.2 mg per day. The majority of reported mean or median intakes of α-tocopherol and all eight isomers together were below recommended intake values in all the countries and regions included in this global review. Applying an RDA of 15 mg/day and EAR of 12 mg/day globally to all populations with a minimum age of 14 years [6], 82% and 61% of all subentries were below the RDA and EAR, respectively. In the Americas, 91% of all subentries were below the RDA and 89% below the EAR. Eighty percent and 55% of the subentries in Europe were found below the RDA and the EAR, respectively. The corresponding proportions in Asia Pacific were 79% below the RDA and 68% below the EAR. Even using the more conservative approach proposed

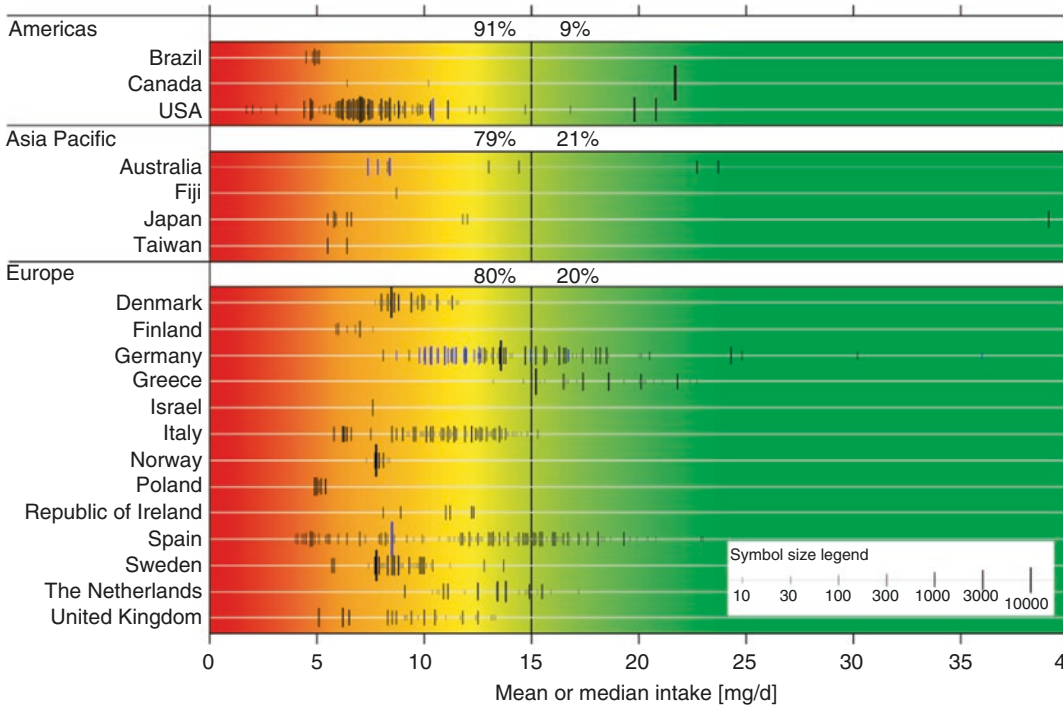

Fig. 13.1 Vitamin E intake by region and country for populations with a minimum age of 14 years. The figure shows all subentries as reported in the publications, with each symbol corresponding to one subentry. The vertical line indicates the RDA of 15 mg/day recommended by IOM. Red, low intake (≤5 mg/day); yellow, moderate intake (6–14 mg/day); green, recommended intake or above (≥15 mg/day) [3]

by the WHO (10 mg/day intake), authors found 83% of the subentries in the Americas, 35% of the subentries in Europe, and 60% of the subentries in Asia Pacific below this intake level. However, the reported low intake levels in different countries do not necessarily reflect the serum concentrations in these countries. Since only a small proportion of the studies provided both nutritional intake data and data on status measured in blood, a correlation between the two data sets was beyond the scope of this review (Fig. 13.1).

The biggest study on intakes in the data set was the pan-European EPIC study with 36,000 participants. The overall mean intake was 11.9 mg/day. It showed an interesting regional difference: higher in the southern countries and lower in the northern ones. This may be related to the food sources, particularly vegetable oils, which are popular in the south [79]. The explanations of these findings regarding the generally low intake may be manifold. For methodological reasons, vitamin E might be underestimated and thus underreported in most of the intake assessments (e.g., misreporting food intake in the past, difficulty of portion size estimation, variability between interviewers) [80]. On the other hand, recent studies about vitamin E stability in vegetable oils suggest that the actual intakes might be even lower than estimated. Commercial vegetable oils, which contain vitamin E, are commonly stored in the supermarket or kitchen in transparent polyethylene terephthalate (PET) bottles (see Chap. 16, Pignitter et al.). Light, temperature, and oxygen availability have been shown to promote rancidity in these vegetable oils. Recent studies demonstrated that storing soybean oil in transparent bottles under household conditions might pose an increased risk for accelerated lipid oxidation [81]. Therefore, the oxidative stability of vitamin E in edible oils is limited, and vegetable oils might contribute less to vitamin E intake than has been thought so far. Many scientists believe that it is difficult for an individual to consume more than 15 mg/day α-tocopherol from food (RRR-α-tocopherol) alone, without increasing fat intake above recommended levels [82].

Vitamin E Serum Concentrations by Region and Country

The few existing reviews on this topic report substantial variation in the prevalence of vitamin E deficiency across countries worldwide, with estimates ranging from 0.7% to 89.0%, depending on cutoff value and study population [83, 84]. In the current systematic review, for circulating α- and γ-tocopherol concentrations, the unadjusted medians were 22.1 μmol/L and 2.2 μmol/L, respectively, and the lipid-adjusted medians were 27.4 μmol/L and 4.9 μmol/L, respectively. For all 8 isomers together, the unadjusted median was 22.1 μmol/L, and the lipid-adjusted median was 22.6 μmol/L (no significant difference, $p = 0.63$ after correction for age group). Globally 13% of the reported values did not reach the functional deficiency threshold concentration of 12 μmol/L, mostly newborns and children up to the age of 12 years. In the Americas, 11% of the reported subentries were in the functional deficiency range. The highest proportion of data points in the deficiency range was found in populations in the Middle East and Africa (27%), but values were also relatively high in Asia Pacific (16%) and Europe (8%). Considering a threshold concentration of 20 μmol/L recommended by some experts [85], 27% of the American, 80% of the Middle East/African, 62% of the Asian, and 19% of the European study

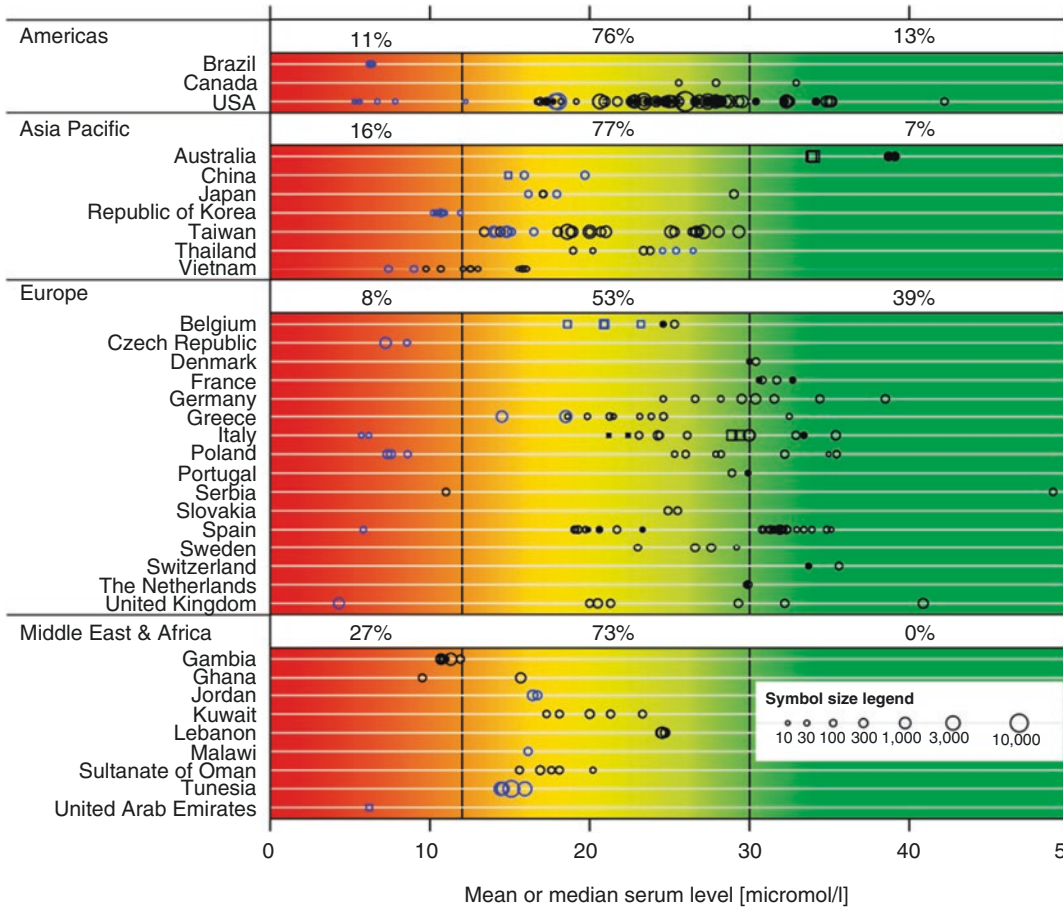

Fig. 13.2 Vitamin E status in different countries by serum concentrations of α-tocopherol. The figure shows all subentries as reported in the publications, with each symbol corresponding to one subentry. o, unadjusted; ●, lipid adjusted; black, mean; □, median; blue, 0–12 years; black, >12 years. Red, concentration in functional deficiency range (≤12 μmol/L); yellow, concentration between functional deficiency and desirable threshold (13–29 μmol/L); green, concentration in desirable range (≥30 μmol/L) [3]

data points were below this serum value. The average serum α-tocopherol concentration of 20 μmol/L can be reached in normal healthy adults who consume a variety of foods, including whole grains, seeds, and nuts. On the other hand, only 21% of the total data points included in this global review reached a desirable mean serum concentration of 30 μmol/L or higher (Fig. 13.2).

The largest study on serum concentrations in the data set is the NHANES, which is also the study with the most publications ($n = 13$) in this data set. Ford et al. (2006) provided details about the statistically significant age gradient within the NHANES ($p < 0.001$), which is in agreement with the present global data set. The older the subjects, the higher the α-tocopherol concentration, even if adjusted for cholesterol [59]. In this review, globally 66% of all subentries ranged between 12 and 30 μmol/L. However, the interpretation of the measured α-tocopherol values is challenging due to the positive correlation with different blood lipids. Various methods to control the confounding effect of blood lipids have been suggested in the literature, but they are not completely effective [86]. The data are very heterogeneous; even within one country values ranging from about 5 to 35 μmol/L were observed. In the total data set a significant difference between the adjusted and unadjusted serum concentrations was observed. However, the difference between the adjusted and unadjusted serum concentrations disappeared in the data set once age group was included as a covariate. The previous difference was again mainly driven by newborns and children, who tend to have lower serum concentrations than adults [87]. Nevertheless, more research is needed to clarify and verify the biological relevance of the α-tocopherol serum range between 12 and 30 μmol/L in both adults and children.

Serum Levels and Health Effects as Possible Marker to Assess Vitamin E Status

When using dietary intake data to evaluate vitamin E status, the respective dietary intake recommendation gives a clear guidance; however, as seen above, there are uncertainties regarding the reporting and accurate information in the databases used to calculate the intakes. In the case of vitamin E serum levels, measurements are much more precise, but there is still a debate going on regarding the desired levels and how they might relate to the health status of a given individual or group. As defined by the US Food and Nutrition Board, the plasma α-tocopherol concentration cutoff value for healthy, adult humans, indicative for vitamin E deficiency, is 12 μmol/L [6]. It has been demonstrated that plasma vitamin E levels less than 8 μmol/L are associated with neurologic disturbances and diseases (e.g., peripheral neuropathy, spinocerebellar ataxia, skeletal myopathy, etc.) and levels less than 12 μmol/L with miscarriage [88] and increased erythrocyte fragility [6], and generally low plasma α-tocopherol concentrations are also associated with increased risk of developing mild cognitive impairment and Alzheimer's disease (AD) [89]. On the other hand, evidence is emerging from various epidemiological studies for plasma vitamin E levels over 30 μmol/l, achievable by nutritional intake, those being linked with decreased odds of noncommunicable diseases (NCDs) [90]. Furthermore, plasma vitamin E levels above ca. 45 μmol/L, as a result of pharmacological interventions, have been associated with therapeutic benefits in certain medical conditions. The α-tocopherol concentration of ≥30 μmol/L as a desirable target for health benefits is further supported by the fact that urine excretion of α-carboxyethyl hydroxychroman (α-CEHC), a metabolite and status marker of α-tocopherol [91], increases when under healthy conditions the α-tocopherol serum threshold of 30 μmol/L is exceeded [6]. In addition, pentane concentration in the exhaled air, a marker of oxidative stress, is inversely related to vitamin E intakes and is very low at α-tocopherol serum concentrations of 30 μmol/L [92]. The beneficial effects associated with these blood levels have been acknowledged by the common recommendations of the German-speaking countries (Germany, Austria, and Switzerland), which have set the desired α-tocopherol serum concentrations at or above 30 μmol/L [8]. Furthermore, as discussed above, calculation of effective vitamin E blood concentrations needs to take into account possible elevated lipid levels too, since in such cases the otherwise adequate vitamin E concentrations may

not be sufficient to protect cell membranes and tissues from oxidative damage [93]. During the past couple of decades, a number of studies have reported determinants of plasma α-tocopherol concentrations, measured by HPLC methods, and provided kinetic models that aimed to correlate vitamin E intakes with plasma levels [94]. Such compartmental models might be useful for the development of markers of functionality or health benefits can be related to it, as shown below [95, 96].

Data from randomized controlled trials (RCTs) among population groups with specific disease conditions such as age-related macular degeneration (AMD), nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH), and Alzheimer's disease suggest that daily doses of vitamin E above the RDA (and corresponding elevated plasma levels) may confer additional health benefits (Table 13.3). These findings indicate that vitamin E supplementation may be safely and efficaciously targeted to selected individuals or high-risk groups, comprising subsets of the general population, for improving immune function, cardiovascular and liver health, and cognitive function, among others. Accumulation of new findings on the use of high-dose preventive or therapeutic supplements suggests the existence of a dual distribution of vitamin E intakes and respective status levels, one that approximates an RDA to maintain general population health and a higher level that may ameliorate the progression of certain population groups with chronic medical conditions, but cannot be obtained from the diet alone. According to these studies, provision of α-tocopherol in a dosage range of 60–800 mg/day may provide immune enhancement in elderly [97]. Results suggest that vitamin E supplementation of the α-tocopherol form at a daily dose of 400 IU can reduce and normalize risk for cardiovascular events in type 2 diabetic patients with a certain (Hp 2-2) genotype [98]. Various expert groups have concluded that vitamin E (α-tocopherol) administered at a daily dose of 800 IU improves liver histology in non-diabetic adults with biopsy-proven NASH and should be considered as a first-line pharmacotherapy for this patient population [99]. In a recent AD trial, the administration of high-dose α-tocopherol (2000 IU/d) for a period of 2 years resulted in slower functional decline and was considered to be generally safe [100]. We still need to understand the mechanisms of these disorders so that we can provide guidance to the public concerning public health measures with a focus on prevention of diseases. These data support the concept that measuring vitamin E serum levels could provide a reference ranging from vitamin E deficiency to suboptimal and desired levels which can be achieved by the diet and which may even provide guidance that at a certain level pharmacological effects could be achieved (Table 13.3). More attention should be given to further explore this opportunity as the vitamin E serum level may be a much more useful marker to assess vitamin E status rather than relying on dietary intake reports.

Table 13.3 The concept of a dual distribution of vitamin E roles in human health. Vitamin E acts as a peroxyl radical scavenger, and therefore damage to biological membranes in individuals susceptible to disease is most probably the reason for benefit in the above examples. Emerging evidence suggests that the lower end of the current adequate α-tocopherol serum concentration rather corresponds to a suboptimal status, since to higher concentrations further health benefits seem to be attributed

Vitamin E role	**Essentiality**		**Health benefits**	
Vitamin E status	**Deficient**	**Suboptimal**	**Desirable**	**Therapeutic**
Vitamin E serum concentration	≤12 μmol/L	13–29 μmol/L	30–44 μmol/L	≥45 μmol/L
Vitamin E intake	<15 IU/day	15–54 IU/day	55–249 IU/day	≥250 IU/day
Health impact	Hemolysis Neurological symptoms Miscarriage	Absence of deficiency Normal erythrocyte stability	Decreased risk of noncommunicable diseases	Immune function Cardiovascular health Liver health Cognitive function
References	[88, 95, 96, 101–108]	[2, 8, 89–91, 95, 97, 109–115]		[95–97, 116–119]

Possible Limitations of the Systematic Review

This systematic review was the first to analyze the globally published scientific literature on vitamin E dietary intake levels and serum concentrations. All subentries were considered as reported in the publications (median, mean, or geometric mean), which provided a comprehensive picture and captured heterogeneity among subgroups, but may also lead to under- or overrepresentation of some studies. Many studies published results according to several predictors: While the NHANES study reported the most data on serum concentrations, differentiated by gender, age group, and race, the EPIC study focused on intake levels, differentiated by country, gender, and age categories that differed from those of NHANES, whereas race was not differentiated. This redundancy along with the differences in reporting among studies has been accepted for the sake of completeness. Also for the same reason, no distinction has been made between representative and nonrepresentative studies. No consideration could be given to the quality of the dietary assessment data or to the standardization of blood assays in different studies. Another factor that could influence the interpretation of data was that supplement use was not always sufficiently reported.

Conclusions

This comprehensive review of vitamin E dietary intake levels and serum concentrations demonstrates that a significant part of reported intake values worldwide are below the recommended level. Similarly, it shows that a considerable proportion of the data points of the global population do not reach the functional deficiency threshold serum concentration for α-tocopherol, particularly in newborns and children. There is also a substantial geographical knowledge gap for Latin America, the Middle East and Africa, and Asia Pacific. While there is still no consensus among scientists regarding desirable α-tocopherol serum concentrations, these recent results based on the reported dietary intake levels and serum concentrations suggest that the vitamin E status of the included population groups can be improved. Possible measures include encouragement of consumption of vitamin E-rich food sources (e.g., vegetables, dairy products, eggs), adequate fortification of food products (e.g., vegetable oils), and supplementation. More accurate understanding of the relationship of vitamin E intakes/dosage to responding serum vitamin E levels and the resulting health benefits is required. Biomarkers are needed to be used in the assessment of vitamin E intake and status. Vitamin E serum levels are considered an encouraging approach to define vitamin E status; however additional evidence is desired to substantiate vitamin E reference values as proposed above. Large-scale studies in children have to be conducted using such validated biomarkers to assess their vitamin E requirements. More information is needed also on the other isoforms of vitamin E. Thus, much needs to be done to study what minimal concentrations are beneficial in the presence of other competing isoforms of tocopherols. Global intake and status data could be useful stepping stones for researchers to combine existing data, to fill in data gaps, and to understand more about the complex field of vitamin E and its impact on human health.

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Chapter 14
Vitamin E Intakes and Status in Toddlers, School Kids and Adolescents: What Do We Know?

Volker Böhm

Keywords α-Tocopherol · Adequate intake (AI) · Recommended Dietary Allowance (RDA) Children · Blood plasma

Key Points

- Vitamin E intake increases with increasing age.
- Infants' intake often meets the reference value, while toddlers and adolescents often ingest less vitamin E than recommended.
- α-Tocopherol contents in plasma/serum of infants, toddlers and adolescents were mostly very low, close to deficiency.
- Data of vitamin E intake and status are controversial.

Introduction

Vitamin E is the major lipid-soluble antioxidant in the cell antioxidant system and is exclusively obtained from the diet [1]. Vitamin E protects polyunsaturated fatty acids and other components of cell membranes and low-density lipoproteins from oxidation by free radicals. It is located primarily within the phospholipid bilayer of cell membranes. The most important form is α-tocopherol. Clinical signs of deficiency occur very rare. But newborn infants, particularly if born prematurely, are vulnerable to oxidative stress. Main reasons may be low body stores of vitamin E, impaired absorption and reduced transport capacity due to low concentrations of circulating low-density lipoproteins at birth. Term infants nearly achieve adult concentrations of vitamin E in plasma within the first week [1].

Newborns had lower vitamin E concentrations in plasma compared to their mothers. These differences were no longer significant when plasma vitamin E concentrations were standardised for phospholipids or total lipids. Vitamin E seems not to pass efficiently through the placenta to the newborn circulation as short-term supplementation of pregnant women before delivery only enhanced the vitamin E status of the mother [2].

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_14

Additional functions, independent of its antioxidant properties, have been reported for α-tocopherol. It has, e.g. a role in modulating gene transcription, and inhibits platelet aggregation and vascular smooth muscle proliferation [3] (see Chaps. 3, 4 and 7).

Reference Values

In 2015, EFSA concluded that average requirements (ARs) and population reference intakes (PRIs) for vitamin E (as α-tocopherol) cannot be derived for adults, infants and children. Thus, EFSA defined adequate intakes (AIs), based on observed intakes in apparently healthy populations with no obvious α-tocopherol deficiency in the EU [4]. Looking into the development of reference values for Europe, in 1993, the Scientific Committee for Food (SCF) considered an amount of 0.4 mg α-tocopherol equivalents (α-TE) per gram of dietary polyunsaturated fatty acids (PUFAs) to fulfil the requirement of children and adults, with a minimal intake of 4 mg α-TE/day for men and 3 mg α-TE/day for women regardless of PUFA intake [4]. Later, different organisations defined Dietary Reference Values (DRVs) for vitamin E in mg α-TE/d. However, for toddlers the situation remained difficult. Often reference values were defined based on the vitamin E concentration of human milk by using mean breast milk consumption (e.g. 0.78 L/d for US values and 0.85 L/d for WHO/FAO values). Table 14.1 presents an overview of DRVs for vitamin E for infants and children [4].

The EFSA Panel on Dietetic Products, Nutrition and Allergies noted uncertainties in the available food composition and consumption data. The Panel considered that there are, at present, insufficient data on markers of α-tocopherol intake/status/function (e.g. plasma/serum α-tocopherol concentration, hydrogen peroxide-induced haemolysis, urinary α-CEHC excretion, markers of oxidative damage) to derive the requirement for α-tocopherol for adults. The Panel also considered that there are no data that can be used to derive the requirement for α-tocopherol for infants or children. Therefore, the Panel proposed to set adequate intakes (AIs) for α-tocopherol for all population groups [4].

Table 14.1 Overview of Dietary Reference Values [mg/d] for vitamin E for infants and children

	D-A-CH (2015)	NCM (2014)	WHO/FAO (2004)	Afssa (2001)	IOM (2000)
Age (m)	6–<12	6–11	7–12	6–12	6–12
All (mg vit. E/d)	4	3	2.7	4	5
Age (y)	1–<4	1–<2	1–3	1–3	1–3
All (mg vit. E/d)	5–6	4	5	6	6
Age (y)	4–<7	2–5	4–6	4–6	4–8
All (mg vit. E/d)	8	5	5	7.5	7
Age (y)	7–<10	6–9	7–9	7–9	9–13
All (mg vit. E/d)	9–10	6	7	9	11
Age (y)	10–<13	10–13	10–18	10–12	14–18
Boys (mg vit. E/d)	13	8	10	11	15
Girls (mg vit. E/d)	11	7	7.5	11	15
Age (y)	13–<15	14–17		13–19	
Boys (mg vit. E/d)	14	10		12	
Girls (mg vit. E/d)	12	8		12	
Age (y)	15–<19				
Boys (mg vit. E/d)	15				
Girls (mg vit. E/d)	12				

Modified from [4]

D-A-CH Deutschland-Austria-Confoederatio Helvetica, *NCM* Nordic Council of Ministers, *WHO/FAO* World Health Organization/Food and Agriculture Organization of the United Nations, *Afssa* Agence française de sécurité sanitaire des aliments, *IOM* US Institute of Medicine of the National Academy of Sciences

For children aged 1 to <3 years, an AI for α-tocopherol was set at 6 mg/d for both sexes. For children aged 3 to <10 years, an AI for α-tocopherol was set at 9 mg/d for both sexes. For children aged 10 to <18 years, AI for α-tocopherol was set at 13 mg/d for boys and 11 mg/d for girls [4].

For infants aged 7–11 months, an AI for α-tocopherol of 5 mg/d was extrapolated upwards from the estimated α-tocopherol intake in exclusively breast-fed infants aged 0–6 months, using allometric scaling (assuming that the requirement for this vitamin is related to metabolically active body mass) and rounding to the closest unit [4].

Intake

In the United States, The Feeding Infants and Toddlers Study (FITS) is one large study (data collection: March to July 2002) looking for the usual nutrient intake of infants and toddlers. The results of this study were published by discussing different aspects. Briefel et al. [5] compared Hispanic and non-Hispanic infants and toddlers. In 2002, they collected 24-h dietary recalls and reported results for 3008 infants and toddlers aged 4–24 months (321 Hispanics and 2637 non-Hispanics). Figure 14.1 presents mean dietary intakes of three age groups (4–5 months, 6–11 months and 12–24 months) compared to the reference values available.

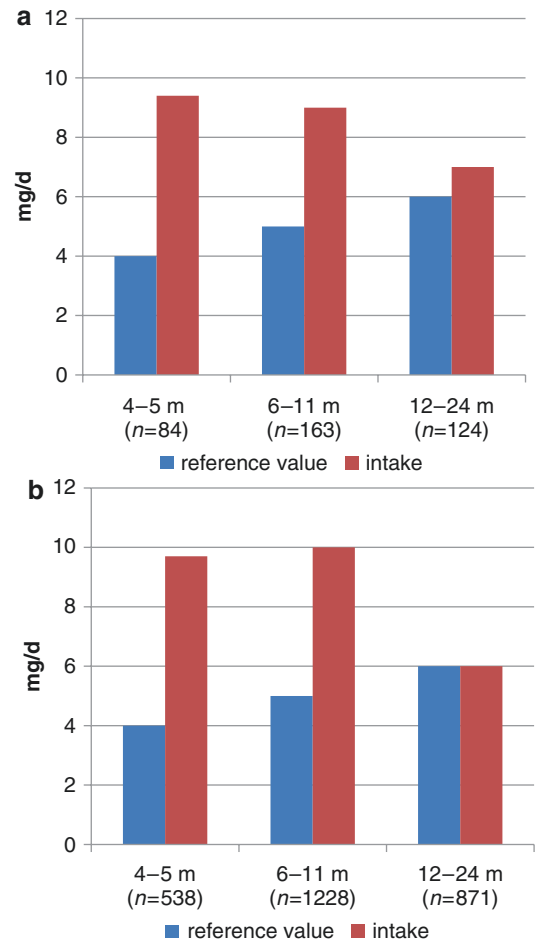

Fig. 14.1 Mean dietary intakes (mg/d) of vitamin E compared to reference values for Hispanic (**a**) and non-Hispanic (**b**) infants and toddlers in the United States. (Modified from [5])

While the intakes for Hispanic and non-Hispanic infants (4–5 months) were comparable (9.4 vs. 9.7 mg/d), there were significant differences for infants aged 6–11 months (9 vs. 10 mg/d: $p < 0.01$) and for toddlers aged 12–24 months (7 vs. 6 mg/d: $p < 0.05$). Except the non-Hispanic toddlers, all groups had higher intakes than the reference values [5].

In 2008, another Feeding Infants and Toddlers Study (FITS) was done with data collection between June 2008 and January 2009. 3273 children aged 0–47 months were assessed by using 24-h dietary recalls [6]. Four age groups (0–5 m, 6–11 m, 12–23 m and 24–47 m) are shown in Fig. 14.2 with vitamin E intakes between 4 and 8 mg/d and compared to the reference values.

Results of FITS 2002 and FITS 2008 were comparable. Intakes of vitamin E were mostly adequate. However, 61% (2002) or 63% (2008) of the toddlers (12–23 months) had intakes less than the Estimated Average Requirement (EAR) [6].

Comparable results were published for a smaller American study investigating infants and toddlers in Baltimore [7]. Seventy-three children aged 0–24 months were assessed between January and February 2008 by using single 24-h dietary recalls (Fig. 14.3). While infants up to 12 months exceeded adequate intake (AI: 4–5 mg/d), the toddlers (13–24 months) failed to meet the recommendation (6 mg/d).

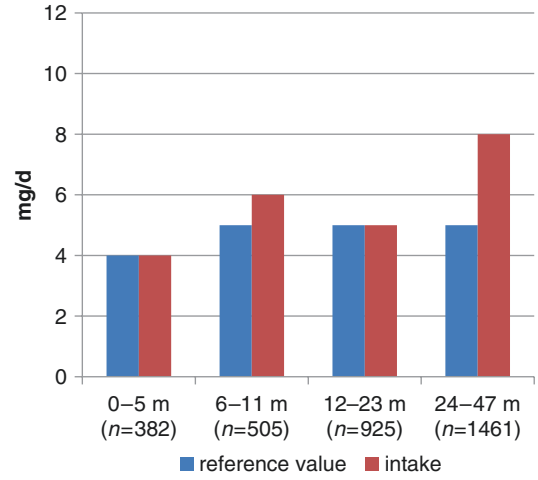

Fig. 14.2 Mean dietary intakes of vitamin E compared to reference values for American infants and toddlers. (Modified from [6])

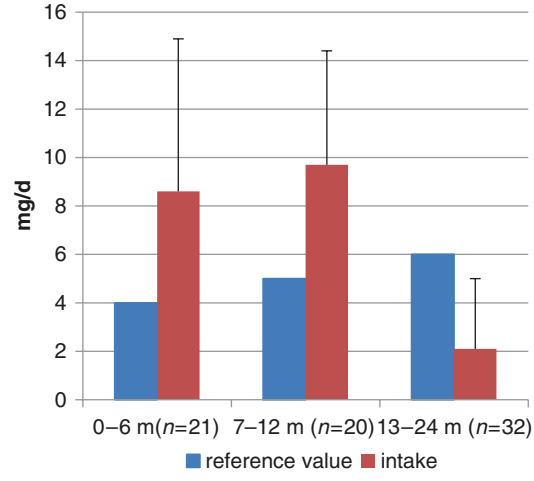

Fig. 14.3 Mean vitamin E (as α-tocopherol) intakes (+ standard deviation) compared to reference values for American infants and toddlers from Baltimore. (Modified from [7])

Recently, results on dietary vitamin intakes by Mexican populations were published [8]. These data of the ENSANUT 2012 cohort (October 2011–May 2012) also give insight in the vitamin E intake of children. The authors used a 24-h dietary recall and presented results for three age groups (1–4 years, 5–11 years, 12–18 years) with relevance for this book chapter. The highest prevalences of inadequate intake were observed for vitamins D and E, independent of age and sex. The rates of inadequacy of vitamin E were >50% in participants <20 years. For preschool children (1–4 years), the vitamin E intake was 5.33 ± 0.06 mg/d (n = 2113). The results for the other age groups are shown in Fig. 14.4. In schoolchildren as well as in adolescents, males had higher intakes compared to females. The RDA values were 1–3 years, 5 mg/d; 4–8 years, 6 mg/d; 9–13 years, 9 mg/d; and 14–18 years, 12 mg/d. Thus, especially older children had vitamin E intakes below the reference value [8].

In Greece, the GENESIS (2003/2004) cohort of 2374 children (aged 1–5 years) investigated dietary intakes by using 3-day food records. The mean intake of vitamin E was 6.4 mg/d. 22.2% of the children had intakes less than EAR [9]. The Spanish study enKid (1998–2000) used two 24-h recalls. The cohort was divided into four age groups (2–5 years, 6–9 years, 10–13 years and 14–17 years). Gender-specific results are shown in Fig. 14.5.

Male infants and toddlers had mean vitamin E intakes between 4.7 and 8.0 mg/d, while female ones had lower vitamin E intakes (4.6–6.4 mg/d). The largest gender difference was seen for the children aged 14–17 years. Reference values were (independent of gender) 2–3 years, 6 mg/d; 4–5 years, 7 mg/d; 6–9 years, 8 mg/d; 10–12 years, 10 mg/d; 13 years, 11 mg/d; 14–15 years, 11 mg/d; and 16–17 years, 12 mg/d. Thus, all mean intake values were below the reference values [10].

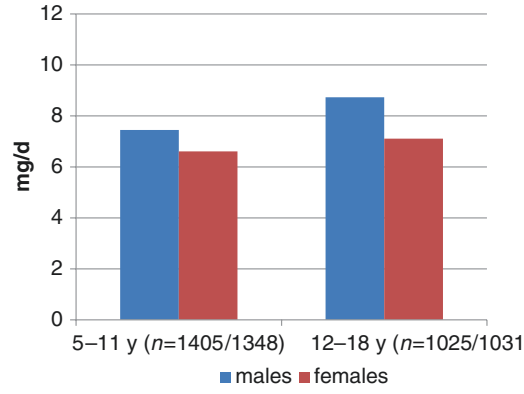

Fig. 14.4 Mean vitamin E intakes for schoolchildren and adolescents of the Mexican ENSANUT cohort. (Modified from [8])

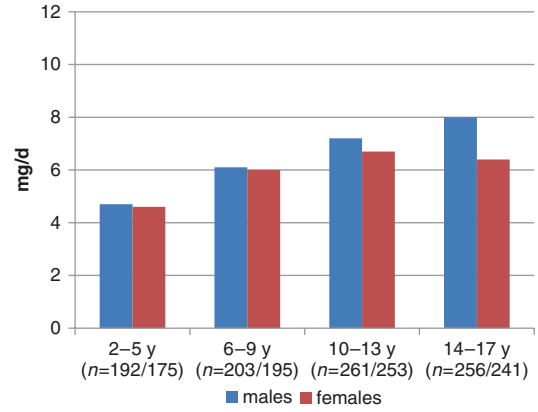

Fig. 14.5 Mean vitamin E intakes for infants and toddlers of the Spanish enKid study. (Modified from [10])

A Polish study (September 2011–May 2012) assessed day menus and food inventory reports in 30 nursery schools in Lodz. The mean vitamin E intake was calculated as 2.6 ± 0.8 mg/d. This study does not allow a comparison of different age groups. However, it showed a very low vitamin E intake [11].

Recently, the intake of fat-soluble vitamins in the Belgian population was investigated by using 1-day food diaries (3–9 years) or 24-h dietary recalls and food frequency questionnaires (10–64 years) [12]. The authors determined the contributions of foods, fortified foods and supplements. The Belgian food consumption survey (n = 3200) was conducted in 2014 and showed beside other results data for the following four age groups: 3–6 years, 7–10 years, 11–14 years and 15–17 years. Usual vitamin E intakes from foods only ranged from 7.0 to 12.2 mg/d with an increase in intake with increasing age (Fig. 14.6). Food was the major contributor to total vitamin intake. The median vitamin E intake from foods only was below the AI in most sex-age groups [12].

Skinner et al. [13] investigated the vitamin E intake of 72 American subjects with data from infancy to age of 60 months for each subject. They used 24-h dietary recalls and 2-day food records. The results for six time points are shown in Fig. 14.7.

At all interview points, mean dietary intakes of vitamin E were in all groups lower than the Recommended Dietary Allowances (RDA). The RDA values of 1989 were 6 mg/d for the age group 1–3 years and 7 mg/d for children of age 4–6 years [13].

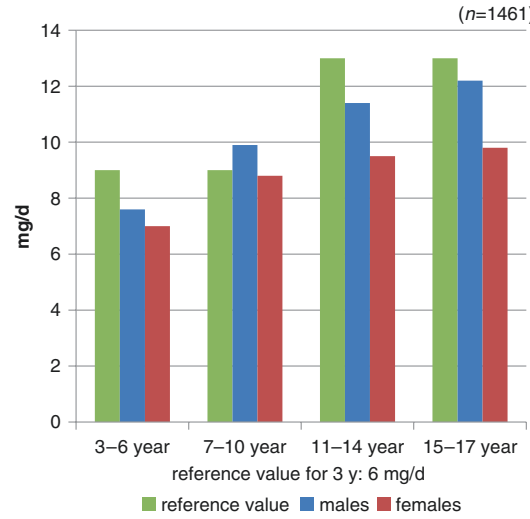

Fig. 14.6 Mean vitamin E intakes compared to reference values for children and adolescents of the Belgian food consumption survey. (Modified from [12])

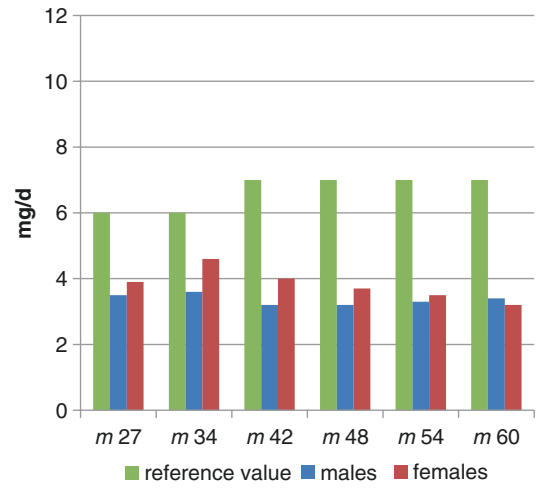

Fig. 14.7 Mean dietary intakes of vitamin E compared to reference values for 72 American children, assessed at the age of 27–60 months. (Modified from [13])

In France, 706 children aged 1–36 months were assessed (January to March 2005) in 11 subgroups by using 3-day weight food records [14]. The mean intakes of vitamin E are shown in Fig. 14.8.

Vitamin E intake decreased with increasing age. Thus, children over 12 months were at a risk of inadequate intake when comparing with reference values [14].

Data from a large multicentre study in Brazil titled "Nutri-Brasil Infância" showed vitamin E intake for children aged 1–6 years with intakes not significantly different between the age groups (Fig. 14.9) [15].

Results on vitamin E intake discussed here were published between 1999 and 2017. While several surveys took place in the United States or Mexico or Brazil, some studies were also done in Europe (Belgium, France and Spain). Vitamin E intake was evaluated within a large range between 2.1 and 12.2 mg/d. Some studies showed intakes comparable for all age groups investigated, while the majority

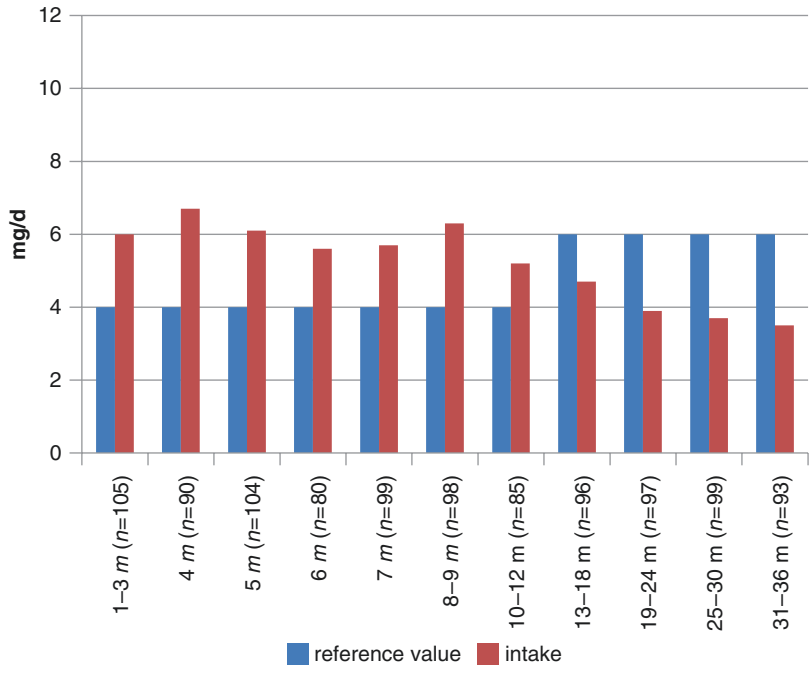

Fig. 14.8 Mean vitamin E intakes compared to reference values for French infants and toddlers. (Modified from [14])

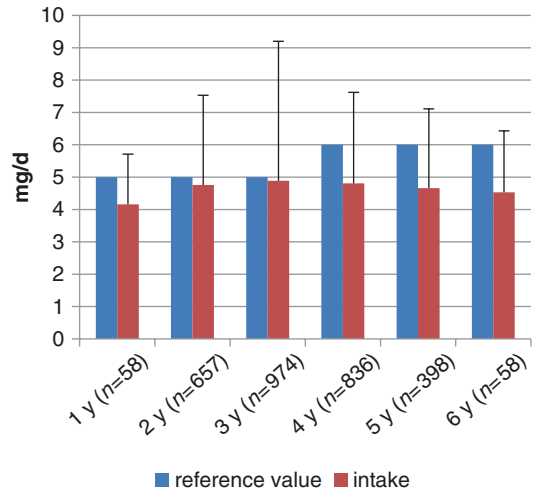

Fig. 14.9 Mean vitamin E intakes (+ standard deviation) compared to reference values for Brazilian children. (Modified from [15])

of surveys led to increased vitamin E intake with increasing age. However, some data also showed the opposite with decreasing intake with increasing age. Thus, data of vitamin E intake are still controversial, making the development of reference values very difficult. While intake data of infants often reflected a good situation, intake of toddlers and older children was mostly calculated below the reference value. One reason might be the change from formula (infants) to normal diet (toddlers and older children).

Status

Vitamin E status is often assessed by determining the concentration of α-tocopherol in blood plasma or serum. In Germany, 90 athletes (35 m, 55 f) and 18 controls (7 m, 11 f), aged 16 ± 2 years, were analysed on their antioxidant status [16]. As there were no significant differences between both groups, data are presented together. The results for four age groups (12–14.5 years, >14.5–16.5 years, >16.5–18.5 years, >18.5 years) are shown in Fig. 14.10. The mean levels of all age groups were above the cutoff point for deficiency (12 μmol/L).

Harik-Khan et al. [17] presented data on vitamin E in serum of 4093 children (6–17 years), selected from the Third National Health and Nutrition Examination Survey (NHANES III) that was done in the United States between 1988 and 1994. Their main focus was comparing children with no asthma diagnosis (n = 3696) to children with asthma diagnosis (n = 397). Children with no asthma diagnosis had mean vitamin E concentrations of 770 ± 171 μg/dL (α-tocopherol: 18.0 ± 4.0 μmol/L), being comparable ($p > 0.05$) to children with asthma diagnosis (767 ± 189 μg/dL; α-tocopherol, 17.8 ± 4.4 μmol/L) [17]. Data of a later NHANES (2001–2004) showed for 1154 Mexican-American children aged 8–15 years slightly higher vitamin E concentrations in serum for females (n = 536: 4.44 ± 0.03 μmol α-tocopherol/mmol cholesterol) compared to males (n = 477: 4.35 ± 0.03 μmol α-tocopherol/mmol cholesterol) [18].

Kumar and Rajagopalan [19] reported data of an intervention study with schoolchildren (5–15 years) in India where a multiple-micronutrient food supplement was used. Baseline data for vitamin E (α-tocopherol) were comparable ($p > 0.05$) for control group (n = 77) with 974 ± 314 μg/dL (22.6 ± 7.3 μmol/L) and experimental group (n = 82) with 911 ± 269 μg/dL (21.2 ± 6.2 μmol/L) [19]. Results for 285 American adolescents (12–17 years) from the Olestra Post Marketing Surveillance Study were reported by Neuhouser et al. [20]. α-Tocopherol concentrations in serum

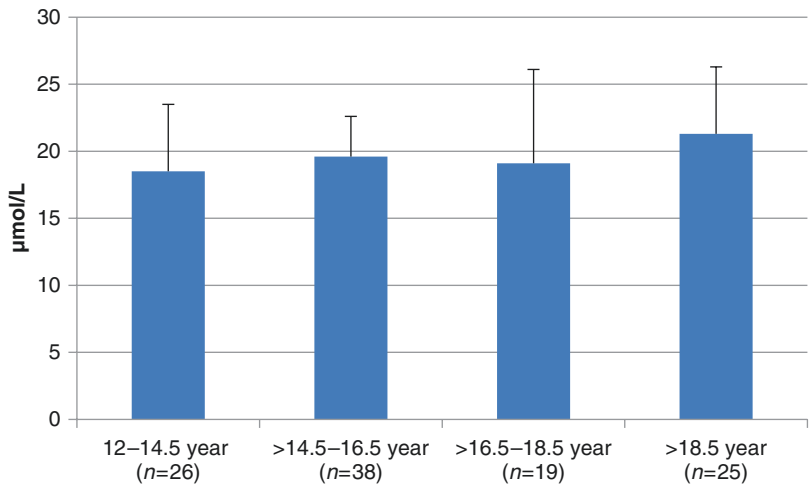

Fig. 14.10 α-Tocopherol concentrations (μmol/L) in blood plasma of German children and adolescents. (Modified from [16])

Table 14.2 Overview of α-tocopherol concentrations (μmol/L) in serum for adolescents in different European countries

Country\ age (year)	9	10	11	12	13	14	15	16	17	18
Austria		20.5 ± 4.9	19.8 ± 18.4	20.7 ± 4.7						
Austria			27.2/24.5 (m/f)							
France		18.3/20.5 (m/f)	18.3/20.5 (m/f)							
France	23.3 ± 4.7	22.7 ± 5.8	23.7 ± 4.9							
France				(19.3/20.7 (m/f)	21.2/21.9 (m/f)					
Great Britain			19.7/20.5 (m/f)	19.2/19.8 (m/f)						
Greece					18.4					
Hungary							29.2 ± 1.0			
Slovakia			29.2/22.9 (v/o)							
Sweden				21.2	14.9	19.1	17.4			

Modified from [22]
m male, *f* female, *v* vegetarians, *o* omnivores

were not significantly different ($p > 0.05$) between sexes. Males ($n = 153$) had α-tocopherol concentrations of 16.9 ± 4.5 μmol/L and females ($n = 132$) concentrations of 17.3 ± 4.4 μmol/L [20]. Another American study investigated 161 adolescents (12–20 years) and analysed concentrations of α-tocopherol in plasma. This study also did not show gender differences ($p > 0.05$) for α-tocopherol concentrations. Males ($n = 81$) had 17.0 ± 3.1 μmol/L and females ($n = 78$) 17.1 ± 2.8 μmol/L [21].

Valtuena et al. [22] recently reviewed various studies on vitamin E concentrations in blood of adolescents, published in Europe between 1981 and 2010. The results are presented in Table 14.2.

Valtuena et al. [22] stated that there were very few published studies on nutritional vitamin status in European adolescents and data were not available for all countries, especially in the Eastern part of Europe. In some countries, the sample was not representative of the general population. Thus, comparability of data is difficult [22].

Results on vitamin status presented here were published between 2001 and 2011. Besides some studies from the United States and India, several studies were conducted in Europe (Austria, France, Germany, Greece, Slovakia and Sweden). Concentrations of vitamin E (mostly as α-tocopherol) were determined between 16.9 and 29.2 μmol/L plasma/serum. As already stated for the intake data, for status as well an increase, a decrease or no change with increasing age was shown. Thus, these data are as controversial as intake data are. However, most results indicate a low status, partly close to deficiency. In addition, status data surprisingly suggest an inadequate intake, while intake data indicate a better supply of vitamin E for some age groups.

Quality and Limits of Existing Studies

Most of the intake surveys have the strength giving results for a high number of children and adolescents, avoiding the bias of studies with very low numbers of volunteers. As the data are from different countries, it is important to take into account the differences in eating behaviour. However, not all countries are represented yet. In addition, the studies used different methods to determine dietary intake of vitamin E. If only one single 24-h dietary recall was done per person, this might not show the usual nutrient intake (day-to-day variability). Other studies used 3-day food records, giving a

better impression of the dietary behaviour. Anyway, the accurate assessment of food intake in children is always challenging [7]. Another important aspect often not considered is the intake of supplements. However, the contribution of supplements to total vitamin E intake was generally low with contributions between 2% and 6% [12]. The consumption of fortified foods may be another difficulty to get accurate intake data. Their contribution to total vitamin E intake was shown to be between 5% and 15% [12]. One strength of intake surveys with high number of volunteers is giving information on nutrient deficiencies among subgroups. Thus, development of strategies for prevention of vitamin deficiencies is easier. A slight drawback of the published results is the use of different age groups in the studies.

Status of vitamin E was also assessed by using different methods. Analysis of plasma/serum was done by using various HPLC methods. Here also, not all the studies used the same age groups, making a comparison difficult. Regarding status data, contents of α-tocopherol in plasma or serum were not always lipid-standardised. In addition, as literature was published in different decades, dietary habits may have changed.

Although inadequate intake of vitamin E was presented by several authors for various subgroups, the reference values for vitamin E have been widely questioned. These values were established on the basis of prevention of hydrogen peroxide-induced haemolysis. However, many healthy people have intakes lower than those recommended [8].

Conclusions and Perspectives

Intake surveys as well as investigations on the concentrations of vitamin E in plasma/serum of children and adolescents were done in several countries. Intake ranged between 2.1 and 12.2 mg/d. The concentrations of vitamin E in plasma or serum, as a marker of the status, were between 16.9 and 29.2 μmol/L. However, especially investigations on the intake of nutrients in children are always a challenge. The data of intake and status presented here are controversial. In many studies, intake values were lower than the reference values. Due to conflicting data and other factors affecting the results, it is yet very difficult to define reference values for the intake of vitamin E. Thus, for Europe EFSA defined Average Intake (AI) values between 5 and 13 mg/d, depending on age.

In future, more dietary intake data as well as status data are needed for specific subgroups to adjust recommendations for vitamin E intake. A recent paper also stated that there is a need to adapt survey methodologies to include intake data from all sources (foods, fortified foods and supplements) [12].

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Chapter 15
Vitamin E Serum Levels and the Challenge to Correct for Lipids: Accounting for the Usual Double Correction for Variance Shared by Total Cholesterol and Fasting Triglycerides Reveals New Insights into the Association with the One-Carbon Pathway

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Keywords Vitamin E · Tocopherol · Cholesterol · HDL · LDL · Triglycerides · Variance · Metabolic syndrome · One carbon metabolism · Vitamin B6 · Vitamin B12 · Folic acid · Vitamin B11 · Homocysteine

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_15

Key Points

- Total cholesterol and fasting triglycerides explain a considerable part, i.e., 59%, of the variation of circulating concentrations of α-tocopherol, whereas they limitedly, i.e., 14%, explain the variation of circulating γ-tocopherol.
- Double correction of circulating vitamin E species for variance shared by total cholesterol and fasting triglycerides may bias interpretation of the associations of vitamin E with parameters belonging to the lipid domain.
- The associations of vitamin E with parameters from biological domains other than lipids do not seem to be materially affected by a possible double correction for total cholesterol and fasting triglycerides.
- In the elderly, circulating α-tocopherol may serve as a biomarker of a healthy diet through its positive associations with B vitamins and inverse association with homocysteine, whereas γ-tocopherol may serve as a marker of an unhealthy diet.

Introduction

Vitamin E has important antioxidant properties, and low vitamin E status is therefore implicated in accelerated development of age-related diseases, including type 2 diabetes and atherosclerosis [7, 13, 23]. Importantly, both these conditions are characterized by high circulating concentrations of fasting triglycerides and total cholesterol, which may complicate evaluation of the association of vitamin E status with these conditions. The reason is that in the circulation, vitamin E is carried by lipoproteins, which usually results in a positive correlation of circulating concentrations of vitamin E with those of fasting cholesterol and fasting triglycerides and the need to one way or another correct for this [32, 33]. This is usually done by dividing circulating vitamin E concentrations by the concentration of total lipids, which is calculated as total cholesterol plus triglycerides [32, 33]. Importantly, because there usually also is a positive correlation between total cholesterol and fasting triglycerides, simple summation of total cholesterol and fasting triglycerides to calculate total lipids and correction for this by dividing vitamin E concentrations may result in an unintended double correction for variance shared by total cholesterol and fasting triglycerides and unwanted weakening, disappearance, or even inversion of actually existing associations. If such an effect would exist, it would predict otherwise positive associations of vitamin E with fasting circulating lipids to become significantly inverse rather than absent after correction for total lipids. In this field HDL cholesterol might be an outlier. Because it contributes to total cholesterol, it is likely positively correlated with total cholesterol, but because of the action of circulating cholesterol ester transfer protein (CETP), it is likely inversely correlated with fasting triglycerides [5, 20], while, because of its antioxidant activity [16–18], one would anticipate a positive correlation with circulating vitamin E concentrations. Here it is quite unpredictable what the effect of correction of total cholesterol and triglycerides will be. Other interesting domains to be investigated from this perspective are components of the metabolic syndrome, including domains of body composition, blood pressure, glucose homeostasis, inflammation, uric acid, and liver enzymes, particularly, alanine amino transferase and gamma-glutamyl transferase, all of which are known to be relatively strongly and positively correlated with fasting triglycerides [1, 2, 12, 27, 34]. Concerning correlations of vitamin E with other circulating vitamins and effects of correction for total lipids with double correction for variance shared by total cholesterol and fasting triglycerides versus adjustment by means of linear regression, circulating concentrations of vitamin A might be interesting to have a closer look at, because in the circulation, vitamin A is also transported by lipoproteins [11, 21]. We hypothesized that the effect of correction for total lipids on the correlation of circulating vitamin E concentrations with circulating vitamin A concentrations will materially differ from the effect of this correction correlations with circulating concentrations of other vitamins. One way to overcome

double correction for variance shared by total cholesterol and fasting triglycerides may be by adjustment of vitamin E concentrations via linear regression rather than correlating variables to the quotient of vitamin E and total lipid concentrations. We therefore set out to compare correlations of circulating vitamin E concentrations, correlations of the quotient of circulating vitamin E concentrations, and linear regression-derived standardized regression coefficients in which associations are adjusted for total cholesterol and fasting triglycerides with circulating concentrations of fasting lipids, components of the metabolic syndrome, and circulating vitamin concentrations.

Materials and Methods

Study Design and Population

The LifeLines Cohort Study is a large observational population-based cohort study and biobank which examines the health and health-related behaviors of more than 167,000 persons [30]. The participants were recruited from the three Northern provinces of the Netherlands between 2006 and 2013. A more detailed description of the LifeLines Cohort study can be found elsewhere [14, 28]. In short, the first group of participants was recruited via local general practices. Participants could indicate whether family members were interested as well. In addition, individuals who were interested in the study had the possibility to register via an online self-registration. Individuals with insufficient knowledge of the Dutch language, with severe psychiatric or physical illness, and those with limited life expectancy (<5 years) were excluded from the study. Participants (>18 years old) completed several questionnaires, including topics such as occurrence of diseases, general health, medication use, diet, physical activity, and personality. Participants were invited to the LifeLines Research sites for a comprehensive health assessment and to allow for storage of biological samples, including plasma, serum, and 24 urine samples in the biobank underlying the LifeLines cohort. All participants provided written consent. The LifeLines Cohort Study was conducted according to the principles of the Declaration of Helsinki and approved by the Medical Ethical Committee of the University Medical Center Groningen, The Netherlands.

Selection of Subjects

For the current study, we selected 1600 subjects with age between 60 and 75 years and required plasma, serum, and 24 urine samples to be available from the biobank of the LifeLines cohort. The 1600 subjects were composed of 400 men and 400 women with low socioeconomic status and 400 men and 400 women with high socioeconomic status.

Dietary Intake

To asses dietary intake and use of vitamin supplements in the Lifelines Cohort, a 110-item semiquantitative baseline food frequency questionnaire (FFQ) assessing food items over the previous month was developed and validated by the Wageningen University using the Dutch FFQTOOL™, in which food items were selected based on the Dutch National Food Consumption Survey of 1997/1998. Seven answers categories were used to assess consumption frequency, ranging from “not this month” to “6–7 days a week.” Portion size was estimated by fixed portion sizes (e.g., slices of bread, pieces

of fruit) and commonly used household measures (e.g., cups, spoons). Energy and macronutrient intake was estimated from the FFQ data by using the Dutch food composition database of 2011 (NEVO). Participants were identified as vitamin supplement users if they consumed one or more vitamin supplements at an average of at least 4 days per week.

Data on Education, Smoking Habits, and General Health

Information about education and smoking was collected from the self-administered questionnaire. Educational level was categorized into four groups: (1) never been to school or elementary school only, (2) lower vocational or secondary schooling, (3) intermediate vocational schooling or intermediate/higher secondary schooling, or (4) higher vocational schooling or university. Since education is more differentiating than income in the egalitarian Dutch population, classification of socioeconomic status was based on educational status and defined as low (never been to school or elementary school only or completed lower vocational or secondary schooling) and high (completed higher vocational schooling or education). Additionally, subjects were classified according to their smoking habits (non-smokers, former smokers, or current smokers).

Clinical Measurements

Anthropometric measurements (weight, height, and waist circumference) and blood pressure were measured by well-trained staff. The anthropometric measurements were measured without shoes. Body weight was measured to the nearest 0.1 kg. Height and waist circumferences were measured to the nearest 0.5 cm. Height was measured with a stadiometer placing their heels against the rod and the head in Frankfort plane position. Waist circumference was measured in standing position with a tape measure all around the body, at the level midway between the lower rib margin and the iliac crest. BMI was calculated as weight (kg) divided by height squared (m^2). Systolic and diastolic blood pressures were measured 10 times during a period of 10 min, using an automated Dinamap Monitor (GE Healthcare, Freiburg, Germany). The average of the final three readings was used for each blood pressure parameter.

Biochemical Measurements

Blood samples were collected in the fasting state, between 8:00 and 10:00 a.m., and subsequently transported to the Central LifeLines Laboratory in the University Medical Center Groningen. The tocopherol species and vitamin A (retinol) were measured simultaneously by means of a validated liquid chromatography tandem-mass spectrometry (LC-MS/MS) assay, essentially as described by Riphagen et al. for vitamin K [26]. Serum levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and total triglycerides were quantified with validated routine enzymatic assays with spectrophotometric detection, all on a Roche Modular P chemistry analyzer (Roche, Basel, Switzerland). On the same analyzer, fasting blood glucose was measured via the hexokinase method. HbA1c was determined in EDTA-anticoagulated whole blood and the thyroid function parameters in plasma by means of routine immunochemical assays on a Cobas Integra 800 CTS analyzer (Roche Diagnostics Netherland BV, Almere, The Netherlands). Insulin resistance was estimated by calculating the triglycerides/HDL cholesterol ratio [25]. Serum uric acid and creatinine were measured via an enzymatic assay with colorimetric detection on a Roche

Modular P chemistry analyzer (Roche, Basel, Switzerland). Hs-CRP was determined by nephelometry (BN II system Siemens, Marburg, Germany). Creatine was quantified by means of LC-MS/MS [4]. Vitamin B6 was measured in plasma as pyridoxal-5′-phosphate with high-performance liquid chromatography (Water Alliance) with fluorescence detection (FP-2020; Jasco Inc.) [31]. Methylmalonic acid [3] and vitamin D3 [6] were determined by LC-MS/MS, as described previously. Other vitamin markers were assessed by commercially available assays on a Roche Cobas chemistry analyzer (Roche, Basel, Switzerland).

Statistical Analyses

All analyses were performed using IBM SPSS Statistics, version 22.0 for Windows software (IBM, Armonk, NY, USA), and GraphPad Prism, version 5.03 for Windows software (GraphPad Software, La Jolla, CA, USA). Total lipids were calculated as the sum of total cholesterol and triglycerides. Kidney function was estimated by the creatinine-based CKD-EPI formula [19]. Baseline data are presented as mean ± SD (normally distributed data), median (25th–75th percentile) (non-normally distributed data), or number (%) (categorical data). Tocopherol species was standardized for total lipids in two ways, i.e., by calculating the quotient of tocopherol and total lipids and by adjusting for total lipids in the linear regression models. Vitamin A was standardized via the quotient method only. All reported probability values are two-tailed, and a $P \leq 0.05$ was considered statistically significant.

Results

We included 802 men and 803 women with a mean age of 66 ± 4 years and BMI of 26.4 kg/m^2. We first investigated the associations of α-tocopherol and γ-tocopherol with each other and with fasting lipids. We found a significant positive association between α-tocopherol and γ-tocopherol (standardized beta = 0.29, $P < 0.001$). In an initial model of associations with fasting lipids, we entered fasting cholesterol and log-transformed fasting triglycerides as potential independent determinants of α-tocopherol, and we found that 59% of the variation in α-tocopherol concentrations could be explained by fasting cholesterol and log-transformed fasting triglycerides, with the highest contribution by total cholesterol (Table 15.1). Other combinations of lipids (models 2–4) did not much better or worse than the combination of total cholesterol and fasting triglycerides. Total cholesterol alone and calculated total lipids explained 48% and 61% of the variance, respectively. In analyses with γ-tocopherol as dependent variable, we found that total cholesterol and log-transformed fasting triglycerides only explained 14% of the variation in γ-tocopherol. Again, other combinations of lipids (models 2–4) did not much better or worse than the combination of total cholesterol and fasting triglycerides. Total cholesterol alone and calculated total lipids explained 9% and 14% of the variance, respectively.

We then proceeded with linear regression analyses in which standardized regression coefficients – which in terms of interpretation are comparable to a correlation coefficient – of age- and sex-adjusted associations of crude α-tocopherol concentrations as independent variable with demographics, dietary intake, use of multivitamin supplements, thyroid function, lipids, kidney function, glucose homeostasis parameters, inflammation, a functional marker for vitamin E status (urinary creatine over creatinine ratio), and status markers of other vitamins are investigated (Table 15.2). In these analyses, in which the association with age was only adjusted for gender and the association of sex was only adjusted for age, we found significant inverse associations with age, male gender, energy intake, free T3, free T4, gamma-glutamyl transferase, homocysteine, and vitamin A per total lipid concentration.

Table 15.1 Associations between tocopherol species and lipids in plasma in the overall study population

	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6	Model 7
α-Tocopherol							
Total cholesterol	0.69***	…	0.64***	…	0.58***	…	…
HDL cholesterol	…	…	…	…	0.14***	0.37***	0.38***
Non-HDL cholesterol	…	…	…	…	…	…	0.55***
LDL cholesterol	…	…	…	…	…	0.50***	…
Triglycerides	…	0.44***	0.34***	…	0.43***	0.54***	0.43***
Total lipids	…	…	…	0.78***	…	…	…
Adjusted R^2	***0.48***	***0.19***	***0.59***	***0.61***	***0.60***	***0.58***	***0.60***
γ-Tocopherol							
Total cholesterol	0.30***	…	0.26***	…	0.23***	…	…
HDL cholesterol	…	…	…	…	0.07*	0.16***	0.16***
Non-HDL cholesterol	…	…	…	…	…	…	0.28***
LDL cholesterol	…	…	…	…	…	0.19***	…
Triglycerides	…	0.27***	0.23***	…	0.28***	0.33***	0.22***
Total lipids	…	…	…	0.38***	…	…	…
Adjusted R^2	***0.09***	***0.07***	***0.14***	***0.14***	***0.14***	***0.13***	***0.14***

Associations between tocopherol species and lipids were tested via univariable and multivariable linear regression analyses of which standardized βs are presented (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Table 15.2 Participant characteristics and their associations with unstandardized, quotient-standardized, and regression-standardized α-tocopherol concentration

		Standardization method		
	N = 1605	None	Quotient	Regression
α-Tocopherol	33.4 [29.3–38.5]	…	…	…
α-Tocopherol/total lipid	5.1 [4.7–5.6]	…	…	…
γ-Tocopherol	1.59 [1.24–2.07]	…	…	…
γ-Tocopherol/total lipid	0.24 [0.19–0.31]	…	…	…
Demographics				
Age, y[a]	66 ± 4	−0.05*	0.03	0.01
Male gender, n (%)[a]	802 (50)	−0.17***	0.01	0.03
Low SES, n (%)	803 (50)	−0.03	−0.05	−0.04
BMI, kg/m^2	26.4 [24.1–29.4]	−0.03	−0.03	−0.04
Waist circumference, cm	101 ± 9	−0.04	−0.03	−0.03
Smoker, n (%)				
Never	547 (34)	…	…	…
Past	853 (54)	0.04	0.02	0.01
Current	191 (12)	0.02	−0.06*	−0.04*
Dietary intake				
Total protein, g/d	70 [57–82]	−0.02	0.07*	0.03
Animal protein	41 [32–50]	−0.01	0.04	0.02
Vegetable protein	28 [22–34]	−0.02	0.08**	0.04*
Total carbohydrates, g/d	201 ± 74	−0.04	0.03	0.01
Total fat, g/d	72 ± 30	−0.02	0.05	0.02
Alcohol intake, g/d	6.3 [0.9–16.4]	0.08**	0.01	0.03
Energy intake, kcal/d	1843 ± 634	−0.02	0.05	0.02
Use of multivitamins, n(%)	175 (11)	0.14***	0.21***	0.14***

Table 15.2 (continued)

Hemodynamics, mmHg				
SBP	133 [122–145]	0.08**	−0.05	−0.01
DBP	75 [69–81]	0.08**	−0.04	−0.01
Thyroid function				
TSH, mU/L	2.4 [1.6–3.3]	0.07	0.11*	0.05
Free T3, pmol/L	5.0 ± 0.6	−0.12*	−0.12*	−0.09**
Free T4, pmol/L	15.9 ± 2.4	−0.10*	−0.11*	−0.08*
Liver parameters, U/L[b]				
ASAT	24 [21–28]	0.02	0.04	0.03
ALAT	21 [16–27]	0.03	0.01	0.01
Alkaline Phosphatase	64 [56–76]	0.01	−0.04	−0.03
Gamma-GT	24 [17–34]	−0.10*	−0.02	0.01
Lipids, mmol/L				
Total cholesterol	5.4 ± 1.1	0.69***	−0.20***	…
HDL cholesterol	1.5 [1.2–1.8]	0.04	0.09**	0.14***
Non-HDL cholesterol	3.9 ± 1.0	0.67***	−0.23***	−0.33***
LDL cholesterol	3.5 [2.8–4.2]	0.61***	−0.21***	−0.26***
Triglycerides	1.1 [0.8–1.5	0.45***	−0.11***	…
Total lipid	6.6 [5.8–7.5]	0.77***	−0.19***	…
Kidney function				
Creatinine, mmol/L	75 [66–85]	0.04	−0.02	−0.01
eGFR, mL/min/1.73m^2	92 [80–105]	0.01	0.04	0.03
Urinary albumin, mg/24 h	5.2 [3.0–9.7]	−0.01	−0.04	−0.02
Uric acid, mmol/L	0.32 ± 0.07	0.07	−0.07	−0.05
Glucose homeostasis				
Glucose, mmol/L	5.2 [4.8–5.7]	−0.04	0.01	−0.01
HbA1c, %	5.8 [5.6–6.0]	−0.04	0.01	−0.01
Inflammation[b]				
High-sensitivity CRP, mg/L	1.5 [0.7–2.9]	0.04	0.12**	0.06*
Vitamins				
Urinary creatine/creatinine ratio	0.014 [0.009–0.053]	0.05*	0.01	0.01
Vitamin B6, nmol/L	75 [37–81]	0.24***	0.27***	0.19***
Vitamin B12, pmol/L	290 [224–362]	0.09***	0.14***	0.09***
Methylmalonic acid, nmol/L	169 [137–214]	−0.04	−0.01	−0.01
Folic acid, nmol/L	16.6 [11.1–24.9]	0.17***	0.27***	0.19***
Homocysteine, μmol/L	13 [11–15]	−0.08**	−0.13***	−0.09***
Vitamin D3, nmol/L	62.5 [47.3–77.1]	0.08**	0.10**	0.07**
Vitamin A, nmol/L	2.0 [1.8–2.3]	0.24***	−0.02	0.04*
Vitamin A/total lipid	0.31 [0.26–0.36]	−0.42***	0.16***	0.01

Associations between α-tocopherol and participant characteristics were tested via multivariable linear regression analyses of which standardized βs are presented (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

[a]Associations were adjusted for age and/or gender, where applicable

[b]Data was available for 613 participants

Abbreviations: *ALAT* alanine aminotransferase, *ASAT* aspartate aminotransferase, *BMI* body mass index, *CRP* C-reactive protein, *DBP* diastolic blood pressure, *eGFR* estimated glomerular filtration rate, *GT* glutamyl transferase, *SBP* systolic blood pressure, *SES* socioeconomic status

We found significant positive associations with alcohol intake, use of multivitamins, systolic blood pressure, diastolic blood pressure, total cholesterol, non-HDL cholesterol, LDL cholesterol, triglycerides, total lipids, urinary creatine/creatinine ratio, vitamin B6, vitamin B12, folic acid, vitamin D3, and vitamin A concentration. In similar analyses, with the quotient of α-tocopherol over total lipids as independent variable, we found significant inverse associations with current smoking, free T3, free T4, total cholesterol, non-HDL cholesterol, LDL cholesterol, triglycerides, total lipids, and homocysteine. We found significant positive associations of vitamin E with intake of total protein, vegetable protein, use of multivitamins, TSH, hs-CRP, vitamin B6, vitamin B12, folic acid, vitamin D3, and vitamin A per total lipid concentration. Finally, we performed analyses in which again crude α-tocopherol concentrations were entered as independent variable, but in which now in addition to age and sex, total cholesterol concentrations and log-transformed fasting triglycerides were added as independent variables, thereby additionally adjusting the associations of α-tocopherol concentrations with listed variables for total cholesterol and fasting triglycerides rather than by correcting them directly by making a quotient. The results of these analyses did not materially differ from the results of the analyses with the quotient, albeit non-lipid-related associations appeared somewhat weaker, while lipid-related associations were somewhat stronger and therefore more inverse, which may indicate that – if the inverted regression coefficients of the association of the α-tocopherol lipid quotient are a sign over overcorrection – this cannot be overcome by means of regression analyses.

Results of analyses for γ-tocopherol are shown in Table 15.3. If the first columns of Tables 15.2 and 15.3 are compared at the level of the lipid domain, it can be seen that age- and sex-adjusted standardized regression coefficients for associations of γ-tocopherol with lipids are positive, but much weaker than for α-tocopherol, and have no significant association with HDL cholesterol. In the further analy-

Table 15.3 Participant characteristics and their associations with unstandardized, quotient-standardized, and regression-standardized γ-tocopherol concentration

	Standardization method		
	None	Quotient	Regression
Demographics			
Age, y[a]	−0.07**	−0.04	−0.06*
Male gender, n (%)[a]	0.08**	−0.05	0.04
Low SES, n (%)	−0.05	−0.05	−0.03
BMI, kg/m^2	0.10***	0.13***	0.09**
Waist circumference, cm	0.09**	0.11***	0.07**
Smoker, n (%)			
Never			
Past	0.08**	0.05	0.06*
Current	0.06*	0.02	0.02
Dietary intake			
Total protein, g/d	−0.04	−0.02	−0.02
Animal protein	−0.03	−0.03	−0.02
Vegetable protein	−0.04	−0.01	−0.01
Total carbohydrates, g/d	−0.07*	−0.05	−0.05*
Total fat, g/d	0.01	0.04	0.04
Alcohol intake, g/d	0.10**	0.05	0.07**
Energy intake, kcal/d	−0.03	−0.01	−0.01
Use of multivitamins, n (%)	−0.16***	−0.16***	−0.16***
Hemodynamics, mmHg			
SBP	0.08**	0.02	0.03
DBP	0.04	−0.02	0.01

Table 15.3 (continued)

Thyroid function			
TSH, mU/L	0.03	0.04	0.02
Free T3, pmol/L	−0.07	−0.05	−0.07
Free T4, pmol/L	−0.15**	−0.15**	−0.14**
Liver parameters, U/L[b]			
ASAT	0.02	0.01	0.02
ALAT	−0.03	−0.05	−0.05
Alkaline Phosphatase	0.01	−0.01	−0.02
Gamma-GT	0.02	−0.05	−0.04
Lipids, mmol/L			
Total cholesterol	0.29***	−0.19***	…
HDL cholesterol	−0.05	−0.04	0.06
Non-HDL cholesterol	0.30***	−0.17***	−0.13
LDL cholesterol	0.24***	−0.19***	−0.22**
Triglycerides	0.28***	−0.01	…
Total lipid	0.37***	−0.14***	…
Kidney function			
Creatinine, mmol/L	0.05	0.01	0.02
eGFR, mL/min/1.73m^2	−0.04	−0.02	−0.02
Urinary albumin, mg/24 h	0.02	0.01	0.01
Uric acid, mmol/L	0.17***	0.12**	0.10*
Glucose homeostasis			
Glucose, mmol/L	0.10***	0.16***	0.10***
HbA1c, %	0.10***	0.16***	0.10***
Inflammation[b]			
High-sensitivity CRP, mg/L	−0.02	0.01	−0.01
Vitamins			
Urinary creatine/creatinine ratio	0.04	0.02	0.03
Vitamin B6, nmol/L	−0.19***	−0.19***	−0.21***
Vitamin B12, pmol/L	−0.19***	−0.18***	−0.19***
Methylmalonic acid, nmol/L	0.07**	0.08**	0.08**
Folic acid, nmol/L	−0.17***	−0.15***	−0.16***
Homocysteine, μmol/L	0.12***	0.09***	0.11***
Vitamin D3, nmol/L	−0.05	−0.06*	−0.04
Vitamin A, nmol/L	−0.05*	−0.08**	−0.07**
Vitamin A/total lipid	−0.24***	0.07**	−0.08**

Associations between γ-tocopherol and participant characteristics were tested via multivariable linear regression analyses of which standardized βs are presented (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

[a]Associations were adjusted for age or and gender, where applicable

[b]Data was available for 613 participants

Abbreviations: *ALAT* alanine aminotransferase, *ASAT* aspartate aminotransferase, *BMI* body mass index, *CRP* C-reactive protein, *DBP* diastolic blood pressure, *eGFR* estimated glomerular filtration rate, *GT* glutamyl transferase, *SBP* systolic blood pressure, *SES* socioeconomic status

ses, we found significant inverse associations with age, total carbohydrate intake, use of multivitamins, free T4, vitamin B6, vitamin B12, folic acid, vitamin D3, vitamin A, and vitamin A per total lipid. We found significant positive associations with male gender, BMI, waist circumference, past smoking, current smoking, alcohol intake, uric acid, fasting glucose, HbA1c, methylmalonic acid, and homocysteine. In similar analyses, with the quotient of γ-tocopherol over total lipids as independent variable, analogous to the analyses with α-tocopherol, we found associations with components of the

lipid domain, if present, to become inverted, and we found significant inverse associations with use of multivitamins, free T4, vitamin B6, vitamin B12, folic acid, vitamin D3, and vitamin A. We found significant positive associations of γ-tocopherol with BMI, waist circumference, uric acid, glucose, HbA1c, methylmalonic acid, homocysteine, and vitamin A per total lipid concentration. Finally, we performed analyses in which again crude γ-tocopherol concentrations were entered as independent variable, but in which now in addition to age and sex, total cholesterol concentrations and log-transformed fasting triglycerides were added as independent variables, thereby additionally adjusting the associations of α-tocopherol concentrations with listed variables for total cholesterol and fasting triglycerides rather than by correcting them directly by making a quotient. The results of these analyses did not materially differ from the results of the analyses with the crude data of γ-tocopherol.

Discussion

We found that in elderly people of the general population, total cholesterol and fasting triglyceride concentrations together explain a sizeable portion of 59% of the variation in α-tocopherol concentrations, while they only explain 14% of the variation in γ-tocopherol concentrations. For both α-tocopherol and γ-tocopherol, we found inversion of standardized regression coefficients from positive values for the crude concentrations of α-tocopherol and γ-tocopherol toward negative values for the total lipid corrected values, which may be indicative of overcorrection, particularly in case of α-tocopherol. Our further analyses with adjustment for total cholesterol and fasting triglycerides by means of linear regression rather than by calculating quotients of α-tocopherol and total lipids and of γ-tocopherol and total lipids revealed that, if overcorrection took place, it could not be solved by this, but, if anything, rather resulted in a higher degree of overcorrection. Interestingly, with γ-tocopherol, which appeared to have much lower strengths of associations with lipid concentrations, a similar pattern was seen, which suggests that overcorrection may not be so important, leaving the conclusion that one should not attempt to interpret associations of α-tocopherol and γ-tocopherol with lipid concentrations but use them to normalize or adjust for and then interpret associations in other biological domains. Irrespective of doing so or not, it appears that the most striking associations of circulating concentrations of α-tocopherol lie in the domain of vitamins in the one-carbon pathway and homocysteine, with particularly strong associations with vitamin B6 and folic acid and less of a sign of independent involvement in metabolism related to vitamin B12, because associations with vitamin B12 are relatively low and an association with methylmalonic acid is absent. These associations were similar irrespective of the way α-tocopherol concentrations were analyzed. Similarly, irrespective of doing so or not, it appears that the most striking associations of circulating concentrations of γ-tocopherol lie in the lipid- and glucose-related components of the metabolic syndrome, with clear and positive associations of γ-tocopherol with BMI, waist circumference, uric acid, fasting glucose, and HbA1c, all consistent with and adverse effect of high exposure to γ-tocopherol. Similar in this line, and consistent for whatever way of analyzing – crude, directly corrected for total lipids or indirectly adjusted via linear regression – γ-tocopherol is inversely associated with concentrations of vitamins in the one-carbon pathway and now not only positively associated with homocysteine but also with methylmalonic acid, indicative of either concomitant poor intake of vitamin B6, vitamin B12, and folic acid, or adverse interference of in the metabolic pathways associated with these vitamins.

We found that total cholesterol, fasting triglycerides, and total lipids represented by the sum of total cholesterol and fasting triglycerides explained around 60% of the variance of α-tocopherol. It should be realized that only 40% of the variance is left for finding other associations and that finding such associations is further hampered by the fact that all variables involved, including measurements of α-tocopherol, total cholesterol, fasting triglycerides, and that of the other variable under investigation, are invariably subject to measurement error and biological variation, which will further limit true

variation available for finding associations. Thus, these associations, particularly because they are consistently found for α-tocopherol concentrations themselves and for α-tocopherol concentrations indexed for total lipids or for total cholesterol and fasting triglycerides, must be relatively robust. We indexed our main analyses for total lipids. This is consistent with a recent publication on vitamin E and the metabolic syndrome [33]. Many studies, however, perform no indexing for circulating lipid concentrations, which obviously may induce findings that are actually driven by associations with circulating lipids rather than with α-tocopherol concentrations [8]. Other studies only index for total cholesterol or fasting triglycerides [35, 36]. It should be noted that indexing by fasting triglycerides likely is inferior to that by only total cholesterol, given the much lower explained variance of α-tocopherol by fasting triglycerides than by total cholesterol that we found, and that no indexing at all or indexing by either only total cholesterol or fasting triglycerides limits possibilities for comparison of findings on associations and effects in literature. We therefore present analyses in which we performed no indexing and in which we indexed for total lipids and for total cholesterol and fasting triglycerides. In these analyses, we found consistent inverse associations for α-tocopherol with free T3, free T4, and homocysteine. We also found consistent positive associations for α-tocopherol with use of multivitamins, circulating concentrations of vitamin B6, vitamin B12, folic acid, and vitamin D3. While it may not directly explain the inverse associations with free T3 and free T4, it seems not unlikely that the other associations can be explained by a higher multivitamin intake that also associates with higher α-tocopherol concentrations. To the best of our knowledge, we are the first to analyze potential associations of α-tocopherol with other vitamins and functional markers thereof. An old study suggests that α-tocopherol has no direct effect on B vitamins [24], but evidence provided by this study may not hold if the design of these studies would be scrutinized to current standards. It can therefore not be excluded that the nature of the associations of α-tocopherol with B vitamins is causal rather than by use of multivitamin supplements. If so, this would imply a potential role of α-tocopherol in transsulfuration or one-carbon metabolism.

Interestingly, we found the association between circulating α-tocopherol and γ-tocopherol concentrations to be positive, while most of the associations of γ-tocopherol with biological variables, if present, were opposite to those that we observed for α-tocopherol. For γ-tocopherol, we found consistent inverse associations with use of multivitamins, free T4, vitamin B6, vitamin B12, folic acid, and vitamin A. We found consistent positive associations for BMI, waist circumference, uric acid, fasting glucose, HbA1c, methylmalonic acid, and homocysteine. Several other studies have reported on opposing associations of α-tocopherol and γ-tocopherol with other biological variables, particularly in the pulmonary domain, and those of inflammation, vitamin D, and anemia, generally in directions that are consistent with beneficial if it concerns α-tocopherol and adverse if it concerns γ-tocopherol [9, 10, 22, 29]. Interestingly, we found the associations for γ-tocopherol in such a way that high γ-tocopherol is consistently associated with higher expression of several important components of the metabolic syndrome, including BMI, waist circumference, uric acid, fasting glucose, and HbA1c. This may indicate a true effect of γ-tocopherol on metabolism but could also be the consequence of the intake of a relatively more unhealthy diet, leading to higher circulating concentrations of γ-tocopherol or high circulating concentrations of γ-tocopherol being a marker for a relatively high intake of corn, soybean, canola, and sesame intake, all products with relatively high contents of γ-tocopherol compared to α-tocopherol [9], and possibly also a marker for an even more Western lifestyle-based diet than average in the Netherlands. This is also underscored by the consistent inverse association of circulating γ-tocopherol concentrations with vitamin supplement use and circulating concentrations of vitamins, even supported by positive associations with functional markers thereof, as represented methylmalonic acid and homocysteine. It could, however, also indicate that γ-tocopherol has adverse biological effects in either the domains of metabolic, syndrome, the transsulfuration pathway and one carbon metabolism or both. This also refers to intriguing and unexplained observation of inverse associations of α-tocopherol and γ-tocopherol with respect to free T3 and free T4. Like for the associations that we found in the domains of the metabolic, syndrome, the B-vitamins and functional

markers thereof, we could find little literature reporting on potential effects or associations of α-tocopherol or γ-tocopherol in the field of thyroid function, except an intriguing report on effects of α-tocopherol and γ-tocopherol on the liver transcriptome of chickens [15]. They found 5-deiodonase type 2 and 5-deiodonase type 3 among the most affected genes in the liver, which might give a hinge toward a true biological effect in humans, which might not only link circulating concentrations of α-tocopherol and γ-tocopherol to free T3 and free T4 but also provide a potential biological link to components of the metabolic syndrome, lipid metabolism, and the transsulfuration and one-carbon metabolism pathways, for which we found associations.

It should be acknowledged as a limitation that our study is cross-sectional in nature, which precludes drawing conclusions about cause and effect relations. Another limitation is the fact that we did not have access to data on α-tocopherol and γ-tocopherol in erythrocyte membranes, which could have given us further information on circulating concentrations of α-tocopherol and γ-tocopherol independent of lipids. A strength of our study is its relatively large size and the large number of clinical variables included, which provides the opportunity of recognizing patterns rather than associations with single biomarkers.

In conclusion, we found intriguing opposing associations of α-tocopherol and γ-tocopherol in the biological domains of metabolic syndrome, thyroid function, and B vitamins involved in the transsulfuration and one-carbon metabolism pathway supported by two important functional markers, namely, methylmalonic acid and homocysteine. We found these opposing associations despite a positive association between α-tocopherol and γ-tocopherol themselves. Our findings support the notion that circulating concentrations of γ-tocopherol, at least in this elderly population of the northern part of the Netherlands, have the characteristics of a biomarker associated with relatively poor health or an unhealthy lifestyle. It also provides the suggestion that α-tocopherol and γ-tocopherol, or their interplay, can have true metabolic effects, potentially by influencing the liver transcriptome and potentially the transcriptome of other tissues, e.g., adipose tissue, which together could affect metabolism in a way like we observed.

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Chapter 16
Stability of Vitamin E in Foods

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Keywords Vitamin E · Stability in foods · Foods storage · Vegetable oil · Adequate vitamin E intake Processing · Cooking

Key Points

- Vitamin E, which is known for its antioxidative capacity, is sensitive to oxygen, temperature, and light.
- For vegetable oils, the maturity of the seeds, the method of pressing, storage conditions, and ways of household usage are critical factors of impact on the foods' vitamin E content.
- Inappropriate storage of vegetable oils can result in vitamin E losses of more than 90% within 3 months.
- As vegetable oils are a rich source of vitamin E, inadequate handling may impact on vitamin E intakes.

Definition and Food Sources of Vitamin E

Besides vitamins A, D, and K, vitamin E belongs to the fat-soluble micronutrients. Vitamin E-active substances are defined as tocopherols, whereby the α-homolog has the highest activity. All tocopherols, namely, α-tocopherol (α-T), β-tocopherol (β-T), γ-tocopherol (γ-T), and δ-tocopherol (δ-T), consist of a chromane ring with a hydroxyl group but vary in their number and position of chromane methyl groups. α-T is often written as RRR-α-T, which stands for three chirality centers with the methyl groups positioned in the R-configuration. For α-T, its biological vitamin E activity is defined as 100%, whereas the vitamin E activity of β-T, γ-T and δ-T is lower than 50%.

α-Tocotrienol (α-T3), β-tocotrienol (β-T3), γ-tocotrienol (γ-T3), and δ-tocotrienol (δ-T3) are also part of the vitamin E group and are characterized by double bonds in their isoprenoid side chain, resulting in a very limited vitamin E activity compared to RRR-α-T.

All these vitamin E homologs can be found in different food sources. Especially vegetable fats, oils, nuts, grains, grain-based products, fruits, and vegetables contain vitamin E, whereof vegetable

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_16

Table 16.1 α-Tocopherol content of different food sources

Food source	α-Tocopherol in mg/100 g
Wheat germ oil	149
Dry roasted sunflower seed kernels	26
Blanched almonds	24
Margarine, 80% fat	15
Hazelnuts	15
Spinach (frozen and cooked)	4
Avocados	3
Radicchio	2
Butter	2
Egg (raw)	1
Cheese	<1
Chicken (raw)	<1

Data from USDA Food Composition Databases [1]

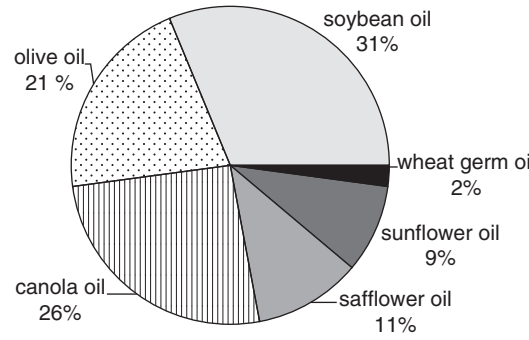

Fig. 16.1 Amount of different edible oils (in g) needed to reach the recommended daily intake of 12 mg α-tocopherol. (Data from USDA Food Composition Databases [1])

oils are considered to be the main dietary sources for meeting the recommendation for the daily vitamin E intake (Table 16.1).

Wheat germ oil, e.g., contains about 149 mg of α-tocopherol per 100 g [1]. Compared to other vegetable oils, this edible oil has the highest amount of total vitamin E (Fig. 16.1). Besides wheat germ oils, α-tocopherol, which has the highest biological activity among the tocopherol homologs, was found to be the predominant form in sunflower, safflower, and olive oils [2].

Although oil seeds are generally rich in vitamin E homologs, Stahl et al. [3] hypothesized that vitamin E from seeds may only be absorbed in low amounts since oil seeds are not easily digested in the human gastrointestinal tract. Therefore, cereals might present a suitable alternative. In the study of Panfili et al. [4], the authors measured a vitamin E concentration of 34 mg/kg dry weight in oats and 23 mg/kg dry weight in barley and soft wheat flakes, clearly demonstrating these foods as valuable sources for dietary vitamin E. However, α-tocotrienol is the most abundant homolog in these cereals, although the contents of tocopherols and tocotrienols greatly vary within different genotypes and growing locations. Compared to cereals and oil seeds as vegetable sources, only little amounts of vitamin E are found in animal products [1]. The study of Murcia et al. [5] showed that consumption of one boiled egg per day could provide approx. 10% of the vitamin E intake recommended per day.

Average Intake Recommendations

Recommendations for the daily vitamin E intake vary within different organizations. According to the WHO, the "best estimates of requirements" for vitamin E for adults in industrialized countries are 10 mg and 7.5 mg of α-tocopherol equivalents/day for men and women, respectively. This corresponds to the nutrient reference values for vitamin E for adults in the Nordic countries, albeit the adequate intake of vitamin E was estimated to 12–15 mg of α-tocopherol equivalents/day in German-speaking countries [6]. Especially oils and nuts, which consist of polyunsaturated fatty acids (PUFAs), are susceptible to lipid oxidation due to their unsaturated bonds. Consequently, the Scientific Committee on Food suggested linking the tocopherol intake to the intake of PUFAs, which is 0.4 mg tocopherol equivalents per gram of dietary PUFAs [6]. Usually, oil seeds high in PUFAs do also contain higher amounts of tocopherol equivalents [7]. All these recommendations are estimated based on population studies, dienoic acid equivalents, and plasma vitamin E concentrations, but there is no globally approved value for the vitamin E requirement. The difficulty for vitamin E recommendations is that there are no specific deficiency symptoms which can be only attributed to the absence of vitamin E, as it is the case with vitamin C and scurvy, for example. Often, the level of vitamin E intake in the population lies within the recommendation range, as it can be observed in Western countries. In the EU, 7.8–12.5 mg and 8.2–16 mg of α-tocopherol are consumed on average by women and men older than 18 years, respectively, as stated in a survey of EFSA NDA Panel [6]. This survey was conducted in Finland, France, Germany, Ireland, Italy, Latvia, the Netherlands, Sweden, and the UK and reported 13 mg of α-tocopherol for men and 11 mg of α-tocopherol for women to be an adequate intake. However, a recent study reported that more than 90% of the US American population does not meet the daily vitamin E requirements, although vitamin E-rich food sources are accessible to the population [8, 9].

Outcome Measures to Evaluate the Nutritional Status of Vitamin E

To evaluate the tocopherol level in the human body, the serum concentration is commonly analyzed after 12–24 h of fasting. For adults, an amount of 0.5–2 mg tocopherols/100 ml plasma (12–46 μmol/l) is recommended according to D-A-CH association [10]. In agreement with these results, a study conducted by Farrell et al. [11] stated that red blood cell hemolysis did not occur at mean α-tocopherol plasma levels of 11.5–14 μmol/l.

The nutritional status of vitamin E is often evaluated by measuring different blood parameters. In particular, the hemolysis rate of erythrocytes as well as the activity of creatine kinase (CK) in the plasma can provide an indication of vitamin E deficiency [12]. Vitamin E protects the highly unsaturated phospholipids in the cell membranes from lipid peroxidation. Peroxidation processes can lead to a decreased membrane integrity and, in turn, to the hemolysis of red blood cells. Once the erythrocyte membranes are destroyed, hemoglobin leaks into the plasma. In muscle cells, a reduced membrane integrity leads to a higher release of creatine kinase. Increased creatine or creatinine levels in the urine can, in addition to increased hemolysis, indicate a tocopherol deficiency [13].

Factors Influencing the Stability of Vitamin E

In general, vitamin E is available through various dietary sources which are accessible to the majority of the population. Nevertheless, losses of vitamin E in the course of heat processing or even during non-heat processing steps should be considered (Fig. 16.2).

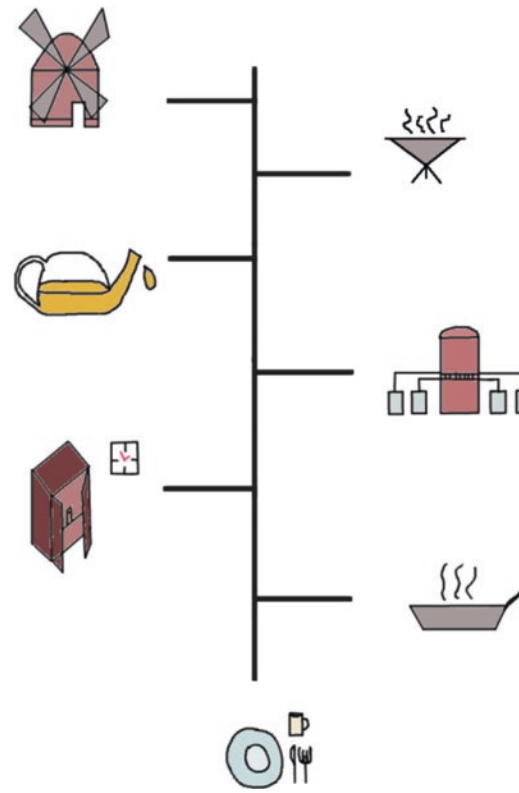

Fig. 16.2 Vitamin E losses in non-heat and heat processed food

Non-heat Processed Food

Plant Species/Varieties

Tocopherol and tocotrienol contents vary within and between plant species and varieties. Abdallah et al. [14] showed significant differences of tocopherol levels within six walnut (*Juglans regia* L.) cultivars. The contents of total tocopherols ranged from 187 mg/kg in the Local gd cultivar to 436 mg/kg in the Lauzeronne cultivar. Tsochatzis et al. [15] examined 12 barley varieties with regard to their contents in tocotrienols and tocopherols. The authors showed a pronounced difference between the varieties, with total tocopherols and tocotrienols ranging from 19 mg/kg to 32 mg/kg. These results are in accordance with a previous study carried out in 2008 by Panfili et al. [16], where 36 barley varieties were examined with respect to their different proportions of the eight vitamin E homologs α-T, β-T, γ-T, δ-T, α-T3, β-T3, γ-T3, and δ-T3.

The tocopherol content in pumpkin seed oil was remarkably influenced by the seed variety in the study of Nakić et al. [17]. The authors compared the tocopherol content of oil extracted from husked pumpkin seed variety (*Cucurbita pepo* L.) with oil samples extracted from a naked seed variety (var. *styriaca*) and found that the oil produced by husked pumpkin seeds contained significantly higher amounts of total tocopherols.

In addition to the variety, the influence of the geographic origin should not be underestimated. This became apparent in the study of Lavedrine et al. [18], where the authors reported a stronger influence on the vitamin E content due to the geographic origin than due to the variety of walnuts.

Impact of Maturity/Harvest Time

As immature seeds for soybean snacking are getting more popular, the time of harvest also needs to be considered. One study, conducted in 2009 [19], monitored the tocopherol content of mature soybeans as well as the amounts of tocopherols in soybeans harvested at three different reproductive stages (Fig. 16.3). Here, immature seeds picked at late reproductive stages showed highest levels of tocopherols, although the free radical scavenging activity and total antioxidant capacity were lower as compared to mature samples. For the tender (immature) seeds, δ-T was the predominant homolog with 70% of the total tocopherol content. Picking the soybeans at later stages also changed the amounts and proportions of the different tocopherol homologs. δ-T decreased, whereas the γ-homolog increased significantly until it reached 57.6% of total tocopherols in mature seeds. Also, α-T increased with later reproductive stages. Therefore, harvesting mature seeds instead of immature ones can contribute to a higher amount of tocopherols.

Impact of Peeling, Pearling, Milling, and Gamma Radiation

Grains and Cereals

The content of vitamin E in cereals and grains is highly influenced by the following actors: pearling process, milling degree, extrusion cooking, malting, baking, and storage conditions. The pearling process is a continuous removing of the seed coat, aleurone, and subaleurone layers which contain the majority of tocotrienols. Furthermore, the germ, which contains the majority of tocopherols, is removed, resulting in a polished grain and the by-products [20].

In the study on 36 barley cultivars by Panfili et al. [16], the authors showed that with progressive pearling steps, parts of the germ as well as of the aleurone layers are removed, with increasing losses of vitamin E, resulting in lowest vitamin E contents in the pearled kernel.

As it was shown in a study by Ha et al. [21], also milling plays an essential role for process-induced tocopherol losses. The authors observed a profound loss of vitamin E with increasing degrees of milling.

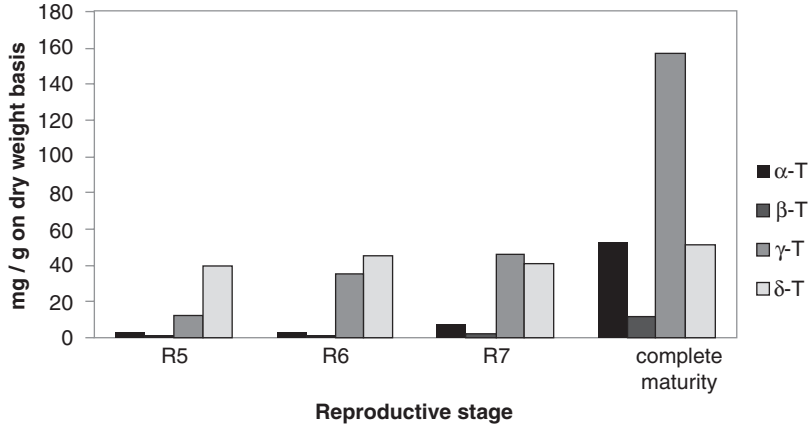

Fig. 16.3 Vitamin E concentration of soybean cultivar (R5 = seeds grow actively; R6 = pod cavity is completely filled but seeds are still green; R7 = beginning of physiological maturity). Different letters indicate statistically significant differences between the means ($P < 0.05$) for each homolog. (Data adapted from Kumar et al. [19])

The total vitamin E concentration in brown rice (no milling) was 37.67 mg/kg. Here, the tocopherol and tocotrienol levels contents decreased continuously from 21.75 mg/kg (5.6% of milling) to 16.91 mg/kg (8.0% of milling) and finally to 11.58 mg/kg (9.6% of milling).

Nuts and Seeds

Similar results were observed in nuts and seeds. The study of De Camargo et al. [22] demonstrated highest losses of 63% of α-T in peeled peanuts exposed to gamma radiation. In general, peeling is a gentler process than pearling. In comparison, only 44.2% and 37.6% of initial α-T levels were lost in in-shell and blanched samples, respectively. Nevertheless, it has to be mentioned that the lowest contents of this tocopherol homolog were found in blanched peanuts due to the removal of the peanut skin as induced by heat exposure. Blanched peanuts had a 40% lower α-T content than peeled samples. In this study, a negative correlation between tocopherol contents and irradiation doses was determined, with the highest decrease of γ-T levels recorded for peeled samples. With respect to storage stability, peeled peanuts were most affected by moderate tocopherol losses after radiation treatment.

Vegetable Oils

From the analysis of Nederal et al. [17], it can be concluded that the tocopherol content of vegetable oil obtained from husk pumpkin seed varieties contained more tocopherol (approximately 651 mg/kg) than the oil gained from the naked seed variety (454 mg/kg). Furthermore, the pressing method influenced the total tocopherol content. Industrially pressed pumpkin seed oils demonstrated significantly lower contents of vitamin E than solvent-extracted oils. Therefore, the usage of whole-grain products and husk seeds may help to exploit higher contents of vitamin E than only using white flour products.

Pressing

In another study of Nederal et al. [23], the authors examined again pumpkin seed oil, but now with the main focus on the influence of three different pressing methods on the total tocopherols. They either used cold-pressed unroasted pumpkin seed oil (UCP, <40 °C), oil pressed from unroasted pumpkin seeds (UP, approx. 90 °C), or oil pressed from roasted pumpkin seeds (110 °C for 45 min, RP). The latter method is used in order to obtain the typical nutty aroma. With regard to the tocopherol contents, the authors also monitored the difference between husk seed varieties and naked seed varieties, as in their previous study in 2006 [17]. Pressing of the husk seed variety led to a tocopherol yield of 776 mg/kg, whereas only 470 mg/kg was obtained with the naked seed variety. Pressing from roasted naked pumpkin seeds was most effective in terms of tocopherol extraction. It showed a 12% higher concentration compared to the unroasted pressed pumpkin seed oil and a 18% higher content than oil obtained from roasted pumpkin seeds. On the other hand, roasting contributed to an increased oxidative stability which may be due to the formation of Maillard-type reaction products acting as antioxidants. Generally, the authors showed that α-T was more susceptible to oxidation and degradation processes than the γ-homolog, whereby γ-T was the predominant homolog in pumpkin seed oils. Another positive aspect of roasting pumpkin seeds was the higher oil yield of approximately 90% after extraction compared to unroasted samples, where only 73% and 48% could be extracted from naked and husk seed varieties, respectively.

The tocopherol contents also vary with the extraction method applied. For canola oils obtained by different extraction methods, Ghazani et al. [24] demonstrated that the highest vitamin E levels were found in solvent-extracted canola oil compared to pressed oil samples. Also Azadmard-Damirchi et al. [25] showed that solvent-extracted canola oil demonstrated a greater amount of total tocopherols compared to cold-pressed oil, whereas hot-pressed canola oil showed higher levels of total tocopherols than cold-pressed samples. These results are in accordance with a previous study [26], where the tocopherol content was compared between rapeseed oils obtained from cold pressing (below 40 °C) and hot pressing methods. Moreover, conditioning at 80 °C for 30 min contributed to a significantly higher yield of α-T. In addition, a 4.4–14.6% higher yield was achieved in hot-pressed oils compared to cold-pressed samples of which approximately 67% of the initial oil content could be extracted. The higher yield can be explained by the conglomeration of small lipid droplets which can be extracted more efficiently than small droplets.

According to these studies, the impact of the different pressing methods on tocopherol concentration is diverse. Nevertheless, the data indicate a positive effect of solvent extraction on the tocopherol yield. Hand in hand with the different pressing methods, also the high impact of temperature needs to be considered for oil extraction.

Reaching the stage where oil is successfully extracted and ready to use, the stability of vitamin E is still influenced by different packaging methods and storage conditions.

Packaging and Storage

Vegetable Oils

A comparison between different packaging materials used for palm oil in Nigeria, namely, metal cans, white plastic bottles, glass bottles, and PET bottles, was carried out by Oluwalana et al. [27]. The authors monitored the changes of vitamin E over a storage period of 120 days in 30 day intervals under different storage conditions: refrigerated (5 °C), in a closed cupboard (27 °C), and under direct exposure to sunlight (35 °C).

Crude palm oil contains a high amount of vitamin E, but approx. 50% of the initial content are lost due to refining processes. In general, a decrease in tocopherol and tocotrienol contents was observed with increasing storage duration and temperature. Higher losses of vitamin E were shown when the oils were stored in the dark at 27 °C compared to refrigeration. Nevertheless, these losses were not as high as those when oil samples were stored under exposure of sunlight, regardless of the packaging material. For all storage conditions, palm oil stored in the metal can demonstrated the highest loss of vitamin E, whereas oil in white plastic bottles showed least pronounced losses (Table 16.2).

The beneficial effects of white plastic bottles might be due to a certain protection against sunlight and heat penetration. Under direct sunlight, vitamin E decreased by approx. 99% in the metal can during 120 days, whereas approx. 93% were lost in white plastic bottles. Storing palm oil in a closed cupboard

Table 16.2 Losses of vitamin E in % and Vitamin E contents in mg/ml after a storage period of 120 days

Packaging material	Refrigerated (5 °C)	Dark (27 °C)	Sunlight (35 °C)
Metal can	94%	92%^{d}	99%^{d}
PET bottle	86%	82%^{c}	99%^{c}
Glass bottle	73%	75%^{b}	94%^{b}
White plastic bottle	60%	62%^{a}	93%^{a}

Mean data from Oluwalana et al. [27]
Different letters indicate statistically significant differences ($P < 0.05$) in each column

Table 16.3 Decrease of γ-tocopherol (in %) in soybean oil after a storage time of 56 days

	Dark		Cold fluorescent light	
Tocopherol homolog	22 °C	32 °C	22 °C	32 °C
γ-tocopherol	69.4 ± 2.10[a]	62.1 ± 6.38[a]	77.3 ± 3.52[c]	85.4 ± 4.11[d]

Data from Pignitter et al. [28]
Different letters indicate statistically significant differences ($P < 0.05$)

at 27 °C led to losses of 92% in metal cans and 62% in white plastic bottles. At refrigerated storage conditions, vitamin E decreased by 94% in the metal can and by 60% in the white plastic bottle.

The influence of cold fluorescent light on the stability of tocopherols in soybean oil was examined in the study of Pignitter et al. [28]. Soybean oils were stored at 22 and 32 °C in the presence of cold fluorescent light or in the dark in PET bottles for 56 days. During 8 weeks of storage, all tocopherol homologs decreased, regardless of the storage conditions. Nevertheless, the highest losses were obtained in soybean oil stored at 32 °C with exposure to light. γ-T levels decreased by approximately 77.3% at 22 °C and by 85.4% at 32 °C when the oil had been exposed to cold fluorescent light. Storing the oil in the dark caused lower losses of tocopherols.

The tocopherol degradation has a major impact on the stability of the edible oils, as it is stated in the study of Player et al. [29]. These authors reported a positive correlation between increasing tocopherol degradation and increasing oxidation processes. In this case, the authors used a storage temperature of 50 °C over a period of 24 days to demonstrate the decomposition of all four tocopherol homologs (α-T, β-T, γ-T, δ-T) in soybean oil. α-T was degraded by 56% during the first 10 days and was completely decomposed after 16 days. In the first 16 days, γ-T and δ-T decreased by 23% and 13%, respectively. The losses reached a maximum of 28% for γ-T and 17% for δ-T after 24 days.

Vitamin E also decreased continuously with the time of storage of up to 35 days and heating temperatures of up to 110 °C in the study of Li et al. [30], which was carried out with fish oil, flax oil, sunflower oil, and palm oil. Fish oil contained the lowest amount of tocopherols and, after 35 days of storage, tocopherols contents were below the limit of detection. Storing sunflower oil for 35 days led to an amount of 141 ppm of tocopherols, compared to the initial content of 236 ppm.

In contrast to the aforementioned studies, no losses of tocopherol were shown in krill oil incubated at 20 °C and 40 °C. Here, vitamin E levels were monitored for 28 days and 42 days. To explain these results, the authors that astaxanthin esters (strong antioxidants) were degraded to the benefit of tocopherols in order to prevent the oil from lipid oxidation [31]. Another study conducted with rapeseed (*Brassica napus* L.) [32] found no difference in tocopherol levels in extracted rapeseed oil stored at 5 °C and 20 °C over a period of 24 weeks. However, with increasing temperatures up to 40 °C, significant losses in opened as well as in closed flasks were reached after 4 weeks already. After 16 weeks of storage, the decomposition continued up to more than 90% of the initial tocopherol content whereof α-T was more susceptible to degradation than its γ-homolog. The authors also investigated the losses in intact rapeseeds and showed lower vitamin E losses compared to ground seeds due to a reduced interaction of air oxygen and lipids. In contrast to the extracted oil, no losses were monitored in intact seeds. Only a slight decrease of tocopherols was shown in seeds stored in open flasks, subjected to air oxygen, at a temperature of 40 °C.

Seeds and Nuts

In order to improve the storage stability, drying of rapeseed is a commonly applied method. As stated in the study of Gawrysiak-Witulska et al. [33], the majority of rapeseed harvested in Poland usually is cleaned and dried in order to achieve a moisture content of 7%. In contrast to high-temperature drying, which was carried out either at 100 °C for 12–15 min, 80 °C for 15–20 min, or 60 °C for

36–42 min, the near-ambient temperature method showed slightly higher tocopherol losses, regardless of the height of the seed layer. This observation may be explained by a longer drying duration of 120–136 h, depending on the initial moisture content of the cultivar, when the seed layer was two meters. Storing the dried seeds at approx. 10 °C for 1 year led to a loss of 23–30% of total tocopherols. With regard to γ-T, the losses in two of the three rapeseed cultivars during storage occurred faster in samples dried at high temperatures than at near-ambient temperatures. Due to the decreased moisture content, also the activity of microorganisms is reduced which results in a higher stability of the seeds.

The moisture content of rapeseeds was also considered in another study of Gawrysiak-Witulska [34]. The authors showed an accelerated decrease of tocochromanols when the temperature and moisture content increased from 25 °C to 30 °C and from 10% to 15.5%, respectively. Chun et al. [35] investigated the change of the tocopherol content in peanuts stored under vacuum and air at 21 °C. Compared to raw peanuts, the roasted samples experienced higher losses, especially for α-T. After 12 weeks of storage under air, approximately 90% of α-T and 70% of γ-T were already lost, and no tocopherols could be detected after 38 weeks of storage. The oxidation processes were delayed when the roasted peanuts were stored under vacuum: after 38 weeks of storage, 26% of α-T still remained. The best results could be achieved with raw peanuts. Here, more than 70% of tocopherols remained after 38 weeks of storage under vacuum and under air. Due to the fact that peanuts are usually consumed after roasting, the study of Silva et al. [36] only monitored the tocopherol losses in roasted samples stored at 40 °C for 84 days. Generally, all tocopherols decreased with storage time, whereby 50% of the initial γ-T and 45% of the initial α-T remained after 84 days.

Also under refrigerated conditions (4 °C), approximately 30% of tocopherols decreased in walnuts stored for 3 months. Nevertheless, a longer storage time up to 9 months did not cause a further decrease in vitamin E [18].

A storage temperature of 0.6 °C and a relative humidity (RH) of 75% during 12 months caused no degradation of tocopherols in the three pecan cultivars examined by Yao et al. [37]. On the contrary, temperatures of 23.9 °C (60–70% RH) contributed to a significant decrease of total tocopherols.

Meat

As tocopherol is also present in meat, the packaging method had been investigated in several studies [38, 39]. Clausen et al. [39] examined the degradation rate of tocopherol in raw beef under modified atmosphere packaging (MAP). In raw meat, the α-T content was very variable, ranging from 0.84 to 4.91 μg/g. Vitamin E decreased with storage time, depending on the packaging type. The following packaging methods were used: (*1*) anaerobic packaging, (*2*) anaerobic packaging with exposure to air (21% O_2) for 2 days, (*3*) anaerobic packaging followed by 6 days of storage under high oxygen (50% or 80% O_2), and (*4*) high O_2 exposure (50% O_2) during the whole storage period. In terms of vacuum packaging, three different systems were used: (a) thermoforming (muscle), (b) thermoforming (steak), and (c) vacuum skin packaging (VSP, steak).

Compared to the average content of vitamin E in raw meat (2.2 μg/g), the anaerobic system (VSP) did not contribute to a high, but yet statistically significant, loss (1.9 μg/g). Following exposure to air, the α-T content continuously decreased each day, reaching 1.7–1.8 μg/g and 1.4–1.5 μg/g meat after 2 days and 5 days, respectively. Storing the meat in a high oxygen atmosphere for 20 days led to the most pronounced decrease of α-T. Therefore, the authors did not recommend a high O_2 atmosphere for storage packaging of meat.

Lagerstedt et al. [38] also described the tocopherol content of M. Longissimus dorsi steaks (beef) under modified atmosphere packaging (MAP, 80% O_2, 20% CO_2) and vacuum packaging. The steaks were frozen either directly or after storage of 5 or 15 days under MAP and/or vacuum. In contrast to vacuum, where α-T was not subjected to degradation, MAP induced a significant decline of

α-T. Samples, which were first packed under vacuum for 5 days and then moved to MAP for the next 10 days, showed the highest decrease of α-T.

A study carried out by Faustman et al. [40] showed a decrease of α-T levels in longissimus lumborum (LL) and psoas major (PM) samples of beef during 4 days of storage at 4 °C. At the same time, the oxidation products α-tocopherolquinone (TQ) and 2,3-epoxy-α-tocopherolquinone (TQE_2) increased, whereby 5,6-epoxy-α-tocopherolquinone (TQE_1) remained stable. Similar observations were made with PM muscles. These results show that tocopherol acts as an antioxidant during oxidation processes in meat as well. In LL steaks, the losses of α-T and accumulation of TQ + TQE_2 were higher in surface samples than in samples drawn from deep portions of LL. This is in accordance with the hypothesis that the exposure of tocopherol to oxidation processes is higher at the surface than in the anaerobic deep part of meat samples.

Bread

When tocopherol contents in the crust and crumb of a whole wheat bread were studied during 5 weeks of storage in modified atmosphere (nitrogen), the loss of tocopherol was higher in the crumb than in the crust. This finding might be explained by a formation of Maillard reaction products which act as antioxidants in the crust [41].

In order to benefit from higher tocopherol contents, it is of great importance to store the food in the absence of air oxygen, e.g., in air-tight bottles or in vacuum. In addition, storage in the dark with a focus on keeping the storage time to a minimum will prolong oxidation processes and protect tocopherols from degradation.

Stability of Vitamin E in Low Heat (<100 °C) Processed Food

The study of Elisia et al. [2] elucidated a significant decrease in tocopherol levels when 14 edible oils were heated at 56 °C over a period of 7 days. More severe losses were monitored when the temperature was set to 95 °C. α-T was the most sensitive tocopherol homolog to deterioration, regardless of the initial concentration. In addition, a positive correlation could be drawn between α-T and the formation of conjugated dienes (primary lipid oxidation products), which suggests a prooxidant activity of this homolog at high concentrations. The losses of vitamin E were also investigated in oils exposed to heat treatment at 95 °C for 1 day. In castor, soybean, and flaxseed oil, almost 100% of the initial α-T content was lost. The losses of this homolog in canola and sunflower oil were 52.4% and 49.1%, respectively, whereby y-T decreased by 12.2% and 30.6%, respectively. Yet, the number of controlled studies which consider low heat processed foods in the context of vitamin E stability is limited in contrast to high heat processed food. This aspect has to be examined in future research.

Stability of Vitamin E in High Heat (>100 °C) Processed Food

Refining

As stated in the study of Gogolewski et al. [42], bleaching, neutralization, and the final deodorization stage can result in losses of tocopherols. During refining processes, the authors monitored a total tocopherol degradation of more than 30% in the rapeseed oils of two subsequent crops. Thereby, δ-T

showed a higher decrease than the α-homolog. The loss of one-third of tocopherols was due to chemical degradation during neutralization and bleaching, whereas two-thirds were destroyed by deodorization (distillation and thermal degradation). These results are in accordance with a previous study of Ferrari et al. [43], in which the highest decomposition rates were noticed after deodorization steps. In corn oil, a twofold decrease was recorded, and also for soybean and rapeseed oils, 1.2–1.5-fold decrease in total tocopherols could be shown during refining. Moreover, the authors also stated that the relative proportions of the tocopherol homologs did not change significantly in the course of refining processes.

Heating

Vegetable Oils

As vegetable oils are used for common cooking methods, the potential losses of tocopherols during these processing methods have also been addressed. Malheiro et al. [44] showed a continuous decrease of vitamin E in the course of microwave heating of olive oil for 1, 3, 5, 10, or 15 min. At the beginning, α-T increased slightly, but after 1 min of microwave heating, the amounts decreased and almost disappeared after 10 min. In virgin olive oil samples, tocopherol contents rose until 3 min of microwave heating. The phenomenon of the initial increase could be explained by a better cleavage of α-T from the matrix compared to non-heated oil.

When olive oil was used for panfrying at 220 °C for 4 min [45], almost 100% of the initial tocopherol disappeared. In contrast, approx. 50% of the tocopherol concentrations remained when sunflower oil was used for frying. When considering the absolute losses in mg/kg, the tocopherol degradation was higher in sunflower oil, very likely due to its higher content of PUFAs compared to olive oil. After frying two slices of bread for 8 min, after preheating the oil for 4 min at 180 °C, less than 30% of vitamin E was degraded, regardless of which oil was used. Other studies showed controversial results [46–48]. Here, the highest loss was already detected during preheating of the oil, ranging from 10% loss of α-T in sunflower oil to 22% in olive oil. The applied temperature was recommended not to exceed 180 °C during preheating time, and the authors also suggested that bread should be put in the oil right after reaching the appropriate temperature. The tocopherol losses also depended on food-oil ratio as the oil, which had been absorbed by the food during frying, and the excess oil, which remained in the pan, was exposed to different temperatures. Tocopherol losses were lower in the bread-absorbed oil. In this study, all oil was absorbed by the bread slices. Therefore, it was assumed that the higher losses of tocopherols in the previous studies [46–48] were caused by the remaining oil in the pan. Tocopherol losses can be prevented to a certain extent by using a minimum amount of oil for frying. In the previously mentioned work of Chiou et al. [48], the impact of fortifying sunflower, olive, and palm oil with olive leaf extract, which did not contain Vitamin E, was studied. Here, higher levels of tocopherols were retained when the oils were fortified with the olive leaf extract before frying. Without supplementation, only 39% (olive oil) to 75% (palm oil) of vitamin E were retained, whereas 52% (olive oil) to 88% (palm oil) of the initial vitamin E content were retained after supplementation. The authors assumed that olive leaf extract protected the vitamin E from degradation.

In another study [49], heating at 150 °C for 6 h caused a loss of 47.5% of α-T when the oil was fortified with chlorogenic acid, instead of a loss of 68% without addition of chlorogenic acid. Also rosemary extract was demonstrated to protect tocopherols from oxidative degradation in cottonseed, soybean, and rice bran oil [50].

Bread

Processing-induced vitamin E loss in bread could be reduced by shortening the kneading time and intensity [51]. A total of 30% of the initial tocopherol content was demonstrated to be lost in the process of breadmaking of whole wheat bread [51]. This result was explained by the incorporation of oxygen during the kneading process and by baking the bread for 25 min at a temperature of 250 °C. In contrast, addition of germ to white wheat flour helped to increase the vitamin E content in industrial bread and wheat products.

Besides the direct oxidation of tocopherols during breadmaking, the enzyme lipoxygenase may also play an important role in lipid oxidation. As whole wheat bread also contains carotenoids, which are better electron donors than vitamin E, the lipoxygenase mostly affects carotenoids rather than tocopherols. Tocopherol in wheat bread decreased by 69% in the study of Nurit et al. [52], due to kneading processes and direct oxidation. In the same study, toasting wheat bread led to a significant increase of tocopherols. According to Nurit et al. [52], this result could not be explained by the drying processes, but by a more efficient extraction and release of tocopherols from the matrix.

The impact of extrusion cooking at 120, 160, and 200 °C on the vitamin E content was investigated for whole-grain wheat, barley, rye, and oats. This hydrothermal processing caused a decrease of 63–94% of vitamin E, depending on the grain cultivar. α-T and α -T3 were most sensitive to deterioration. A further study [53] examined the influence of extrusion of rice bran at 110, 120, 130, and 140 °C, with a post-extrusion holding time of 0.3 and 0.6 min and a subsequent storage time of 1 year. The highest level of vitamin E during storage could be retained in raw rice bran, a by-product of rice milling. Vitamin E decomposition rose with increasing extrusion temperature and holding time. This finding might be due to higher mechanical damage and pro-oxidative conditions during extrusion. After 1 year of storage at 22–26 °C, the total loss of vitamin E reached 73%.

Eggs

A study carried out by Murcia et al. [5] compared the extent of tocopherol losses in egg yolk subjected to common cooking methods, frying, and microwave heating. The authors came to the conclusion that the degradation of tocopherol was higher after microwave heating than after traditional boiling methods for 3 and 10 min. All the different tocopherol homologs were reduced by up to 50% during frying and microwave heating. Cooking methods are not the only factor which influences the vitamin E content in eggs. As shown in the study of Toyosaki T [54], it is also relevant, if eggs from hen or Silky fowl are used. The loss of vitamin E was higher in fried dough made with hen egg than with Silky fowl egg when stored in the dark for 12 days at a temperature of 50 °C. Although this study did not mention the type of food given to the animals, others demonstrated a clear influence of the feed ingredients on the egg composition. Omega-3 fatty acid-rich microalgae [55], flaxseed, and fish oil [56] were used for the feeding of laying hens in order to increase the omega-3 fatty acid content in the eggs. The resulting content of docosahexaenoic acid (DHA) increases the oxidative vulnerability of the egg yolk, making vitamin E more likely to decrease upon thermal treatment.

Vegetables and Legumes

With regard to the coating of vegetables, Miyagawa et al. [57] investigated if there is a difference in tocopherol levels between coated tempura-fried and non-coated french-fried potatoes. The tempura coating consisted of wheat flour, egg, and water, and both methods were fried in a mixture of soybean and rapeseed oil at 180 ± 10 °C over 17 min. A total of 52.5% of tocopherols were lost in the course

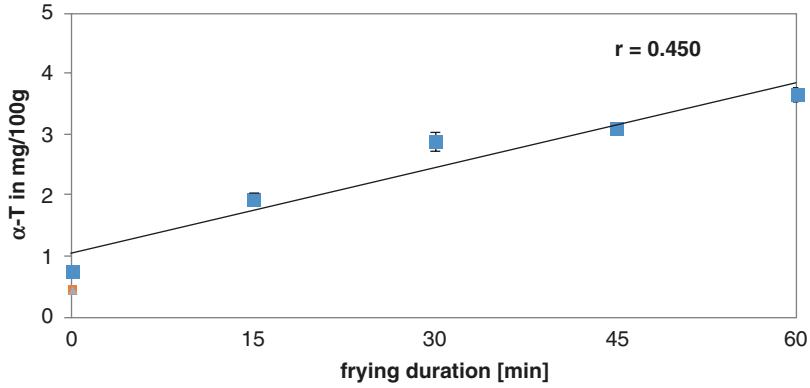

Fig. 16.4 Tocopherol content of spinach leaves fried at 250 °C in sunflower oil. (Data adapted from Zeb and Nisar [58])

of french-frying, whereas only 16.8% were degraded during tempura-frying. The authors assumed that the coating protects tocopherol from decomposition processes.

Another study, carried out in 2017 [58], showed an increase in α-T in spinach leaves with increasing frying time from 15 to 60 min (Fig. 16.4). The α-T level rose from approx. 0.73 mg/100 g (non-fried spinach leaves) to 2.87 mg/100 g (30 min) and finally reached 3.64 mg/100 g (60 min) (Fig. 16.4).

The leaves were fried in sunflower oil at 250 °C and also contributed to a higher oxidative stability of the frying oil. This finding is in accordance with a study conducted with potatoes, green peppers, zucchinis, and eggplants fried in virgin olive oil [47]. These raw vegetables contained very low tocopherol levels, but their α-T content was 6–30 times higher after frying. The authors also noted that tocopherol was more stable when it had been absorbed by the vegetables compared to tocopherol in the oil which remained in the frying pan. However, another study [48] found no significant difference of tocopherol contents between the oils which were absorbed by french-fried potatoes and the remaining oil in the pan. Generally, the tocopherol level was significantly higher in fried potatoes than in raw samples, whereas panfrying contributed to a loss of vitamin E in the different oil samples.

It is also important the vegetables are in a fresh or frozen condition before heat treatment. This was also demonstrated in the study of Bernhardt and Schlich [59], where the vitamin E content was higher in the frozen samples of red pepper and broccoli compared to the fresh samples. The various cooking methods, namely, boiling, stewing, steaming (approx. 100 °C), pressure steaming (approx. 120 °C), and microwave heating, had no significant effects when frozen vegetables were used. On the contrary, the content of α-T increased significantly in fresh broccoli during all cooking methods, but it was still lower than in the frozen samples. For fresh red pepper, no increase in tocopherols could be observed.

Different preparation methods of three flageolet-type beans and two dry seed beans were examined in the study of Słupski and Lisiewska [60]. The authors showed that boiling fresh beans led to a decrease in total tocopherols of approximately 13–28%, depending on the cultivar. A reduction of 17–31% in vitamin E activity was shown after boiling fresh beans. Beans undergoing blanching, followed by freezing (treatment I), experienced a reduction of total tocopherols by 16–43% compared to raw beans. Sterilized seeds (treatment III) contained 15–62% less vitamin E than the raw samples. Generally, the loss was greater compared to boiled seeds and frozen products. However, converting it to dry matter, no significant changes between canned and fresh beans were observed. Beans, which were boiled after freezing (treatment II), contained approx. 8–40% less total tocopherols and 12–43% lower vitamin E activity than the raw material after this treatment. Twelve months of storage led to a significant decrease of 36–53% for treatment I, 13–52% for treatment II, and 23–49% for treatment

III. In general, sterilized products retained more tocopherols than frozen ones when converted to dry matter, but this is not relevant for human nutrition as dry beans are usually not consumed.

Meat

Sabolová et al. [61] investigated the effect of thermal processing on tocopherol concentrations in minced meat and came to the conclusion that α-T degraded with increasing temperature and time of baking (200 °C for 10 min + 180 °C for 50 min; 250 °C for 50 min; or 200 °C for 10 min + 180 °C for 100 min). In the study of Kalogeropoulos et al. [46], tocopherol contents were monitored in eight finfish fried in virgin olive oil. Their low initial α-T level (<0.1 mg/100 g) significantly increased after frying and reached between 1.93 and 2.97 mg/100 g. They noted that the α-T in the frying oil was similar to that absorbed by the fish.

In order to keep tocopherol losses to a minimum, cooking should be carried out with lower heating temperatures and shorter heating times. However, frying of foods which contain low initial amounts of tocopherols can help to increase their tocopherol content by absorbing tocopherols from the oil used for frying, as reported by several studies.

Roasting

Vegetable Oils

Besides the cooking methods, also the roasting processes before oil extraction can be relevant with regard to tocopherol yields. In the study of Lee et al. [62], α-T in safflower seeds increased progressively with rising temperatures (140–160 °C) and was significantly higher than in unroasted samples. A possible reason could be an improved release of tocopherols due to damaged cell membranes. Oils from roasted mustard seeds showed higher amounts than oils from unroasted samples in the study of Vaidya and Choe [63]. During heating up to 160 °C, vitamin E decreased, but the losses were not as high as in the unroasted mustard seed oil. This finding is not in accordance with the observations made in the study of Shresta and De Meulenaer [64], in which 90 min of roasting mustard seeds at 180 °C caused a loss of 85% in α-T and a decrease of 40% of the γ-homolog. On the other hand, α-T was significantly higher in roasted rapeseeds than in the unroasted samples, but γ-T slightly decreased. Comparing this outcome with that of the study of Siger et al. [65], the results from Shresta and De Meulenaer [64] are contradictory. Here, α-T contents decreased with roasting processes. The decrease of γ-T occurred slower with rising temperature and time, and 15 min of roasting at 180 °C even caused an increase of γ-T compared to unroasted rapeseed oil. The authors assumed that different oil processing techniques (cold pressing vs. solvent extraction) could be the reason for these discrepancies. Another study conducted with sunflower seeds reported a significant decrease of tocopherols due to an increasing microwave roasting time from 5 to 15 min [66]. Other studies, where rapeseeds were roasted between 73 °C and 165 °C, reported no differences in tocopherol content between roasted and non-roasted samples [67–69]. However, no direct comparison between these studies is valid as different seeds and heat treatments were used.

Nuts

McDaniel et al. [70] tried to obtain similar surface colors of peanuts by a different combination of roasting time and temperature. In order to get a dark surface color, peanuts were roasted for 71 min at 147 °C, but when treated at 187 °C, the roasting time could be reduced to approximately 12 min.

Table 16.4 Impact of roasting on total tocopherols

Study	Roasting temp.	Roasting time (min)	Seed type	Increase of total tocopherol (%)	Decrease of total tocopherol (%)	No change in total tocopherol
Spielmeyer et al. [68]	73–161 °C	1.5–9	Rapeseed			X
Siger et al. [65]	160 °C	10	Rapeseed		2	
Lee et al. [62]	Up to 160 °C	19	Safflower	24		
Wakamatsu et al. [67]	165 °C	5	Rapeseed			X
Wijesundera et al. [69]	165 °C	5	Rapeseed			X
Shresta and De Meulenaer [64]	165 °C	10	Mustard (two varieties)		9–16	
Vaidya and Choe [63]	165 °C	30	Mustard	7		
Shresta and De Meulenaer [64]	165 °C	30	Rapeseed			X
Anjum et al. [66]	2450 MHz	10	Sunflower (two varieties)		15–20	

Tocopherols decreased continuously with increasing roasting temperatures between 147 °C and 177 °C. The tocopherol level remained higher when the peanuts were roasted at lower temperatures up to 167 °C and darker surface colors.

Roasting peanuts at 166 °C for 7 and 21.5 min contributed to higher losses of α-T than roasting for 77 min at the same temperature in the study conducted by Davis et al. [71]. After 4 days of storage at approx. 85 °C, the α-T level was higher than in raw peanuts. Seven minutes of roasting also caused the lowest amounts of γ-T in peanuts, which were slightly above 4 mg/100 g-seed weight. Both the samples which were roasted for 77 min and the oil extracted from the raw peanuts were higher than 8 mg/100 g-seed weight after 4 days of storage.

As summarized in Table 16.4, the outcome of roasting processes on tocopherol concentration is heterogenous. On the one hand, some studies point to an increased tocopherol content due to roasting, while others reported no significant influences or even a decrease of vitamin E content. Here, it can be assumed that the different seed varieties, roasting temperatures and times, as well as the usage of either an electric or microwave oven may contribute to this controversial outcome.

Outlook: Measures to Improve the Stability of Vitamin E in Foods

In order to achieve an adequate vitamin E intake, the diet should be balanced and varied, including vegetable oils. Additionally, adequate processing of vitamin E-rich products can help to prevent tocopherol losses to a certain extent. Vegetable oils should be stored in the dark at low temperatures and, if possible, in the absence of air oxygen. As shown in several studies, vacuum packaging of meat or nuts can be beneficial for the stability of vitamin E. Also during breadmaking, it can be advantageous to reduce the kneading time in order to inhibit a high incorporation of oxygen. Furthermore, it is recommended to keep the storage time to a minimum and consume the products soon after purchase.

Although not all of the food-processing technologies can be optimized by the consumer individually, almost every processing step has a high impact on tocopherol stability.

Harvesting seeds at complete maturity can be advantageous for the vitamin E content, even though the amounts vary within and between plant species and varieties.

As the aleurone layer and the germ, which contain the majority of vitamin E, are continuously removed with an increasing milling degree, the consumption of whole wheat bread should be favored. A study [51] also suggested to add vitamin E to white wheat flour. In terms of vegetable oils, husk seeds have a positive impact on the tocopherol content. Unfortunately, the oil yield gained from pressing husk seeds as well as using the cold-pressing method was found to be lower compared to naked seeds and pressing at higher temperatures. In order to inhibit the activity of microorganisms in rapeseeds, the moisture content is reduced by drying. There were only slight differences between near-ambient and hot temperature drying, which were both negligible compared to other processing steps such as refining. As near-ambient temperature drying is more energy-saving, albeit accompanied by slightly higher tocopherol losses, this method should be preferred. Deodorization steps during refining also lead to high vitamin E degradation; therefore, the use of crude oils should be favored.

In general, high heating temperatures and extended heating times are reported to accelerate vitamin E degradation processes. Frying, on the other hand, can contribute to an increase of tocopherols in products with initial low vitamin E levels, when vitamin E-rich vegetable oils are used for frying. Nevertheless, it is important to use as much oil as required but as little as possible for frying. Several studies also showed that high temperatures can lead to a better release of tocopherols from the matrix and, with regard to seed roasting, a beneficial effect on the oil stability could be observed due to the formation of Maillard-type reaction products which act as antioxidants [17]. Moreover, the oil yield can be increased after roasting the seeds. Therefore, roasting is recommended, albeit it is important to minimize the temperature and duration as several studies showed a decrease of tocopherols.

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Part III
Safety of Vitamin E and Interactions with Other Nutrients and Drugs

Chapter 17
Vitamin E and Mortality: A Critical Perspective of the Conflicting Meta-analysis Outcomes

Wolfgang Köpcke

Keywords Vitamin E supplementation · All-cause mortality · Meta-analysis · Meta-regression

Key Points

- Vitamin E intake is considered to be safe up to an established upper tolerable intake (UL) level of 300 mg/day by the European Food Safety Authority to 1000 mg/day by the Institute of Medicine.
- Nonetheless, concerns have arisen over the safety of high, chronic intakes of vitamin E as administered in vitamin E supplementation trials to test the efficacy of vitamin E in reducing all-cause mortality and fatality from cardiovascular disease and cancer in adult populations.
- In this meta-analysis which includes 68 studies with a vitamin E supplementation ranging from 16.5 to 5000 IU/d, the results show that with all different methods tested, there is no significant evidence for an increase in all-cause mortality caused by vitamin E supplementation.
- The results of this new meta-analysis might give guidance not only to the nutrition community but also to the health professionals so it is assured that emerging health benefits of vitamin E will be brought to the patients and are not hampered by publications which are no longer reflecting the current status of evidence.

Background

When evidence from individual trials is inconclusive or contradictory, a meta-analysis can enhance understanding of an issue by combining evidence from multiple trials. Yet, meta-analyses may also be contradictory or inconclusive since any given meta-analysis on a topic need not contain the same individual studies or employ the same statistical methodology as any other on that same subject. A 2004 meta-analysis [1], for example, found that the risk of all-cause mortality increased slightly but significantly for those subjects taking at least 400 IU/d vitamin E. In this meta-analysis, 19 clinical trials were included.

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_17

A meta-analysis in 2009 [2] included 29 studies. The results showed that the causal relationship of vitamin E supplementation and increased mortality is questionable. Different methodological approaches of meta-analysis yield contradictory results. Thus none of these results can be regarded to supply evidence in a statistical sense. In particular high-dose vitamin E supplementation cannot be regarded proved to increase mortality.

In 2011, a meta-analysis with 57 studies [3] was published. Based on this meta-analysis, supplementation with vitamin E appears to have no effect on all-cause mortality at doses up to 5500 IU/d.

In 2007 and 2012, meta-analyses [4, 5] were published. Based on this analysis, vitamin E seems to increase mortality. The selection of studies and the used methodology have been criticized heavily [6].

Material and Methods

In 2011, a meta-analysis with 57 studies [3] was published. Inclusion criteria were (1) peer review (indicated by publication in a peer-reviewed journal), (2) randomized treatment conditions, (3) comparator arms, (4) adult participants (excluding pregnant women), (5) parallel or factorial designs, and (6) the assignment of participants to supplemental vitamin E, taken orally, alone or in combination with other drugs and supplements for at least 1 year.

In our current meta-analysis, we used the same inclusion criteria. We added seven studies from the 2012 meta-analysis [5] – studies 04,12,25,39,51,57, and 60 – and four new published studies, studies 16,58,67, and 61. Thus, we performed a meta-analysis with 68 studies. Including the additional trials, dosages of vitamin E supplementation ranged from 16.5 to 5000 IU/d. Table 17.1 shows the data source underlying the statistical analysis of the present paper.

Table 17.1 Data source underlying the meta-analysis

Nr.	Study	Year	Vitamin E dosage (IU/d)	Vitamin E		Control		Follow-up in years	Mean age in years	% male
				Deaths	Participants	Deaths	Participants			
01	Avenell	2005	10	8	456	4	454	1	72	96
02	Girodon	1997	15	12	41	13	40	2	84	25
03	Girodon	1999	15	100	361	106	364	2	83	25
04	Bonelli	1998	30	1	147	0	157	5	57	62
05	CTNSARC	2009	30	77	510	81	510	9	68	55
06	Blot	1993	33	1018	14,792	1109	14,792	5.25	55	50
07	Hercberg	2004	33	76	6481	98	6536	7.54	49	39
08	Chandra	1992	44	0	48	2	48	1	74	73
09	Pike	1995	45	1	24	0	23	1	69	28
10	Wright	2006	50	6709	14,564	6671	14,569	6.1	58	100
11	Li	1993	60	157	1657	167	1661	6	54	44
12	Liu	2007	74	96	379	97	384	1.6	85	30
13	Takamatsu	1995	136	1	74	0	73	6	47	37
14	Meydani	2004	200	39	311	44	306	1	85	34
15	ARMDS	1996	200	2	39	2	32	1.5	72	93
16	Belch	2008	200	115	640	80	636	6.7	60	63
17	You	2001	200	38	1706	43	1705	3.25	47	51
18	Graat	2002	272	3	336	5	316	1	73	50
19	Salonen	2003	272	19	390	4	130	6	60	49
20	Cook	2007	300	502	4083	493	4088	9.4	61	0

Table 17.1 (continued)

21	Lee	2005	300	636	19,937	615	19,939	10.1	55	0
22	GISSI	1999	330	488	5660	529	5664	3.5	59	85
23	Roncaglioni	2001	330	72	2231	68	2264	3.6	64	58
24	Bukin	1997	400	0	18	0	18	1	55	39
25	Collins	2003	400	1	26	1	26	2.5	67	98
26	Mooney	2005	400	1	142	0	142	1.25	37	55
27	Milman	2008	400	11	726	12	708	1.5	69	48
28	De Waart	2001	400	0	109	1	109	1.8	60	10
29	McKeown-E	1988	400	4	96	3	89	2	58	66
30	Bairati	2006	400	102	273	77	267	3	63	79
31	Hodis	2002	400	2	177	1	176	3	56	45
32	Lonn	2005	400	799	4761	801	4780	4.5	66	74
33	Lippman	2008	400	717	17,767	760	17,766	5.46	62	100
34	AREDS	2001	400	251	2370	240	2387	6.3	68	45
35	Sesso	2008	400	841	7315	820	7326	8	64	100
36	Greenberg	1994	440	15	433	29	431	4	61	79
37	Antoniadi	2008	500	1	33	2	25	1	60	40
38	de la Maza	1995	500	5	37	4	37	1	49	85
39	Manuel-Y-	2004	500	1	12	0	12	4.5	51	86
40	Richer	2004	500	0	30	3	60	1	75	97
41	Wluka	2002	500	1	67	0	69	2	64	42
42	Magliano	2006	500	9	205	17	204	4	64	45
43	McNeil	2004	500	20	595	11	598	4	66	44
44	Pathak	2005	578	54	64	64	72	2	58	83
45	Stephens	1996	600	68	1035	52	967	1.4	62	84
46	Plummer	2007	600	10	990	7	990	3	48	47
47	Takagi	2003	600	10	51	16	42	5	63	40
48	CLIPS	2006	660	7	185	4	181	2	66	77
49	Chylack	2002	660	9	149	3	148	3	68	41
50	MRC/BHF	2002	660	1446	10,269	1389	10,267	5	67	70
51	Garbagnati	2008	700	1	34	3	38	1	65	65
52	Bugianesi	2005	800	0	28	0	82	1	42	83
53	Fang	2002	800	0	19	0	21	1	51	88
54	Boaz	2000	800	31	97	29	99	1.42	64	69
55	Brown	2001	800	1	84	1	76	3	53	7
56	Waters	2002	800	16	212	6	211	3	65	0
57	Tam	2005	800	1	20	1	19	2.67	46	0
58	Sanyal	2010	800	1	84	0	83	1.85	45	40
59	Desnuelle	2001	1000	34	144	35	144	1	64	55
60	Mezey	2004	1000	4	25	5	26	1	58	66
61	Sano	2016	1000	28	168	16	169	3	54	60
62	Stevic	2001	1200	3	16	6	12	1	57	75
63	Singh	2007	1200	1	42	2	43	2	59	74
64	Sano	1997	2000	19	170	22	171	2	73	35
65	Marras	2005	2000	154	399	142	401	2.6	61	66
66	Petersen	2005	2000	5	257	18	512	3	73	54
67	Dysken	2014	2000	26	152	31	152	2.27	78	97
68	Graf	2005	5500	31	83	28	77	1.5	58	65

We used different approaches of meta-analysis including fixed and random effect models, categorical dose-response models, models adjusted by covariates, and continuous dose-response models with linear splines. Mathematical details were published in 2009 [2].

Data analyses of the present paper were performed using the statistical package R version 3.4.1 [7].

Results

Figure 17.1 is a forest plot with all studies. The plot contains the following information for every study: study number, first author, number of deaths in the vitamin E group, total number of participants in the vitamin E group, number of deaths in the control group, total number of participants in the control group, a graph of the odds ratio with 95% confidence interval, the odds ratio, the 95% confidence interval, the weight for the fixed effect model, and the weight for the random effect model.

On the bottom of the plot, you find the total numbers of study participants: 124,836 in the vitamin E group and 124,925 in the control group. A heterogeneity test with a p-value of $p = 0.36$ is shown. This nonsignificant result means that the random effect model could be used. One could see that the random and fixed effect models are nearly equal. The numbers in Table 17.2 show the results with more digits. The odds ratios, the 95% confidence intervals, and the p-values indicate that there is absolutely no difference between vitamin E and control treatment.

The forest plot shows that the studies 10 and 50 have a weight of more than 10% of the complete meta-analysis. Therefore, in a sensitivity analysis, the meta-analysis was performed without study 10 and without studies 10 and 50. In both cases, the heterogeneity test did not change. Therefore, the random effect model was adequate, and the results are nearly identical with the all-studies meta-analysis. In the further analyses, we did not remove studies 10 and 50.

Table 17.3 shows the results of the continuous dose-response model. The linear increase of log(vitamin E dose) is not significant. If possible covariables (length of follow-up, mean age, percent of males) are added to the model, the linear increase remains not significant. None of the covariables prove to be significant. So biased effect estimates due to confounding could be ruled out.

In order to analyze the dose-response relationship of vitamin E supplementation and mortality, additionally a categorical model could be used. In this case, certain fixed cut point separating high and low dosages has to be determined. Table 17.4 shows the results for cut points 300, 400, 500, 600, and 700.

None of the cut points show a significant result. As in the continuous model, all covariables did not change the results.

Discussion

Vitamin E intake is considered to be safe up to an established upper tolerable intake level (UL) of 300 [8]–1000 mg/day [9] (depending on the regulatory authority) and, more generally, in amounts naturally occurring in foods or in multivitamin supplements as acknowledged by expert groups of the IOM, the EFSA, and UK's Expert Group on Vitamins and Minerals (EVM) [9]. Nonetheless, concerns have arisen over the safety of high, chronic intakes of vitamin E as provided in many stand-alone vitamin E supplements, stemming from an accumulation of evidence from vitamin E supplementation trials over the past 30 years to test the efficacy of the vitamin in reducing all-cause mortality and fatality from cardiovascular disease and cancer in North American and European adult populations. The design, inclusion of trials, and methods applied to address safety of vitamin E in the published meta-analyses vary considerably, and some are dating back more than a decade. Given the emerging

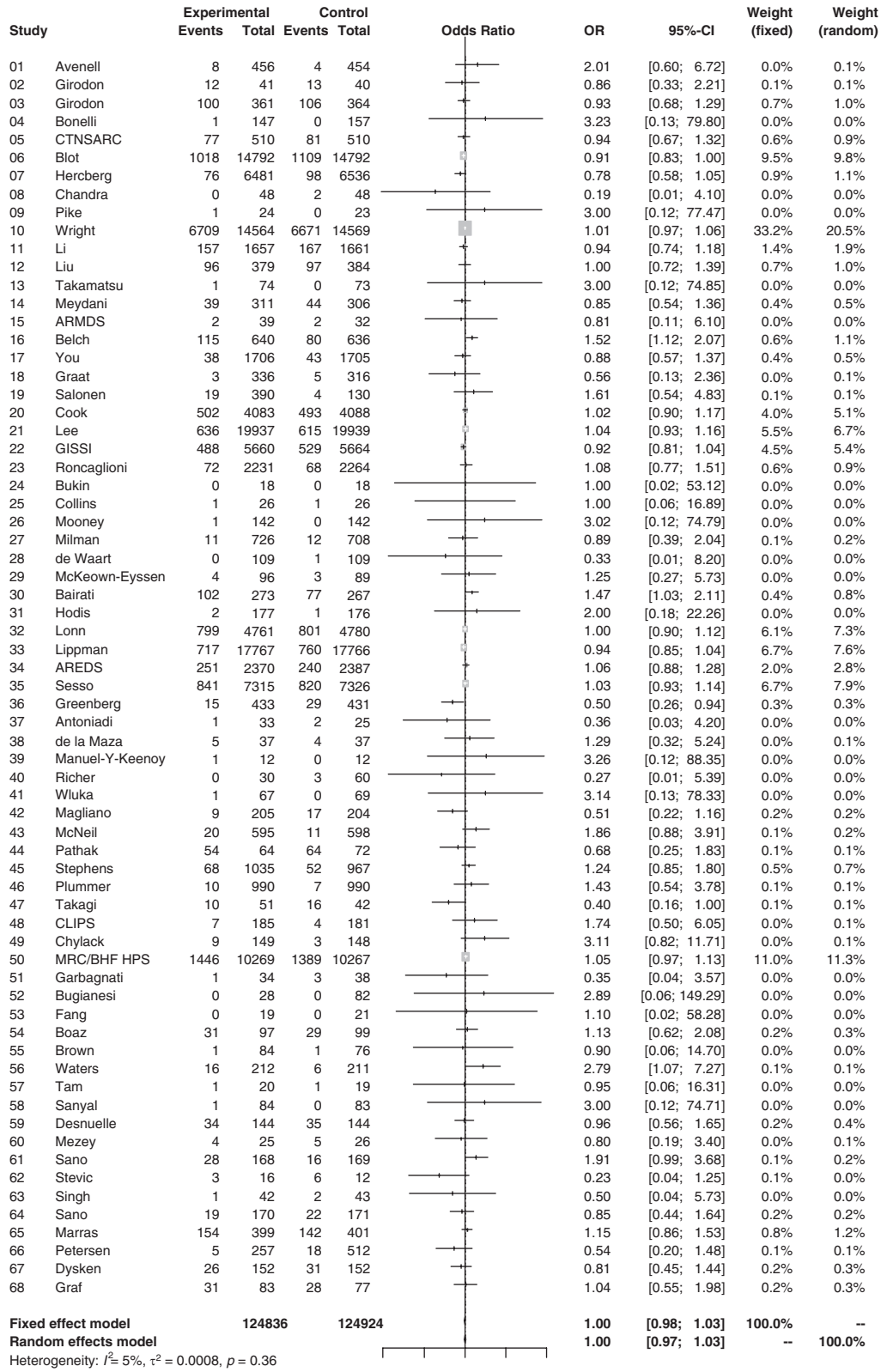

Fig. 17.1 Forest plot all studies

Table 17.2 Sensitivity analysis for fixed and random effect model

	Fixed effect model			Random effect model			Residual heterogeneity
Studies	Odds ratio	95% confidence interval	*p*-value	Odds ratio	95% confidence interval	*p*-value	*p*-value
All studies	1.002	[0.976; 1.029]	$p = 0.8851$	1.001	[0.971; 1.032]	$p = 0.9431$	$p = 0.3569$
All studies without study 10	0.997	[0.965; 1.031]	$p = 0.8739$	0.999	[0.961; 1.039]	$p = 0.9710$	$p = 0.3321$
All studies without studies 10 and 50	0.987	[0.953; 1.024]	$p = 0.4885$	0.992	[0.952; 1.033]	$p = 0.6968$	$p = 0.3548$

Table 17.3 Continuous dose-response model

Model	Odds ratio	95% confidence interval	*p*-value
All studies	1.024	[0.996; 1.053]	$p = 0.0960$
All studies with covariables	1.024	[0.993; 1.057]	$p = 0.1301$
Covariable follow-up	1.007	[0.988; 1.026]	$p = 0.4850$
Covariable mean age	1.001	[0.994; 1.007]	$p = 0.7992$
Covariable percent of males	1.000	[0.999; 1.001]	$p = 0.8549$

Table 17.4 Categorical dose-response model

	Low dose			High dose		
Cut point	Odds ratio	95% confidence interval	*p*-value	Odds ratio	95% confidence interval	*p*-value
300	0.977	[0.925, 1.032]	$p = 0.2804$	1.016	[0.973, 1.060]	$p = 0.3039$
400	0.984	[0.943, 1.028]	$p = 0.3039$	1.023	[0.974, 1.076]	$p = 0.2561$
500	0.991	[0.959, 1.024]	$p = 0.3410$	1.057	[0.980, 1.139]	$p = 0.1356$
600	0.991	[0.959, 1.024]	$p = 0.3383$	1.060	[0.982, 1.145]	$p = 0.1254$
700	0.999	[0.968, 1.031]	$p = 0.3967$	1.070	[0.896, 1.278]	$p = 0.2958$

evidence on the beneficial role of vitamin E to reduce, for instance, the risk of nonalcoholic fatty liver disease (NAFLD) [10], cardiovascular disease in patients carrying a particular genotype [11], and even improving the condition of patients with Alzheimer's disease [12], many of which are reported to occur at intakes/doses which are a multiple of the current RDA, we deemed it critical to revisit the evidence addressing the safety of vitamin E relating to all-cause mortality. In our current meta-analysis which includes 68 studies with a vitamin E supplementation ranging from 16.5 to 5000 IU/d, the results show that with all different methods tested, there is no significant effect of different dosages and of possible covariables (length of study, mean age, percent of males). Thus, on the basis of the published trials, there is no clear evidence for an increase in all-cause mortality caused by vitamin E supplementation, and vitamin E supplementation seems to be safe even at intakes/doses which are several times higher than the current RDA. It would go beyond the scope of this chapter to discuss the shortcomings and limitations of all the previous meta-analyses; however, the nature of some of these publications which have been quoted more frequently is being highlighted below.

Findings from 19 population-based RCTs that delivered 17 to 2000 IU of vitamin E daily were summarized in a meta-analysis in 2005, pointing to a significant 4% increased risk of all-cause adult mortality with dosages ≥400 IU/d while there was evidence of a lower all-cause mortality risk in trials that tested consumed dosages ≤150 IU/d [1]. Further breakdown of the risk by vitamin E dose and

adjustment for other vitamin and mineral supplements revealed that the increased risk of death was statistically significant only at a very high dose of 2000 IU/day, which is many times the recommended amount. Bjelakovic et al. reached a similar conclusion in a meta-analysis of 46 primary or secondary prevention trials with low risk of bias in 2013, finding a nonsignificant 2% increased risk of all-cause mortality in adults associated with use of vitamin E alone (across a daily dosage range of 10–5000 IU) versus placebo and a significant 3% increased risk in trials employing a dosage of vitamin E that was above the RDA [13]. Again, experts raised serious doubts about the conclusions as they were drawn from flawed meta-analysis pooling data from trials with heterogeneous populations (healthy and diseased individuals) and different methodologies [14].

Many human long-term studies with higher doses of vitamin E have not reported any adverse effects: three other meta-analyses that combined the results of randomized controlled trials designed to evaluate the efficacy of vitamin E supplementation for the prevention or treatment of cardiovascular disease found no evidence that vitamin E supplementation up to 800 IU/day significantly increased or decreased cardiovascular disease mortality or all-cause mortality [2, 15–17]. A meta-analysis of 68 randomized trials found that supplemental vitamin E, singly or in combination with other antioxidant supplements, did not significantly alter the risk of all-cause mortality [18].

In conclusion, when discussing the safety of vitamin E using all-cause mortality as an endpoint, it appears to be prudent to consider the totality of evidence and to bear in mind as well that vitamin E is an essential micronutrient. This means that it must be crystal clear that vitamin E at the recommended daily intakes (RDA) is safe, and it is important that these intakes are being met. In addition, there is emerging and encouraging evidence that vitamin E can reduce the risk of some of the so-called noncommunicable diseases like NAFLD, CVD, and even Alzheimer's disease at doses which are substantially higher than the RDA. For these patients, from a real-life perspective, an individualized risk-benefit analysis should be considered for each setting and indication [19]. The results of this new meta-analysis might give guidance not only to the nutrition community but also to the health professionals, so it is assured that emerging health benefits of vitamin E will be brought to the patients and are not hampered by publications which are no longer reflecting the current status of evidence. After all, it is encouraging that an essential micronutrient such as vitamin E has two target applications – (i) meeting daily intakes and (ii) possibly reducing risk of NCDs.

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Chapter 18
Vitamin E-Drug Interactions

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Keywords Nutrient-drug interactions · Tocopherols · Tocotrienols · Pharmacokinetics Pharmacodynamics

Key Points

- Vitamin E-containing food supplements are often taken by persons who also take prescription drugs.
- Potential nutrient-drug interactions thus need to be considered.
- Nutrients in general can alter the pharmacokinetics and/or the pharmacodynamics of drugs by a number of mechanisms.
- There is no evidence for vitamin E-drug interactions at vitamin E intakes achievable by diet in the scientific literature.
- High-dose (≥300 mg/d) supplementation of vitamin E, especially of α-tocopherol, may lead to interactions with aspirin, warfarin, tamoxifen, and cyclosporine A.
- For the majority of drugs, interactions with vitamin E, even at high doses, have not been observed and are unlikely to occur.

Introduction

Isolated micronutrients, in the form of dietary supplements, are widely consumed, with some variation between countries. In the United States, 52% of adults (NHANES 2011–2012) and 72% of persons older than 65 years report the use of food supplements [1]. In Germany, only 28% of the population regularly take dietary supplements [2]. Thirty-four percent of American and 11% of German adults use food supplements containing vitamin E [1, 2]. Vitamin E supplements may contain doses of up to 1000 IU per serving, which is 45–60 times higher than the RDA of 22 IU per day for adults in the United States [3] or 16–22 IU per day recommended by the German, Austrian, and Swiss

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_18

nutrition societies [4]. Given that older subjects are not only more inclined to consume vitamin E-containing dietary supplements (see above) but also to suffer from age-dependent disorders and may thus be taking one or more prescription drugs, the potential interactions of vitamin E with drugs are of rising concern.

Definition of Nutrient-Drug Interactions

While our diet contains macromolecules to satisfy our energy needs, it also contains many minor components such as vitamins, minerals, and phytochemicals. Some of these can affect the efficacy of concurrently ingested drugs. Consuming them as part of a normal diet can lead to nutrient-drug interactions, but the risk is potentiated when isolated compounds are consumed at high doses. There are two major forms of nutrient-drug interaction: pharmacokinetic and pharmacodynamic nutrient-drug interactions.

A *pharmacokinetic nutrient-drug interaction* occurs when a compound present in our food increases or decreases the uptake, metabolism, or excretion of a concurrently administered drug. For example, consuming 200 mL of grapefruit juice before the oral administration of midazolam, a sedative, is sufficient to markedly increase the peak plasma concentration of the medication [5]. If a compound increases drug bioavailability or decreases drug clearance, higher plasma concentrations can increase side effects, especially when there is a narrow therapeutic window. On the other hand, compounds can decrease the bioavailability, or increase the clearance, of drugs. This leads to reduced concentrations of the drug at its site of action, ultimately impairing the efficacy of the drug. An example of a nutrient-drug interaction that reduces the bioavailability of a drug is the herbal remedy St. John's Wort. Supplementing St. John's Wort extract for 2 weeks (900 mg/d) can significantly decrease the bioavailability of an oral dose of the heart medication digoxin [6]. Both grapefruit and St. John's Wort are examples of pharmacokinetic drug interactions.

A *pharmacodynamic nutrient-drug interaction* directly influences the target of the drug (e.g., an enzyme, cellular receptor, transcription factor, etc.), a required cofactor or another part of the drug's mechanism of action, and leads to a direct modulation of the drug's efficacy. An example of a pharmacodynamic nutrient-drug interaction is the combination of vitamin K supplements with the anticoagulation drug warfarin. Warfarin inhibits vitamin K epoxidase, an enzyme required for the recycling of vitamin K and the maturation of vitamin K-dependent coagulation factors in the liver [7]. By increasing the amount of vitamin K in the diet, the anticoagulant effect of warfarin can be compensated and the drug's activity be impaired. Indeed, patients with low basal vitamin K status are at risk for anticoagulant treatment failure with sudden changes in vitamin K intake [8].

Pharmacokinetic Interactions of Vitamin E and Drugs

An Introduction to Drug Metabolism

About 1200 million years ago, the eucarya branch of the phylogenetic tree of live started to branch out into different subgroups, including animals and plants [9]. One can assume that shortly after the separation, animals discovered plants as a source of energy and nutrients, much to the dismay of the stationary plants. To avoid their fate as food, plants developed new metabolites that would render them toxic or unpalatable to their opponents. But food was scarce, and animals coevolved enzymes and transporters to render these compounds harmless, and soon an arms race between plants and animals had begun. Today, the enzymes and transporters involved in the metabolism of phytochemicals are

called xenobiotic or drug-metabolizing enzymes and are responsible for detoxification of food-based compounds but also play a role in endogenous and drug metabolism. These proteins are especially important for lipophilic compounds that cannot be readily excreted in their native form.

In the 1940s, Richard Tecwyn Williams proposed a simple scheme for classifying drug metabolism reactions: phase I and phase II. Phase I enzymes introduce functional groups, such as a hydroxyl group, and make the xenobiotic more reactive for the next step during which phase II enzymes use these reactive groups as a docking site for hydrophilic moieties, such as glutathione or glucuronic acid [10]. Examples for enzymes catalyzing phase I reactions are the cytochrome P_{450} mixed-function monooxygenases (CYP), alcohol dehydrogenase (ADH), and monoamine oxidase (MAO). Phase II enzymes include UDP-glucuronosyltransferases (UGT), glutathione-*S*-transferases (GST), and sulfotransferases (SULT). Some authors have even proposed the term phase III to describe the selective cellular export of xenobiotics and/or their phase II conjugates [11]. The classification into phases has been questioned, as many compounds do not neatly follow such a linear chain of phase I and phase II reactions and phase III transport, but may only undergo one or two of them [12]. However, in this chapter we will follow this classification system, aware that it is not perfect, as a guideline to describe the effects of vitamin E on the metabolism of drugs.

Interactions Affecting Intestinal Uptake and Metabolism

The human intestine is lined with transporters and enzymes to maximize nutrient uptake and to defend against foreign and potentially damaging compounds. A prominent example of such a defense mechanism is the family of multidrug resistance proteins (MDR), also known as ATP-binding cassette transporters. One member of this family, P-glycoprotein (P-gp, also known as MDR1 or ABCB1), is expressed on the apical surface of enterocytes [13] and actively transports substrates from the enterocyte back to the lumen. P-gp thus limits the bioavailability of its substrates [14], which in turn will affect their plasma concentrations and thus their biological (therapeutic) activity. Induction of intestinal P-gp is most likely the underlying mechanism for the observed pharmacokinetic nutrient-drug interaction between St. John's Wort and digoxin [6].

The regulation of P-gp expression and activity is complex and involves multiple pathways. Both can be regulated transcriptionally, by posttranslational modifications, or through changes in membrane fluidity [15]. Transcription, again, is regulated by multiple factors including heat shock transcription factor 1 (HSF1), nuclear factor Y (NF-Y), and the pregnane X receptor (PXR) [15]. Research on how different vitamin E congeners regulate P-gp has focused on the transcriptional activation of PXR. In vitro, the activation of a nuclear receptor can be measured by using reporter gene assays. Here the nuclear receptor of interest is co-expressed with a reporter gene, typically luciferase, and the luciferase expression is then monitored as luminescence emitted upon addition of the luciferase substrate luciferin. The four T, and T3, have been tested in a reporter gene assay for their potency to activate PXR in intestinal LS 180 cells. None of the four T but all of the four T3 (10 μmol/L, 48 h) activated PXR and induced the expression of P-gp mRNA [16, 17]. In the same cell line and assay, one of the metabolites of α-T, α-13'-COOH, was a potent activator of PXR [17]. To investigate if activation of PXR and induction of P-gp mRNA translate to increased function, P-gp activity can be determined by measuring the efflux of the fluorescent substrate Rhodamine 123. γT3 (25 μmol/L, 48 h) and the αT metabolite α-13'-COOH (20 μmol/L, 48 h) increased P-gp protein expression and activity in cells [17, 18]. Interestingly, although αT3 activates PXR [16, 17] and induces P-gp mRNA expression [16], these changes are not paralleled by an increase in protein expression or activity [17].

Besides efflux transporters, the intestine also expresses phase I and phase II enzymes to facilitate the functionalization and conjugation of lipophilic compounds. The interaction between grapefruit juice and the CYP3A4 substrate midazolam [19] is a classic example of a nutrient-drug interaction

mediated by intestinal xenobiotic enzymes, leading to a higher bioavailability of midazolam [5]. Expression of CYP3A4 in LS180 cells remained unchanged in response to all four T and T3 [16]. Accordingly, intestinal CYP3A4 protein expression was unaltered in guinea pigs supplemented with RRR-αT for 6 weeks (250 mg/kg diet vs. 20 mg/kg diet, 6 weeks) [20]. Taken together, there is no evidence to suggest that any of the eight congeners can affect intestinal CYP3A4 expression or activity. Besides phase I metabolizing enzymes, the intestine also expresses phase II enzymes such as glutathione-S-transferase. NAD(P)H-quinone oxidoreductase (NQO1) inactivates reactive quinones, generated through phase I reactions, to less reactive hydroquinones, while GST conjugates either the parent compound or a phase I metabolite to glutathione. In intestinal cells (Colo205), NQO1 activity was dose-dependently increased by αT (0.0001–10 μmol/L; 48 h), while GST activity was unaffected [21]. Another phase II enzyme UGT1A1, which belongs to the family of UDP-glucuronosyltransferases (UGT), glucuronidates their substrates to increase water solubility. γT3 and all the T did not influence UGT1A1 mRNA expression in LS180 cells, while it was increased twofold by α-, β- ,and δ-T3 (10 μmol/L; 24 h) [16]. In accordance with the in vitro data, αT did not affect total UGT activity in rats, which received 200 mg/kg αT, compared to control rats [22]. Whether the increase of UGT mRNA expression by α-, β-, and δ-T3 translates to relevant effects on activity in vivo remains to be elucidated.

In vitro studies suggest that high-dose γT3 and the long-chain metabolite α-13'-COOH have the potential to upregulate intestinal P-gp expression and activity, but the relevance in vivo is unknown. α-13'-COOH could theoretically be present in the intestine through biliary excretion, especially after high-dose supplementation with αT, and this should be further investigated. In vitro studies also showed modest effects on NQO1 and UGT1A1 activity, but no in vivo studies indicate that T or T3 alter intestinal drug metabolism through these two enzymes.

In summary, there is limited evidence from in vitro experiments to suggest a potential interaction of vitamin E congeners and metabolites with the drug efflux transporter P-gp, while interactions with intestinal phase II enzymes seem unlikely. Further studies are needed to investigate the effect on P-gp in vivo.

Interactions Affecting Hepatic Uptake and Metabolism

Before compounds can be modified or conjugated by the liver, they need to pass the hepatocyte membrane. Depending on their molecular structure and physicochemical properties, some (small lipophilic) compounds can penetrate the membrane, while others (larger and hydrophilic compounds) need the help of transporters. One of these transporters in the liver is the organic anion transporting polypeptide C (OATP C also known as OATP1B1 or OATP2). OATP C is expressed in the sinusoidal membrane of hepatocytes [23] and is responsible for the uptake of a diverse set of substrates. In addition to drugs, such as the lipid-lowering pravastatin [24, 25], OATP C is also responsible for the uptake of endogenous compounds such as 17-β-glucuronosyl estradiol [26].

Daily subcutaneous αT injections (100 mg RRR αT/kg bodyweight) reduced hepatic OATP C mRNA expression in rats after 1 week of treatment compared to control animals [27, 28]. 100 mg RRR-αT/kg bodyweight is a very high dose and would amount to 7 g RRR-αT in a 70 kg human. This dose would be 5000 IU/d, and thus outside the realm of physiological, and even pharmacological, doses. Feeding guinea pigs the lipid-lowering drug atorvastatin, an OATP C substrate, and either 20 or 250 mg/kg/diet αT did not alter hepatic OATP C protein expression, the plasma concentration, or the pharmacodynamics of the drug [20]. The differences in finding between the two aforementioned animal experiments may be caused by the difference in dose, species, or the route of administration. Injection of high doses of αT may lead to a higher production of long-chain metabolites that may exert functions different from the parent compound. Although high doses of subcutaneously administered

αT did decrease hepatic OATP C mRNA expression, there is currently no evidence for clinically relevant drug interactions with oral supplementation. Besides αT, none of the other congeners have been tested.

Interactions Affecting Hepatic Phase I Metabolism

T and T3, like many lipophilic compounds, are substrates for the hepatic cytochrome P_{450} system (CYP). CYP are part of the hepatic phase I metabolism and oxidize, reduce, or hydrolyze drugs. The most likely CYP family member involved in vitamin E metabolism is CYP4F2, which hydroxylates the terminal methyl group and initiates vitamin E metabolism [29, 30]. CYP3A4 another member of the cytochrome P_{450} enzyme family is involved in the metabolism of approximately 50% off all drugs [31]. As CYP3A4 plays such an important role in drug metabolism, numerous studies have investigated the effect of vitamin E on CYP3A4 expression in vitro and in vivo.

One of the nuclear factors regulating CYP3A4 expression is PXR [32]. In a reporter gene assay in the human hepatoma cancer cell line HepG2, all T3 activated PXR up to tenfold (50 μmol/L), while T showed a maximum two-time activation (50 μmol/L) [16, 33]. The lower activation potential of T than T3 also translates to changes in PXR target gene expression CYP3A4. In human primary hepatocytes, T3 (10 μmol/L) induced CYP3A4 mRNA threefold to fivefold, while T had no effect [16]. RRR-αT and all-rac-αT also did not increase CYP3A4 mRNA expression after 7 days of incubation with doses up to 300 μmol/L in HepG2 cells [34]. The latter experiment found 13 CYP expressed in HepG2 cells, and the only CYP that dose-dependently increased was CYP20A1, an orphan CYP that has yet to find its substrate [34]. The in vitro studies suggest that T3, but not T, may increase CYP3A4 gene expression, but until now, no data on protein expression or activity exists.

In vivo the effect of high-dose supplementation as well as vitamin E deficiency on CYP3A4 expression has been investigated. The murine homologue of CYP3A4, Cyp3a11, is decreased in animals fed with a vitamin E deficient compared to a sufficient diet (2 vs. 20 mg RRR-αT /kg diet) for 3 months [35]. Feeding them more than the sufficient diet (20 vs. 0.200 mg RRR-αT /kg/diet) for 9 months did not alter the expression of CYP3A4 mRNA [35]. Vitamin E deficiency for up to 9 months in rats (<2 mg or 60 mg RRR-αT/ kg diet) did not affect any of the 33 CYP, including CYP3A4, investigated [34]. Feeding mice with very high doses of all-rac-αT acetate (1000 mg/kg diet) induced hepatic CYP3A4 mRNA expression fourfold compared to control mice on a standard diet (35 mg all-rac-αT acetate/kg diet) [36]. 1000 mg/kg/diet for a mouse is a nonphysiological dose that may have little relevance for humans. Although T3 show a higher potency to induce CYP3A4 expression in vitro, only γT3 has been investigated in vivo, and it did not affect the expression of Cyp3a11 mRNA after 7 days of oral supplementation (250 μg/d) [35]. All the studies above measured the effect as changes of mRNA transcription, which may not necessarily translate to altered protein expression or enzyme activity. Protein expression of CYP3A4 was unaltered in guinea pigs supplemented with RRR-αT (20 mg/kg diet compared to 250 mg/kg diet) for 6 weeks [20]. The same study also investigated if the CYP3A4-mediated metabolism of atorvastatin as well as its lipid-lowering effect would be affected by αT. No changes in atorvastatin metabolite concentrations (a marker of CYP enzyme activity) or the ability to reduce circulating lipids were observed. This study strengthens the in vitro data that αT does not affect CYP3A4 expression and more importantly that there is no interaction between T and CYP3A4 substrates.

The most compelling evidence for an absence of an interaction of αT with CYP3A4 comes from human studies. There was no change in the activity of the lipid-lowering drugs simvastatin or lovastatin, which are both CYP3A4 substrates, when they were taken together with 400 mg/d RRR-αT acetate for 8 weeks [37]. The same study also estimated CYP3A4 activity by determining urinary cortisol and its CYP metabolite 6-β-hydroxycortisol. In accordance with the absence of changes to the

efficiency of drugs, there was also no effect of αT on CYP3A4 activity [37]. Midazolam, a sedative, is a CYP3A4 substrate and is considered to be a good probe for its activity [38]. In healthy human volunteers, the pharmacokinetics of intravenous midazolam were investigated before and after 3-week supplementation with 750 IU αT or placebo [39]. As the drug was intravenously administered, the intestine was circumvented and only hepatic CYP3A4 activity determined. The area under the plasma concentration-time curve (AUC) for midazolam did not differ between subjects receiving αT and placebo indicating that CYP3A4 activity was unchanged by vitamin E [39].

In summary, there is no evidence for vitamin E-drug interactions due to alterations in hepatic CYP-mediated phase I metabolism in animals and humans. T3 induce CYP, especially CYP3A4, in vitro, but up to now, only one of the T3 was investigated in vivo where it did not affect CYP3A4 mRNA expression. While interactions between αT and CYP3A4 are unlikely, clinically relevant interactions between the other congeners and T3 cannot be ruled out, and further studies are warranted.

Interactions Affecting Hepatic Phase II Metabolism

Just like intestinal cells, hepatocytes also express phase II enzymes to conjugate either the parent compound or the phase I metabolites to polar compounds, such as glucuronic acid, sulfate, glutathione, or amino acids, to aid biliary and urinary excretion. mRNA expression of UGT, the enzyme we have already discussed in the intestine section, is unaffected by all eight vitamin E congeners in primary human hepatocytes (10 μmol/L; 24 h) [16]. In accordance, 2 weeks of supplementation with αT (200 mg αT/kg/diet) in rats did not affect hepatic UGT activity [22]. In contrast to UGT activity, GST activity was upregulated in rats supplemented with αT for 10 days (2500 mg/kg/diet) [40]. Similarly, GST mRNA expression in mice supplemented for 4 months (1000 mg all-rac-αT/kg) was also increased [36]. Both studies employed very high doses of αT which raises the question if GST activity and expression would be altered under normal dietary conditions. As both studies that observed an increase in phase II metabolism were conducted at concentrations that are unrealistic to occur in the human body upon oral supplementation, there is no convincing evidence that αT or T3 influence phase II metabolism.

Interactions Affecting Hepatic Export

P-gp, the transporter we have already discussed in the section dealing with intestinal metabolism, is also expressed in hepatocytes [13] and secretes substrates, including vitamin E and its metabolites [41], into the bile duct. In primary human hepatocytes, none of the eight T or T3 (10 μmol/L; 24 h) induced P-gp mRNA expression [16]. Hepatic P-gp mRNA expression did increase (1.9-fold) in mice fed with a high-dose all-rac-αT acetate diet (1000 mg/kg vs. 35 mg/kg diet) for 4 months [36]. Elevating hepatic T levels even further by daily subcutaneous injections of RRR-αT (100 mg/kg bodyweight) increased P-gp mRNA expression by 3.6–10-fold, depending on the solvent system used [27]. The same experiment also showed a corresponding increase of P-gp protein expression by about threefold [27]. High doses of αT induced P-gp mRNA as well as protein expression in a mouse model. The doses, however, again were very high and unlikely to be achievable in humans.

In summary, although an interaction of αT with hepatic P-gp cannot be excluded, especially at very high doses, it seems to be unlikely under physiological conditions.

Potentially Clinically Relevant Pharmacokinetic Interactions

Tamoxifen and Vitamin E

As reported by the WHO, breast cancer is the number one cause of death in women worldwide. Breast cancer can be divided into cells that express the estrogen receptor (ER+) and are thus responsive to estrogen or cells that do not express the receptor (ER-). ER+ breast cancer can be treated with the estrogen receptor modulator tamoxifen [42], but many patients develop resistance [43]. Tamoxifen inhibits proliferation in the ER+ cell lines MCF-7 and T47D. If the cells are treated with tamoxifen and αT (10 μmol/L; 24 h), proliferation is no longer inhibited, and the cells become resistant to tamoxifen [44, 45]. In a small prospective trial, seven breast cancer patients treated with tamoxifen received 400 mg/d αT acetate for 30 days. In five of the seven patients, the supplementation with vitamin E reduced the plasma concentration of tamoxifen, with four of the patients dropping below the minimum therapeutic concentration [46]. Tamoxifen is metabolized by the liver, with CYP2D6 and CYP3A4 playing a major role [47], and as the plasma concentrations of the parent drug decreased after supplementation, a pharmacokinetic interaction is feasible. CYP3A4 seems to be an unlikely candidate as in vitro and in vivo research suggests that this CYP3A4 is not affected by vitamin E supplementation (see "Interactions Affecting Hepatic Phase I Metabolism"). Interactions may be mediated through CYP2D6 or some other unknown pathway. Given that tamoxifen itself is a prodrug and needs to be metabolized into its active form, it is unclear if the reduced concentration of the parent compound necessarily implies a reduction in therapeutic efficacy. Even though the cell culture data suggested a possible loss of activity with vitamin E supplementation, there is no in vivo evidence to support this. Nonetheless, it might be undesirable for women on tamoxifen therapy to take vitamin E supplements. Additional sufficiently powered clinical trials are required to investigate this interaction further.

Cyclosporine A

Transplant patients receive the immune-suppressant cyclosporine A to prevent graft rejection. Nephrotoxicity is a common side effect of cyclosporine A and is believed to be mediated by an increase in oxidative stress in the kidney [48]. To investigate if this side effect could be alleviated by antioxidants, renal transplant patients were supplemented daily with 800 IU αT, 1000 mg vitamin C, and 6 mg β-carotene or placebo for 6 months. Surprisingly, the antioxidant cocktail did not affect markers of oxidative stress but led to a decrease of the cyclosporine A plasma concentration by 24% [49]. Three-month treatment with a similar cocktail (1000 mg vitamin C and 300 mg αT) also decreased cyclosporine A plasma concentration in renal transplant patients [50]. A 30% reduction in cyclosporine A plasma concentrations, compared to placebo, was observed retrospectively in heart transplant patients supplemented for 3 months with vitamin E and C (800 IU vitamin E and 1000 mg vitamin C/day) [51]. All the above studies investigated vitamin E and C in combination, making it difficult to judge which one is affecting cyclosporine pharmacokinetics. One study investigated the effect of a 6-week supplementation with 800 IU/d αT on the pharmacokinetics of 5 mg cyclosporine A in healthy volunteers. Glomerular filtration rate and peak plasma concentrations were not affected, but the area under the plasma concentration-time curve significantly decreased (21%) compared to baseline [52]. All recorded time points, except the maximum plasma concentration, were lower after vitamin E supplementation, suggesting that a pharmacokinetic interaction with αT may have occurred. Cyclosporine A is primarily metabolized by CYP3A4 [53], but as outlined above,

interactions mediated by CYP3A4 are unlikely. Supplementing more than 300 mg/d αT has been shown to affect the pharmacokinetics of cyclosporine A in four independent clinical trials [49–52]. The underlying mechanism for the pharmacokinetic interaction between cyclosporine A and vitamin E remains unclear. Patients receiving cyclosporine A should avoid taking high-dose vitamin E supplements, or drug concentrations should be closely monitored and the dose of the drug adjusted accordingly.

Pharmacodynamic Interactions of Vitamin E and Drugs

The activity of a drug can be influenced by decreasing or increasing its metabolism and thereby altering the amount of drug available at the target site. These potential pharmacokinetic interactions have been described in the previous section. A nutrient can also directly alter the pharmacodynamics of a drug by interacting with the drug target, cofactors, or independent targets such as signaling molecules. A prominent example would be the combination of a coumarin derivative with vitamin K as described in the section "Definition of Nutrient-Drug Interactions".

Interactions Affecting Blood Coagulation

The US Food and Drug Administration (FDA) states in their consumer fact sheet for vitamin E: "Taking vitamin E with a blood-thinning medication such as Coumadin can increase anti-clotting activity and may cause an increased risk of bleeding." Coagulation is usually initiated upon injury or damage to a vessel when collagen, which is normally hidden underneath the epithelium, is exposed. Circulating platelets can adhere to the exposed collagen via surface receptors (glycoprotein Ia/IIa) and release, together with the injured endothelium, signaling molecules to activate additional platelets as well as the intrinsic and extrinsic coagulation pathways.

The intrinsic coagulation cascade is triggered by contact of coagulation factor XII with collagen, while the extrinsic pathway is triggered by the release of tissue factor (TF) from the damaged endothelium. Both cascades include several enzymes which are synthesized in the liver as precursors. Maturation of these factors involves vitamin K-dependent carboxylation by the γ-glutamyl carboxylase. Platelet adhesion at the side of injury is stabilized by fibrin, also known as factor Ia. The fibrin precursor fibrinogen is polymerized by thrombin, which itself is activated by cleaving its precursor prothrombin (factor II). Platelets contain granules which they secrete upon activation. One of the components of the granules, thromboxane A2 (TXA_2), induces platelet aggregation by linking glycoprotein Ia/IIa with fibrinogen, forming a dense network. TXA_2 is a metabolite of arachidonic acid that is synthesized via the cyclooxygenase (COX) intermediate PGH_2 [54]. The rate-limiting enzyme in the synthesis of TXA_2 is COX [55], which is also the target for aspirin [56], explaining the reduced platelet aggregation after aspirin treatment.

Vitamin E and Acetylsalicylic Acid

Vitamin E has been suggested to inhibit platelet aggregation and multiple studies have investigated either isolated platelets from patients supplemented with vitamin E or platelets preincubated with αT. Platelet activation involves multiple pathways, and agonists include ADP, TXA_2, collagen, and adrenaline. In vitro, platelets can be activated by either one of the agonists above or phorbol myristate

acetate (PMA). PMA is a protein kinase C (PKC) activator which, among other things, activates platelet granular secretion and TXA_2 synthesis.

TXA_2 and PGD_2 formation from arachidonic acid was dose-dependently decreased in isolated human platelets pretreated with 0.02–16.2 μmol/L RRR-αT, which may suggest changes in COX activity [57] that could ultimately lead to a reduction of platelet aggregation. Indeed, human platelets preincubated with 500 μmol/L RRR-αT showed an inhibition of arachidonic acid or PMA-induced aggregation compared to non-treated platelets [58]. Although 500 μmol/L is a very high αT concentration, the intracellular concentrations of αT in the incubated platelets were comparable to those of platelets isolated from humans supplemented with 800 mg αT/d for 14 days [59]. Platelets isolated after supplementation for 14 days with 267, 533, or 800 mg αT/d showed less aggregation after in vitro induction [59]. In a subset of two patients from this study, PKC activity was decreased after 2 weeks of supplementation with 800 mg/d RRR-αT [59]. Inhibition of PKC has been previously linked to αT [60–62] and may be the underlying molecular mechanisms.

Ex vivo platelet adhesion was also dose-dependently reduced by all-rac-αT in 12 healthy subjects (400, 800, and 1200 mg/d, 2 weeks each, total of 6 weeks) when exposed to collagen. The same study also observed that in women, collagen-induced platelet aggregation was significantly reduced, while ADP or adrenalin showed no effect [63].

Acetylsalicylic acid (aspirin), an analgesic, antipyretic, and anti-inflammatory drug, reduces platelet aggregation by inhibiting COX-mediated TXA_2 formation [64]. Combining αT with aspirin may lead to a synergistic decrease in platelet aggregation. Aggregation of platelets isolated from patients treated with aspirin (300 mg every other day) combined with up to 1200 mg all-rac-αT/d did not differ from platelets isolated from patients receiving only aspirin or only all-rac-αT [63]. Gingival bleeding on probing was significantly higher in a subset of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study population receiving aspirin and αT for 5–7 years than in subjects only receiving aspirin [65]. While this may be an undesirable synergism in some situations, in the case of ischemic events that involve a platelet aggregation, the synergistic effect may be desirable. Indeed, patients receiving αT and aspirin after an ischemic event had a lower risk for recurring ischemic events than patients that only received aspirin [66].

In summary, vitamin E and acetylic acid seem to act synergistically on the reduction of platelet aggregation, and while this may be desirable in some situations, like in patients at risk for CVD or stroke, it can be undesirable, as in cases of increased gastrointestinal bleeding, a known side effect of aspirin [67]. The mechanism of this interaction seems to involve protein kinase C-mediated changes in platelet aggregation, but more research is needed to understand the interaction in detail.

Vitamin E and Warfarin

Besides inhibition of protein kinase C, vitamin E may indirectly affect coagulation by altering vitamin K metabolism. As vitamins E and K are both metabolized via cytochrome P_{450} enzymes, perhaps even the same isoform CYP4F2, interactions are possible [68–70]. As mentioned earlier, several coagulation factors (II, VII, IX, and X) are carboxylated by hepatic γ-glutamyl carboxylase, an enzyme that requires vitamin K as a cofactor. To carboxylate coagulation factors, vitamin K is oxidized. The oxidized form is then recycled through the enzyme vitamin K epoxide reductase. Warfarin, an anticoagulant, inhibits vitamin K epoxide reductase, thus reducing the amount of vitamin K available for carboxylation of factors II, VII, IX, and X [71]. The combination of warfarin with vitamin E might thus potentiate the anticoagulatory activity of the drug, if vitamin E influences vitamin K metabolism. A reduced coagulation activity was observed in rats injected for 7 days with intramuscular all-rac-αT (1 IU/g bodyweight) combined with warfarin injections, when compared to rats treated with the agents individually [72]. Coagulation activity in patients receiving warfarin and 4 weeks of either

100–400 IU/day all-rac-αT (n = 12) [72] or 800–1200 IU/day vitamin E (congener not specified) (n = 13) remained unchanged [73]. Although both trials are limited in sample size, they do suggest that doses up to 1200 IU/day do not lead to changes in coagulation in patients on warfarin therapy. Even though interactions are unlikely, vitamin K status and coagulation activity in patients intending to start vitamin E supplementation while on warfarin therapy should be monitored closely to account for individual differences.

A Positive Nutrient-Drug Interaction: Vitamin E Reverses Ritonavir-Induced Overexpression of CD36

At the end of 2015, over 36 million people were infected with the human immunodeficiency virus (HIV), which causes acquired immunodeficiency syndrome (AIDS) (WHO). HIV infection is usually treated with multiple antiretroviral drugs that inhibit the replication of the virus. An example of such an inhibitor is ritonavir, which blocks HIV-1 protease, which is involved in viral maturation [74]. While such antiretroviral therapy reduces HIV-related morbidity and mortality, side effects include dyslipidemia [75] and an increased risk for cardiovascular disease [76]. HIV protease inhibitors have been shown to promote the formation of atherogenic lipoproteins and endothelial dysfunction [77]. A key process in the development of atherosclerosis is the aberrant accumulation of lipids in macrophages [78]. Increased accumulation can be explained by increased uptake of lipids through scavenger receptors, such as CD36. CD36 mediates the uptake of oxidized lipoproteins and other lipids into macrophages and promotes foam cell formation. Downregulation of CD36 in macrophages is thus a potential target for reducing the antiretroviral therapy-induced higher risk for atherosclerosis. In vitro experiments with THP1 monocytes demonstrated that ritonavir-induced overexpression of CD36 can be reversed, if the cells are concurrently incubated with RRR-αT (50 μmol/L; 24 h) [79]. There is evidence that vitamin E is involved in the regulation of CD36 even in the absence of an inducing factor, such as ritonavir. Rats fed a diet deficient in vitamin E (<1 mg total tocopherols/kg diet) showed an increased expression of CD36 when compared to animals fed a diet sufficient in vitamin E [80, 81]. In guinea pigs, CD36 protein expression was induced by a high-fat diet (21% fat by weight), and this induction was reversed by feeding 250 mg αT per kg diet [82]. Patients receiving ritonavir as part of their treatment of HIV infection may thus benefit from αT supplementation, especially if they are vitamin E deficient. However, further research is needed to confirm these findings in humans and to establish if this interaction is clinically relevant (Fig. 18.1).

Conclusion

In summary, there is no evidence of clinically relevant nutrient-drug interactions with vitamin E at physiological concentration in the scientific literature. In vitro, tocotrienols showed a higher potential to activate nuclear receptors and to increase the expression of xenobiotic enzymes and transporters than tocopherol, but in vivo studies are missing. The current data is summarized in Fig. 18.1. Based on available in vitro data, vitamin E, at very high doses, might potentially lead to interactions with aspirin, warfarin, tamoxifen, and cyclosporine A. Patients receiving these drugs and wishing to start vitamin E supplementation should consult their physician and monitor plasma concentrations of the active drugs or their activities to avoid treatment failure or adverse events.

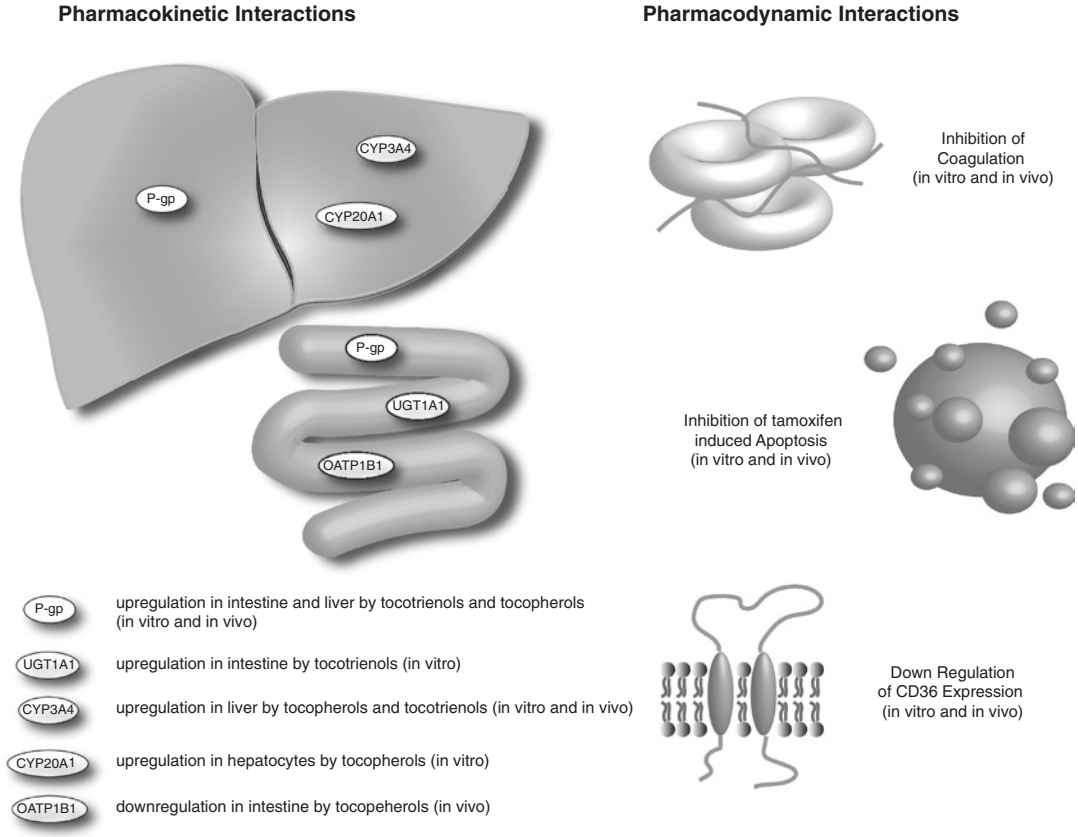

Fig. 18.1 Potential targets of vitamin E that could alter the pharmacokinetics or pharmacodynamics of a drug investigated in cell culture, animal, or human studies. P-glycoprotein (P-gp) is upregulated by tocotrienols in intestinal cells and human primary hepatocytes, while tocopherols show no effect. Hepatic P-gp is also upregulated by α-tocopherol in mice. UDP-glucuronosyltransferase 1A1 (UGT1A1) is upregulated by tocotrienols but not tocopherols in intestinal cells. Cytochrome P450 (CYP) mixed-function monooxygenase 3A4 (CYP3A4) expression is upregulated by α-tocopherol in primary human hepatocytes. In animal experiments, hepatic CYP3A4 activity is inconsistently affected and unchanged in humans. CYP20A1 is dose-dependently upregulated in human hepatocytes by α-tocopherol. Hepatic organic anion-transporting polypeptide (OATP1B1) in animal experiments is either downregulated or unaffected by α-tocopherol. Blood coagulation is reduced by α-tocopherol in experiments carried out in vitro, in animals and in humans. Apoptosis induced by tamoxifen is inhibited by α-tocopherol, and supplementation in humans reduces blood tamoxifen concentrations. α-Tocopherol reduces cluster of differentiation 36/fatty acid translocase (CD36) in vitro and in animal experiments. For experimental details please refer to the accompanying text

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Chapter 19
Vitamin E: Interactions with Vitamin K and Other Bioactive Compounds

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Keywords Vitamin E · Vitamin K · Coagulation · Nutrient-nutrient interactions · Warfarin *Ginkgo biloba*

Key Points

- For almost 50 years, an untoward hemorrhagic reaction accompanied by reductions in vitamin K-dependent clotting factors has been recognized in patients receiving the anticoagulant warfarin and taking high doses of supplemental vitamin E.
- The hemorrhagic effects of high doses of supplemental vitamin E may be exacerbated by co-consumption of dietary bioactive components possessing anticoagulant activity, such as *Gingko biloba* and garlic.
- The mechanism(s) of action underlying the documented adverse interaction between vitamin E and vitamin K remains to be elucidated.

Introduction

Understanding the complexity of dietary patterns and food synergies requires the elucidation of the pharmacodynamics of essential micronutrients and their interactions with other nutrients and dietary bioactive components. Regrettably, critical nutrient-nutrient interactions are often overlooked in observational studies and are challenging to address in randomized clinical trials [1]. Interestingly, one of the first observed adverse nutrient-nutrient interactions was between vitamin E and vitamin K [2]. We present below the potential mechanisms and clinical relevance of how other nutrients and bioactives may influence the functions of vitamin E.

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_19

Vitamin K

Vitamin K Forms and Structures

Vitamin K is a class of fat-soluble compounds containing a methylated naphthoquinone ring with an isoprenoid side chain, the length and saturation of which can vary. Phylloquinone (vitamin K_1) has a saturated side chain containing four isoprenoid units (Fig. 19.1). Menaquinones (collectively referred to as vitamin K_2) have an unsaturated side chain that varies in length from 4 to 13 isoprenoid units. The nomenclature of menaquinones refers to the length of the side chain, which is used to identify the individual menaquinone forms. For example, menaquinone-4 (MK4) has a four-unit side chain. Phylloquinone is plant-based, found in green leafy vegetables and vegetable oils, whereas menaquinones are found in animal-based and fermented foods [3]. Poultry, beef, and some dairy foods contain MK4, because some animal feeds contain menadione (vitamin K_3), which needs to be converted to MK4 to have vitamin K activity [4]. Long-chain menaquinones (i.e., MK7–MK13) are bacterially synthesized and found in some cheeses, meats, and other foods that undergo fermentation [5, 6]. Bacteria in the lower intestine can also synthesize long-chain menaquinones, but their bioavailability is uncertain [7].

Role in Coagulation

Vitamin K was discovered in the 1930s when Danish biochemist Henrik Dam observed that chicks fed a diet stripped of sterols suffered from lethal hemorrhaging. Dietary cholesterol repletion did not resolve the problem because in removing sterols from the diet, other fat-soluble compounds had also been removed. One of these compounds had anti-hemorrhagic activity and was later identified as vitamin K [3]. Of note, both phylloquinone in the form of alfalfa and menaquinones in the form of fermented fish meal were found to have anti-hemorrhagic activity. It has since been established that the function of all forms of vitamin K is as an enzymatic cofactor in the posttranslational carboxylation of certain proteins that contain glutamic acid (Glu) residues, also known as vitamin K-dependent

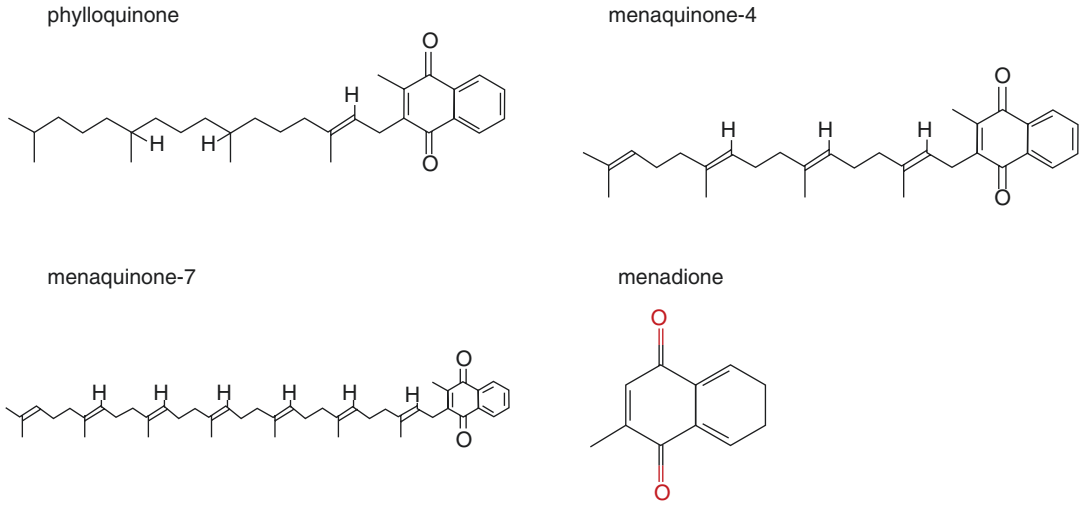

Fig. 19.1 Molecular structure of vitamin K

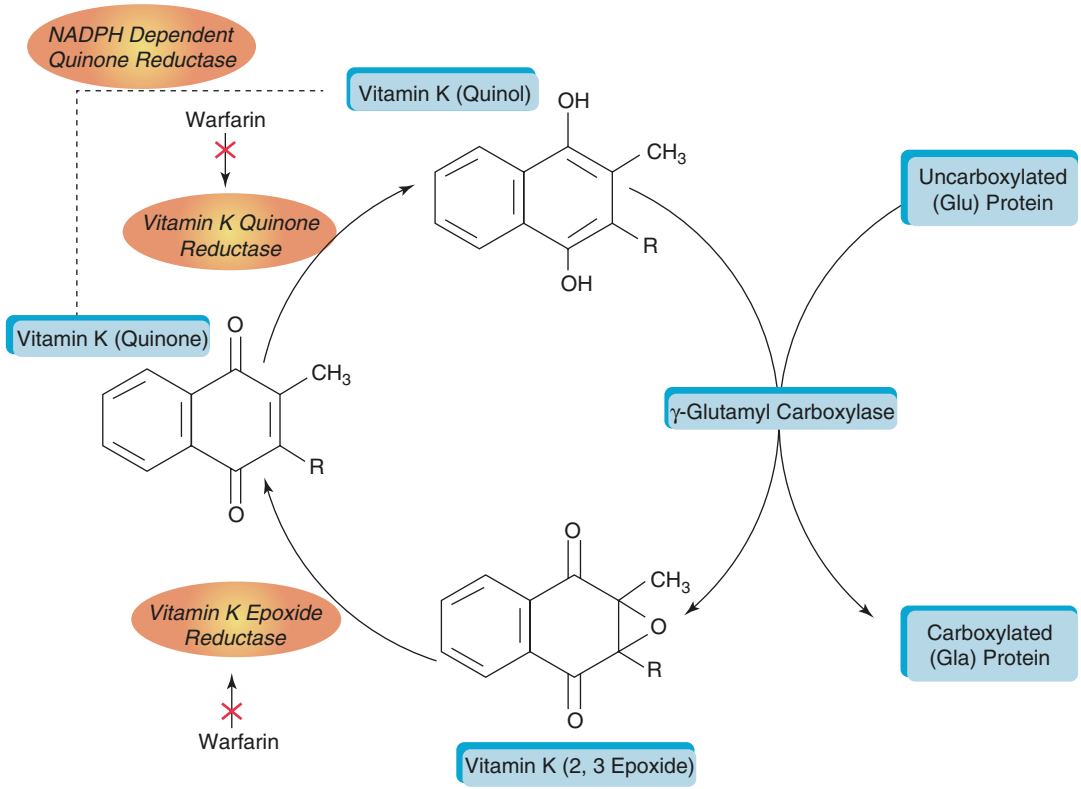

Fig. 19.2 The carboxylation of vitamin K-dependent proteins, known as the vitamin K cycle [8]

proteins (Fig. 19.2) [8]. Carboxylation of Glu residues confers function to these proteins [9]. Because Factors II, VII, IX, and X and proteins C and S are vitamin K-dependent proteins, vitamin K is essential for maintaining normal coagulation. Coumarin derivatives, such as warfarin, inhibit the vitamin K 2,3-epoxide reductase (VKORC1) and therefore inhibit carboxylation of vitamin-dependent proteins, including those involved in coagulation (Fig. 19.2) [8]. When vitamin K-dependent clotting proteins are not carboxylated, clotting is impaired [3].

Vitamin K and Vitamin E Interaction

Historical Context

Although both nutrients were discovered in the early 1900s, and both were linked to coagulation, the interaction between vitamin E and vitamin K was only revealed in the early 1970s when vitamin E supplement use became widespread in the general population. In the form of a case report, the first clinical evidence of an interaction of vitamin E with vitamin K was in a patient treated with warfarin who was taking high amounts of vitamin E (800–1200 IU/d) and experienced signs of hemorrhage accompanied by reductions in vitamin K-dependent clotting factors. These outcomes improved when vitamin E supplementation was discontinued [10]. Since then, multiple case reports have been reported, but the underlying mechanism have yet to be elucidated, despite focused research efforts addressing differential potential mechanisms.

Proposed Mechanisms

Since the interaction of vitamin E with vitamin K is known to result in abnormal coagulation, initial studies to identify the purported mechanism focused on the carboxylation of clotting proteins. In rats treated with warfarin and given high doses of vitamin E (specific form unknown) intramuscularly, prothrombin (also known as Factor II) activation was reduced compared to rats given warfarin without vitamin E [11, 12]. Reduced prothrombin is indicative of impaired coagulation and reduced carboxylation of Glu residues due to vitamin K antagonism [3]. In chicks, a high α-tocopherol diet prolonged prothrombin time, which is a direct measure of anticoagulation. This was reversed by a high phylloquinone diet [13]. Based on these results, the focus shifted to a potential mechanism leveraging the structural similarities of vitamins E and K. Specifically, Dowd and Zheng tested the ability of α-tocopherol and the vitamin E quinone (a vitamin E metabolite) to inhibit the vitamin K-dependent γ-carboxylase using rat liver microsomes. Vitamin E quinone is structurally similar to the vitamin K hydroquinone (Figure 19.2- vitamin K cycle). They found the vitamin E quinone bound to the γ-carboxylase, thus impeding carboxylation, whereas α-tocopherol did not [14]. However, the relevance of this finding to the anticoagulation effects of vitamin E is uncertain because the physiological importance of vitamin E quinones has yet to be established [2, 15].

When the focus on structural similarities failed to support a plausible mechanism supporting the interaction, the research shifted to the common metabolic pathways shared by vitamins E and K. Emerging evidence suggests the same transport-protein-mediated processes are involved in their absorption into enterocytes [16]. In Caco-2 cells, phylloquinone uptake was found to be a saturable process (indicative of transport protein involvement) that was reduced by increases in α-tocopherol [17]. Conversely, α-tocopherol uptake was reduced by phylloquinone [18]. CD36, a membrane protein that mediates intestinal fatty acid uptake [19], and scavenger receptor B1 (SRB1), a lipid transporter also implicated in enterocyte lipid trafficking [20], are reported to be involved in both vitamin E and vitamin K absorption, hence a potential target for uncovering the mechanism by which vitamin E interacts with vitamin K. When SRB1- and CD36-mediated lipid transports were blocked in Caco-2 cells, α-tocopherol and phylloquinone uptake were reduced [17, 21]. In vivo evidence from genetically modified mice also supports the involvement of CD36 and SRB1 in intestinal α-tocopherol and phylloquinone absorption [17, 21]. Niemann-Pick C1-Like 1 (NPC1L1), an important protein for intestinal cholesterol absorption, is also implicated in vitamin E and vitamin K absorption. NPC1L1 overexpression increased both α-tocopherol and phylloquinone uptake by Caco-2 cells [22–24]. Furthermore, ezetimibe, a cholesterol-lowering medication that blocks NPC1L1, inhibited both α-tocopherol and phylloquinone uptake [16, 22, 24]. Results of rodent experiments also support a role for NPC1L1 in intestinal α-tocopherol and phylloquinone absorption [16, 22, 24]. When ezetimibe and warfarin were co-administered to rats, liver phylloquinone concentrations were reduced and prothrombin time was prolonged. However, ezetimibe did not alter liver or serum warfarin concentrations, indicating the prolonged prothrombin time was not due to a difference in warfarin availability but rather due to a decrease in liver vitamin K stores needed to carboxylate clotting proteins. When extended to a clinical patient population through a retrospective chart review, prolonged prothrombin time was noted in patients treated with warfarin and ezetimibe, but not in patients treated with ezetimibe without warfarin. While the observed interaction of vitamin E with vitamin K at the level of intestinal absorption may have clinical implications for individuals taking ezetimibe and warfarin, these observations do not clarify how the interaction could be occurring at the level of absorption of these two nutrients [22]. The amounts of α-tocopherol and phylloquinone that are absorbed are not similar in magnitude in that the former is consumed in milligram doses, whereas the latter is consumed in microgram doses, and the circulating concentrations of α-tocopherol are up to 10,000-fold greater than that of phylloquinone [25]. Therefore, it is not clear how increasing vitamin E intakes through diet and/or supplements would have a threshold effect beyond which there was an adverse interaction [26–30].

After absorption, chylomicrons transport both vitamin E and vitamin K to the liver. In the liver, both undergo ω- then β-oxidation of their side chain as they are metabolized [31, 32]. Cytochrome P4F2 (CYP4F2) catalyzes the ω-oxidation step for both nutrients [33–36]. As hepatic vitamin E stores increase, CYP enzymes are upregulated to prevent vitamin E overaccumulation [31]. There is currently no evidence of a similar regulation for vitamin K in the liver given that the turnover of vitamin K is far more rapid. Vitamin E and vitamin K can both stimulate the pregnane X receptor (PXR), a nuclear receptor that regulates CYP expression [37–39]. It has been proposed that CYP enzyme upregulation in response to excess vitamin E could promote vitamin K catabolism, thereby reducing vitamin K liver stores and promoting anticoagulation [2]. However, α-tocopherol injection did not affect urinary vitamin K metabolite excretion in rats [40]. In this same experiment, CYP4F2 and CYP3A expression decreased in response to α-tocopherol. If excess α-tocopherol promoted vitamin K catabolism, an upregulation of these CYPs would have been expected [40]. In a kinetic experiment using insect microsomes expressing human CYP4F2, α-tocopherol incubation also did not increase vitamin K catabolism [41]. Based on the available evidence, the adverse effect of vitamin E on vitamin K and coagulation status does not appear to be due to increased vitamin K catabolism in the liver.

An alternate mechanism through which vitamin E could interfere with vitamin K involves the conversion of phylloquinone to MK4. Menaquinone-4 appears to be the preferential form of vitamin K in some extrahepatic tissues [42–44]. MK4 can be converted from dietary phylloquinone, through what is thought to be a two-step process: (1) side chain removal from phylloquinone to form menadione in the intestine and (2) prenylation of menadione to form MK4 in peripheral tissues, catalyzed by the prenyltransferase enzyme UBIAD1 [45–47]. Vitamin E supplementation has been hypothesized to interfere with side chain removal of phylloquinone and/or conversion of menadione to MK4, either of which would reduce MK4 formation [2, 40, 48]. In male rats fed an α-tocopherol-supplemented diet for 12 weeks, MK4 concentrations in the kidney, testes, and brain were lower than in rats fed a vitamin E-restricted diet [49]. The brain and kidney phylloquinone concentrations responded similarly. However, α-tocopherol supplementation did not affect liver MK4 or phylloquinone, suggesting an overall effect of high α-tocopherol intakes on extrahepatic tissue vitamin K concentrations. To test whether vitamin E affected MK4 production by interfering with phylloquinone side chain removal, Farley et al. fed rats diets containing phylloquinone or menadione for 2.5 weeks and subcutaneously injected with α-tocopherol daily for the last 7 days [40]. Whether the diet contained phylloquinone or menadione, α-tocopherol reduced MK4 concentrations in the brain, lung, kidney, heart, and plasma. In a subsequent experiment using deuterium-labeled phylloquinone as the sole dietary source of vitamin K, α-tocopherol injection reduced total vitamin K (the sum of labeled and unlabeled MK4 and phylloquinone) in rat kidney, lung, and brain. However, the relative amount of labeled MK4 in each tissue did not change in response to α-tocopherol [50], which is a further indication that α-tocopherol affects overall vitamin K concentrations in extrahepatic tissues, but not the conversion of phylloquinone to MK4. The only tissue in which α-tocopherol appeared to influence the phylloquinone to MK4 conversion was the heart [50]. Phylloquinone is normally the primary vitamin K form in the heart [43, 44], so the importance of reducing the conversion of phylloquinone to MK4 in heart tissue is uncertain. An overall effect of α-tocopherol on extrahepatic tissue vitamin K was also found when rats were fed diets containing the same amount of phylloquinone, which was the only form of vitamin K in the diet, with varying amounts of α-tocopherol for 6 weeks. Phylloquinone concentrations in the kidney, brain, heart, lung, skeletal muscle, and testes all dose-dependently decreased as dietary α-tocopherol increased. MK4 in the kidney, heart, and brain also decreased in response to increasing α-tocopherol intake. However, when the diet contained MK4 instead of phylloquinone, the tissue vitamin K concentrations did not change in response to α-tocopherol [48]. Similar responses were noted when phylloquinone or MK4 were administered orally with α-tocopherol. Collectively, these experiments indicate high α-tocopherol intakes can reduce extrahepatic tissue vitamin K concentrations, but this is not limited to the conversion of phylloquinone to MK4. It is plausible the observed effect of vitamin

E on phylloquinone and MK4 in extrahepatic tissues is due, in part, to an effect of vitamin E on vitamin K intestinal absorption. However, this needs to be tested in experiments designed to do so. Furthermore, the functional consequences of the apparent effect of vitamin E on extrahepatic tissue vitamin K have not been determined. Finally, the studies of vitamin K-vitamin E interaction to date have focused on α-tocopherol with phylloquinone. It is not known if α-tocopherol interacts with menaquinones, other than MK4. In one study, γ-tocopherol was not found to effect vitamin K in tissues to the same extent as α-tocopherol [48]. Therefore further research is needed to determine if other forms of vitamins E and K similarly interact, which may shed light on the mechanism underlying this interaction.

Clinical Evidence to Date

Following the initial case study reporting the interaction of vitamin E with vitamin K in a patient using warfarin, clinical studies ensued that revealed that an adverse interaction with vitamin E does not appear to be limited to warfarin treatment [10, 51, 52]. In two separate randomized-controlled vitamin E supplementation trials conducted in adults not taking warfarin, serum PIVKA-II (protein induced by vitamin K absence-factor II, a measure of uncarboxylated (nonfunctional) prothrombin) increased to levels indicative of impaired coagulation in those who received 1000 IU/d α-tocopherol for 12 weeks. PIVKA-II did not change in those who received placebo. In these same trials, plasma phylloquinone and serum undercarboxylated osteocalcin (a vitamin K-dependent protein in the bone) did not change in response to vitamin E supplementation, suggesting the interaction between vitamin E supplementation and vitamin K occurs in the liver [51]. Of note, while elevated PIVKA-II levels indicated an adverse coagulation protein response to vitamin E supplementation, the clinical measures of coagulation known as the international normalized ratio (INR) were unchanged. To change INR, one requires more than 60% of all prothrombin to have reduced γ-carboxylation of the Glu residues, hence is highly insensitive measure of vitamin K status [53]. However, these data do indicate that the adverse interaction is subtle in otherwise healthy individuals and requires a sensitive biomarker of vitamin K status to reveal an adverse effect. Along this line of thought, warfarin-treated patients taking 100–400 IU/d vitamin E did not have altered coagulation profiles as measured by clinical measures of clotting [11]. At the time of the study, there was no capacity to measure PIVKA-II; hence it is not known if the adverse coagulation effect of vitamin E does not occur when intakes are consistent with dietary recommendations [29] or if the interaction was insufficient to change clinical measures of coagulation. In a survey of warfarin users in Canada, 24% reported taking vitamin E supplements. Yet vitamin E supplement use was not associated with out-of-range INR or abnormal bleeding [54]. Supplement dose was not reported in this study nor was PIVKA-II measured. Similarly, in a convenience sample of warfarin-treated patients, INR did not change in response to 4 weeks of supplementation with 800–1200 IU/d vitamin E [55]. The generalizability of this finding is uncertain, however, because nearly half of enrolled participants did not complete the study. Nonetheless, while the majority of evidence indicates adverse effects with high doses, the use of vitamin E-containing supplements by warfarin-treated patients merits close monitoring.

Interactions Between Vitamin E and Other Bioactive Compounds

While the preponderance of research on vitamin E interactions has focused on its interaction with vitamin K, there are also suggestions of interactions with other bioactives. For example, the hemorrhagic effects of high amounts of vitamin E may be exacerbated by consuming it with other dietary

bioactives that have anticoagulant activity, like *Ginkgo biloba* and garlic [56]. The exact mechanisms are not known, and it is assumed that there is an additive anticoagulant effect with simultaneous consumption. Similar to warfarin, chamomile also contains coumarin derivatives, so an interaction between vitamin E and chamomile is biologically plausible. To date no case studies have been reported; hence this putative interaction is still speculative [57]. More recently, it has been reported that α-tocopherol inhibits the antibacterial properties of ursolic acid, a plant secondary bioactive [58]. This is based on in vitro studies; hence its application to humans is unclear.

It has been suggested that vitamin E supplementation would interfere with the effect of niacin on HDL cholesterol based on results in the HDL-Atherosclerosis Treatment Study (HATS) [59]. In this trial, treatment with simvastatin-niacin reduced coronary artery stenosis and clinical events. However, the improvement was attenuated when simvastatin-niacin was combined with antioxidant vitamins (which consisted of α-tocopherol, vitamin C, β-carotene, and selenium) [59]. Addition of antioxidants also attenuated the increase in HDL cholesterol (specifically HDL_2) in response to niacin treatment [60]. However, α-tocopherol supplementation alone for 2 years did not alter HDL profile [61], suggesting this interaction is not attributable solely to vitamin E.

Vitamin E functions as an antioxidant, stopping the production of reactive oxygen species when lipids are oxidized. Because vitamin C can be used to regenerate the antioxidant capacity of α-tocopherol, vitamin E and vitamin C are thought to be mutually important with respect to reducing oxidative stress [62]. Although vitamin E-vitamin C supplementation trials have not reported a beneficial effect of these two nutrients on oxidative stress-related disease outcomes [63–65], some have attributed the null findings to study design limitations [66]. In addition, both vitamins E and C have been associated with longer telomere lengths, a measure of delayed senescence [67]. Whether these nutrients have a synergistic effect on delayed senescence is unknown. Similarly, although there have been some suggestions that vitamin E exacerbates the symptoms of selenium deficiency, a recent study in weanling rats indicated that vitamin E deficiency did not alter biomarkers of selenium status [68].

Conclusions

Food-based guidance for promotion of health requires an understanding how nutrients interact with each other to function. Vitamin E has been linked to multiple nutrient-nutrient interactions. Although several mechanisms have been put forward, the exact mechanism underlying the documented adverse interaction between vitamin E and vitamin K remains to be clarified. Furthermore, while this interaction can be clinically meaningful [10, 51], data from large-scale studies are lacking. While other interactions have been reported between vitamin E and bioactives, the evidence is weak and interpretation of the findings is hampered by the lack of known underlying mechanisms.

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Part IV
Benefits of Vitamin E on Human Health and Disease

Chapter 20
Clinical Cardiovascular Disease Trials: The Vitamin E Case

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Keywords Vitamin E · Oxidative stress · Cardiovascular disease · Stroke · Myocardial infarction Haptoglobin · Diabetes

Key Points

- Vitamin E has been widely studied as a potential treatment for both primary and secondary protection of cardiovascular disease.
- While epidemiological observations suggest vitamin E to reduce the risk of cardiovascular disease (CVD), the results of most randomized clinical studies did not confirm these findings.
- Many reasons have been discussed to explain the inconsistent findings on vitamin E and the risk reduction/prevention of CVD.
- The failure to identify and select patient groups that are under the specific oxidative stress conditions that would best benefit from vitamin E antioxidant therapy appears to be key to explain the lack of consistent evidence.
- Haptoglobin 2-2 haplotype (Hp 2-2) is a risk factor for CVD, and evidence is emerging from clinical trials showing that diabetic patients homozygous for Hp 2-2 will benefit from vitamin E therapy.
- The possible role of vitamin E in the risk reduction/prevention of CVD should be revisited by designing appropriate clinical studies.

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide. Motivated by the oxidative hypothesis, antioxidant therapy, including vitamin E, has been extensively tested for the primary and secondary protection of CVD. The oxidative hypothesis proposes that CVD develops partly because

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_20

oxidative modifications of lipoproteins promote macrophage foam cell formation and activation and in turn atherosclerosis [1, 2]. As mentioned in previous chapters, vitamin E encompasses a group of eight lipophilic molecules (α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol) that act as antioxidants by scavenging free radicals and singlet oxygen [3–5]. In this chapter studies are summarized performed with α-tocopherol. Vitamin E became a popular candidate for antioxidant therapy because it is recycled by other natural antioxidants, which prevents the accumulation of oxidized vitamin E molecules [6]. Furthermore, the mechanism by which vitamin E prevents oxidation of low-density lipoprotein (LDL) was already well studied [7]. On top of that, epidemiological evidence already existed showing that high amounts of dietary vitamin E reduced the risk of CVD [8, 9]. These reasons provided ample motivation to test vitamin E therapy in prospective clinical trials to investigate the potential protection it could provide against CVD. Numerous clinical trials have looked at the role of vitamin E in cardiovascular disease (CVD). The use of vitamin E as an intervention has been evaluated in both primary and secondary prevention of CVD.

Primary Prevention

The Virtamo et al. study in 1998 was one of the first randomized controlled trials (RCT) to evaluate the role of vitamin E in the primary prevention of CVD [10]. Twenty-seven thousand two hundred seventy-one Finnish smokers between the ages of 50 and 69 were randomly assigned to one of four groups: vitamin E (50 mg/day), B-carotene (20 mg/day), both, or placebo. Patients were treated daily for 5–8 years (median = 6.1). All enrolled patients had no history of myocardial infarction (MI) at the onset of the study and were followed up with the primary endpoint of first MI. Ultimately, a nonstatistically significant reduction in the incidence of MI of 4% was noted for those who received vitamin E as compared with other treatment groups.

The results of the Collaborative Group of the Primary Prevention Project were published by de Gaetano et al. in 2001. The main goal of this study was to evaluate the efficacy of vitamin E (300 mg/day) and aspirin (100 mg/day) in the prevention of CVD events. This was assessed in a population of 4495 individuals with cardiovascular risk factors. The trial was terminated prematurely after a median follow-up of only 3.7 years on the basis of newly emerging data from other trials that revealed the benefits of aspirin therapy in primary prevention of CVD. At that time, there was no evidence to suggest that vitamin E had played a significant role in the reduction of cardiovascular disease [11]. A follow-up evaluation of the Primary Prevention Project was published in 2003. At this point, 1031 patients with diabetes were evaluated for the primary CVD outcomes. Although nonsignificant reductions in CVD were noted in diabetic patients taking aspirin, there were no notable findings for the diabetic patients taking vitamin E, just as in the parent population [12].

The Women's Health Study was a massive RCT of 39,876 healthy individuals that was conducted between 1993 and 2004 at Brigham and Women's Hospital and Harvard Medical School. This study evaluated the effects of low-dose aspirin vitamin E on the incidence of cancer and cardiovascular disease [13]. Results of the study were published in 2005. All of the study participants were previously healthy female health professionals at least 45 years of age who were followed up for an average of 10.1 years. Trial participants were randomly assigned to low-dose aspirin, vitamin E (600 IU), or placebo cohorts using a 2 × 2 factorial design. Ultimately, the primary endpoint was cancer or a composite endpoint of first major cardiovascular event consisting of nonfatal stroke, nonfatal MI, or CV death. There was a total of 482 major CV events in the vitamin E group as compared with 517 in the placebo group – a 7%, yet nonsignificant, risk reduction. Breaking down the composite CV outcome into its constituents revealed no real impact of vitamin E administration on incidence of myocardial infarction or stroke as compared with placebo. However, vitamin E was found to be effective in decreasing CV death, seeing a statistically significant risk reduction of 24%

(RR, 0.76%; 95% CI, 0.59–0.98; $P = 0.03$). The study also noted no significant changes on incidence of cancer. Yet despite the decreased CV mortality, the authors concluded that the results did not indicate the recommendation of vitamin E as an intervention in the prevention of either CVD or cancer in healthy women [13].

A subsequent secondary analysis of data from the Women's Health Study published in 2012 examined the role of vitamin E as antioxidant therapy in prevention of heart failure (HF), noting the potential role of oxidation in the pathogenesis of HF [14]. There were 220 incidents of heart failure during the study period with no observable therapeutic effect on the overall risk of HF. Subgroup analysis for heart failure with normal ejection fraction (HFnEF) and heart failure with reduced ejection fraction (HFrEF) revealed a significantly decreased hazard ratio for patients with HFnEF (HR, 0.59%; 95% CI, 0.38–0.92; $P = 0.02$) but no statistically significant effect on HFrEF. Overall, the study concluded that there was no appreciable effect of vitamin E therapy on the prevention of heart failure. However, the authors noted that the decreased hazard ratio observed in the small cohort of patients with HFnEF required confirmation in additional studies with larger populations of these patients.

Another RCT, the Physicians' Health Study, ran from 1997 to 2007 and evaluated the role of vitamin E and vitamin C interventions on the incidence of major cardiovascular events – a composite outcome consisting of nonfatal myocardial infarction, nonfatal stroke, and CVD death [15]. Enrolled in the study were 14,641 males over the age of 50. Of this population, 5.1% (754) were found to have CVD at the time of selection. Patients in this randomized, double-blind study were selected to take vitamin E (400 IU every other day), vitamin C (500 mg/day), or placebo for an average follow-up of 8.0 years. The only notable result of data analysis from this study was that vitamin E therapy was associated with an increased risk of hemorrhagic stroke (HR, 1.74; 95% CI, 1.04–2.91; $P = 0.036$). Ultimately, the authors concluded against recommending the use of vitamin E or vitamin C in the primary prevention of CVD in males over the age of 50.

The St. Francis RCT looked at the primary prevention of cardiovascular disease by examining the progression of coronary artery calcium score (CAC) following intervention [16]. CAC is a useful tool for the evaluation of subclinical atherosclerosis. Through its use, CAC enables one to assess the CVD risk of patients, thereby better enabling a targeted treatment regimen in the prevention of cardiovascular events [17]. One thousand five men and women who were asymptomatic at patient selection with a coronary calcium score at or above the 80th percentile were randomized to receive vitamin E (1000 IU/day), vitamin C (1 g/day), or atorvastatin (20 mg/day). In addition, each patient also received low-dose aspirin (81 mg/day). After a mean follow-up of 4.3 years, there were no significant findings with regard to affecting clinical outcomes or the progression of coronary calcium scores. The Vitamin E Atherosclerosis Prevention Study (VEAPS) looked specifically at the role of vitamin E in preventing the progression of atherosclerosis but not at specific clinical outcomes [18]. The population for this study included men and women over the age of 40 with LDL cholesterol levels in excess of 3.37 mmol/l, equivalent to 130 mg/dl. These patients were all without clinical signs and symptoms at the study onset and were randomized to receive vitamin E (400 IU/day) or placebo. The primary endpoint was assessed using computer image-processed ultrasonogram to evaluate the rate of change of the intima-media thickness (IMT) in the wall of the common carotid artery. Following an average of 3 years of follow-up, no reduction was noted in the rate of progression of IMT due to vitamin E supplementation. However, importantly, vitamin E was found to significantly reduce the amount of circulating oxidized LDL in the blood leading to lower oxidized LDL susceptibility. Chronic kidney disease (CKD) is a risk factor for the development of CVD. In the Antioxidant Therapy in Chronic Renal Insufficiency (ATIC) study, patients with mild-to-moderate CKD were evaluated using a particular treatment regimen on carotid IMT, endothelial function, and renal function [19]. Patients were randomized to a treatment or placebo group. The treatment group received pravastatin for 6 months, at which point vitamin E was added. Following another 6 months of combined treatment, homocysteine-lowering therapy was added. The results of this stepwise combination therapy were significant reductions in IMT and endothelial dysfunction, thereby reducing the risk of developing cardiovascu-

lar disease. But although there was a significant decrease in albuminuria, the therapy did not affect renal function.

The results of a study published in 2015 by Luzia et al. examined the therapeutic role of fish oil with and without vitamin E therapy in the development of oxidative stress and cholesterol in Brazilian women transitioning through menopause [20]. It has been suggested that reduced levels of estrogen in postmenopausal and perimenopausal women lead to increased risk for CVD. Seventy-four women with an average age of 51.6 years (+/−7.8) were assigned to receive fish oil + vitamin E, fish oil alone, or placebo for 90 days. Plasma levels of markers for dyslipidemia, of hypercholesterolemia, and of oxidative stress were recorded at baseline, at 45, and at 90 days. Included among these are TBARS, or thiobarbituric acid reactive substances, which are a marker that is produced by the damage caused by oxidative stress [21]. In the fish oil + vitamin E group, there was on average a decrease in total cholesterol (230 mg/dL vs. 260 mg/dL) and low-density lipoprotein cholesterol (LDL-C) as compared with fish oil alone (135 mg/dL vs. 165 mg/dL). However, average TBARS were elevated when compared to the use of fish oil alone (0.05 umol/L vs. 0.04 umol/L). All three measures were decreased in the fish oil + vitamin E group as compared with placebo. Ultimately the authors concluded that although addition of vitamin E to fish oil supplementation decreased the CVD risk factors of total cholesterol and LDL-C, there was an increase in oxidative stress compared to fish oil alone.

In Table 20.1 are the main features and outcomes of the primary prevention trials summarized.

Secondary Prevention

In 1992 the results of a double-blind, placebo-controlled trial that tested whether α-tocopherol prevents restenosis following percutaneous transluminal coronary angioplasty (PTCA) were published [22]. After successful PTCA, 100 patients received α-tocopherol (1200 IU/day) or placebo for 4 months. Plasma lipids, lipoproteins, apolipoproteins, α-tocopherol, retinol, β-carotene, and lipoperoxide concentrations were compared to baseline levels. No difference in any blood test parameter was observed except for α-tocopherol. Despite this, 50% of the control group had restenosis (defined as stenosis ≥50%), whereas only 34.6% of those who received vitamin E had restenosis. However, statistically this failed to provide evidence of a significant difference between the two groups, possibly because of the small sample size.

In 1996 the results of the Cambridge Heart Antioxidant Study (CHAOS) were published [23]. This was the first study to assess robust clinical outcomes associated with vitamin E intervention in the setting of secondary prevention to CVD. The primary endpoints of this study were nonfatal MI and CV death. This double-blind, placebo-controlled study divided 2002 patients with angiographic coronary atherosclerosis into three groups and followed them for a median of 510 days. Five hundred forty-six patients were given 800 IU/day of α-tocopherol, 489 were given 400 IU/day of α-tocopherol, and 967 were given a placebo. Those who received α-tocopherol treatment had a significantly lower risk of nonfatal MI (14 vs. 41; RR = 0.23 [0.11–0.47]; $p = 0.005$). However, there was a nonsignificant excess of cardiovascular deaths in the α-tocopherol group compared to the placebo (27 vs. 23; RR = 1.18 [0.62–2.27]; $p = 0.61$). The authors concluded that vitamin E provided secondary protection for nonfatal MI but could not explain the excess of CVD-related deaths in the vitamin E group.

In 1999, the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico (GISSI) study was published [24]. This study divided 11,324 patients who survived a recent MI into groups that received n-3 polyunsaturated fatty acids (PUFAs) (1 g/day), vitamin E (300 mg/day), vitamin E and PUFAs, or neither. Treatment with PUFA, but not vitamin E, significantly lowered the risk of the combined primary endpoints, death, nonfatal myocardial infarction, and stroke (RR decrease 10% [95% CI 1–18] by two-way analysis and 15% [2–26] by four-way analysis). The effect of the combined treatment of vitamin E and PUFA was determined to not be significantly different than that of

Table 20.1 Characteristics of trials evaluating the use of vitamin E in the primary prevention of cardiovascular disease

Study	Number of patients	Patient characteristics	Median follow-up duration	Interventions	Relevant outcomes
ATIC	93	Mild-to-moderate chronic kidney disease (CKD)	1.5 years	α-Tocopherol, pravastatin, homocysteine-lowering therapy (added in a stepwise manner) vs. placebo	Carotid IMT: 0.68–0.63 mm vs. 0.65–0.71 mm ($P < 0.001$) Urine albumin: mean 34% reduction eGFR: 32–35 ml/min/1.73m^2 vs. 35–33 ml/min/1.73m^2 ($P = 0.89$)
Luzia et al.	74	Brazilian perimenopausal females	90 days[a]	Fish oil + α-tocopherol or fish oil alone vs. placebo	Fish oil + vit. E: TBARS 0.05 (0.03 SD) umol/L, total cholesterol 230 (31 SD) mg/dL, LDL-C 135 (33 SD) mg/dL Fish oil: TBARS 0.04 (0.04 SD) umol/L, total cholesterol 260 (47 SD) mg/dL, LDL-C 165 (34 SD) mg/dL Placebo: TBARS 0.06 (0.04 SD) umol/L, total cholesterol 244 (38 SD) mg/dL, LDL-C 147 (34 SD) mg/dL
PHS II	14,641	Male, >50 years old	8 years	α-Tocopherol (400 IU every other day) or vitamin C (500 mg/day) vs. placebo	Major cardiovascular events (MI/nonfatal stroke/CVD death): HR 1.01 (0.90–1.13) Hemorrhagic stroke: HR 1.74 (1.04–2.91)
PPP	4495	At least 1 CV risk factor	3.6 years	α-Tocopherol (300 mg/day) and aspirin (100 mg/day) [note: 2 × 2 design]	CV death, MI, stroke: RR 1.07 (0.74–1.56)
St. Francis	1005	Coronary calcium score (CCS) > 80th percentile	4.3 years	α-Tocopherol (1000 IU/day), vitamin C (1 g/day), or atorvastatin (20 mg/day) vs. placebo Aspirin (81 mg/day)	Atherosclerotic CVD event rate: 6.9% vs. 9.9% ($P = 0.08$) No effect on progression of CCS ($P = 0.80$)
VEAPS	353	LDL cholesterol > 3.37 mmol/L	3 years	DL-α-tocopherol (400 IU/day) vs. placebo	Carotid IMT change: 0.0023 ± 0.0007 vs. 0.0040 ± 0.0007 ($P = 0.08$)
Virtamo et al.	27,271	Finnish male smokers between 50 and 69 years old	6.1 years	α-Tocopherol (50 mg/day), β-carotene (20 mg/day), or both vs. placebo	First MI and CV death: RR 0.98 (0.87–1.10)
WHS	39,876	Female health professionals > 45 years old	10.1 years	α-Tocopherol (600 IU) or low-dose aspirin vs. placebo [note: 2 × 2 design]	CV death, MI, stroke: RR 0.93 (0.82–1.05) Heart failure: 0.93 (0.71–1.21)

[a]Final data was recorded at 90 days

PUFA alone for the primary endpoint and for fatal events. Therefore, the study concluded that the primary end points of CV death, nonfatal MI, and stroke were not affected by vitamin E treatment.

In 2000 the results of the Heart and Outcomes Prevention Evaluation (HOPE) study were published [25]. This study included 2545 women and 6996 men over the age of 55 that were at high risk for CV events due to history of pre-existing CVD or diabetes. These patients were assigned to one of four treatment groups that included vitamin E (400 IU/day), the angiotensin-converting enzyme (ACE) inhibitor ramipril, both vitamin E and ramipril, or neither. These groups were followed for a mean of 4.5 years. The primary endpoint outcomes of CV death, nonfatal MI, and stroke did not differ between the four groups. This led the authors to conclude that vitamin E had no effect on CV events in high-risk patients. In 2005, the HOPE TOO (the ongoing outcomes) results were published and also showed that patients who received vitamin E (400 IU/day) had no difference in cancer incidence, cancer deaths, MI, stroke, and CV-related deaths compared to patients given a placebo [26]. Among all HOPE patients, there were no significant differences in the vitamin E group and the placebo group for major cardiovascular events, 1022 (21.5%) vs. 985 (20.6%), respectively (RR, 1.04; 95% CI, 0.96–1.14; $P = 0.34$). Surprisingly, patients in the vitamin E group had a higher risk of heart failure (RR, 1.13; 95% CI, 1.01–1.26; $P = 0.03$) and hospitalization for heart failure (RR, 1.21; 95% CI, 1.00–1.47; $P = 0.045$). The authors concluded that in patients with vascular disease or diabetes mellitus, long-term vitamin E supplementation does not prevent major cardiovascular events and may actually increase the risk for heart failure.

There have also been many studies that have assessed the ability of vitamin E and vitamin C together to provide secondary prevention to CVD. The Women's Angiographic Vitamin and Estrogen (WAVE) study was a randomized controlled trial published in 2002 [27]. Four hundred twenty-three postmenopausal women with at least one coronary stenosis (15–75%) were given hormone replacement therapy (HRT), antioxidant therapy (vitamin E 400 IU/day and vitamin C 500 mg BID), or placebo. After a mean follow up time of 2.8 years, coronary progression was worse with HRT vs. HRT placebo (0.047 vs 0.024 mm/y) and for those receiving antioxidant vitamin therapy vs. vitamin placebo (0.044 vs 0.028 mm/y). Death, nonfatal MI, or stroke occurred more in HRT and antioxidant therapies compared to their placebos in 26 HRT patients vs. 15 HRT controls (26 vs. 15, HR, 1.9; 95% CI, 0.97–3.6) and in 26 vitamin patients and 18 vitamin controls (26 vs. 18, HR, 1.5; 95% CI, 0.80–2.9), respectively. Looking specifically at death, more patients died compared to their placebo controls in the HRT group (14 vs. 8, [HR], 1.8; 95% confidence interval [CI], 0.75–4.3) and in the vitamin group (16 vs. 6, HR, 2.8; 95% CI, 1.1–7.2). The authors concluded that in postmenopausal women with coronary disease, neither HRT nor antioxidant therapy provides cardiovascular benefit and that both are associated with a potential for harm.

In the Heart Protection Study, also published in 2002, 20,536 adults aged 40–80 with coronary disease, other occlusive arterial disease, or diabetes were randomly divided to receive vitamin E (600 mg/day), vitamin C (250 mg/day), β-carotene (20 mg/day), or placebo [28]. There were no significant differences in all-cause mortality in the vitamin group vs. the placebo (1446 [14.1%] vs. 1389 [13.5%]) or in deaths due to vascular (878 [8.6%] vs. 840 [8.2%]) or nonvascular (568 [5.5%] vs. 549 [5.3%]) causes. There were also no significant differences between the two groups in the numbers of nonfatal myocardial infarction or coronary death (1063 [10.4%] vs. 1047 [10.2%]), nonfatal or fatal stroke (511 [5.0%] vs. 518 [5.0%]), or coronary or non-coronary revascularization (1058 [10.3%] vs. 1086 [10.6%]). The authors concluded that in high-risk individuals, vitamin E did not produce any significant reduction in 5-year mortality, vascular disease, or any major outcome.

One study that did show evidence for a benefit from vitamin E therapy was the Secondary Prevention with Antioxidants of Cardiovascular Disease in End-Stage Renal Disease (SPACE) study [29]. Oxidative stress is implicated in the development of long-term complications in hemodialysis patients including atherosclerosis and CVD [30]. Vitamin E has been used during dialysis as part of the dialysis membrane to protect against the oxygen free radicals generated during the

hemodialysis [31]. This study was designed to test whether the role of vitamin E could be expanded to further decrease their risk of CVD. The hemodialysis patients with CVD were randomized to receive vitamin E (800 IU/day) or a placebo. The patients who received vitamin E had RR of 0.46 after a median of 519 days for a composite MI, ischemic stroke, peripheral vascular disease, and unstable angina. However, they showed no decrease in mortality compared to the placebo. The positive results of this study suggest that there might be patient groups that would benefit from daily vitamin E therapy.

In Table 20.2 are the main features and outcomes of the secondary prevention trials summarized.

Table 20.2 Characteristics of trials evaluating the use of vitamin E in the secondary prevention of cardiovascular disease

Study	Number of patients	Patient characteristics	Median follow-up duration	Interventions	Relevant outcomes
CHAOS	2002	Angiographic coronary atherosclerosis	510 days	α-Tocopherol (800 IU/day or 400 IU/day) vs. placebo	Nonfatal MI: RR 0.23 (0.11–0.47; $P = 0.005$) CV death: RR 1.18 (0.62–2.27) CV death + nonfatal MI: RR 0.53 (0.34–0.83; $P = 0.005$)
DeMaio et al.	100	Successful PTCA	4 months	DL-α-tocopherol (1200 IU/day) vs. placebo	Restenosis: 34.6% vs. 50% ($P = 0.06$)
GISSI	11,324	Recent (≤3 months) MI	3.5 years	n-3 PUFA (1 g/day), α-tocopherol (300 mg/day), both vs. placebo	CV death, MI, stroke (vitamin E): RR 0.95 (0.86–1.05)
HOPE	9541	Pre-existing CVD or diabetes, > 55 years old	4.5 years	α-Tocopherol (400 mg/day), ACE-inhibitor (ramipril) vs. matching placebo [note: 2 × 2 design]	Vitamin E CV death, MI, stroke: RR 1.05 (0.95–1.16)
HOPE TOO	3994	Pre-existing CVD or diabetes, > 55 years old	7.0 years	α-Tocopherol (400 mg/day) vs. placebo	CV events: RR 1.04 (0.96–1.14) Heart failure: RR 1.13 (1.01–1.26; $P = 0.03$)
HPS	20,536	Adults (aged 40–80) with coronary disease, occlusive arterial disease, or diabetes	5.0 years	DL-α-tocopherol (600 mg/day) + vitamin C (250 mg/day) + β-carotene (20 mg/day) vs. placebo	CV events: RR 1.00 (0.94–1.06)
SPACE	196	Hemodialysis patients with CVD	519 days	α-Tocopherol (800 IU/day) vs. placebo	MI, ischemic stroke, peripheral vascular disease, unstable angina: RR 0.46 (0.27–0.78; $P = 0.016$)
WAVE	423	Postmenopausal women with coronary artery stenosis	2.8 years	Hormone replacement therapy vs. placebo and α-tocopherol (400 IU/day) + vitamin C (500 mg BID) or matching placebos [note: 2 × 2 design]	Death, nonfatal MI, stroke: HR 1.5 (0.80–2.90)

Conclusion and Future Directions

After countless large prospective studies, there was a lack of consistent evidence supporting the utility of vitamin E therapy in primary or secondary protection of CVD. Furthermore, in 2005, a meta-analysis of 19 clinical trials that used vitamin E by itself or in combination with other antioxidants and drugs was published. In total, the 19 clinical trials included 135,967 participants, and dosages ranged from 16.5 IU/day to 2000 IU/day (median of 400 IU/day) [32]. The meta-analysis concluded that vitamin E did not provide primary or secondary protection and actually increased the all-cause mortality in patients who received any type of vitamin E therapy. Collectively, these studies have failed to provide sufficient evidence for the use of vitamin E to prevent cardiovascular disease in the general population. There are many potential reasons antioxidant therapy has failed to demonstrate consistent findings that would indicate use in the prevention of CVD. Chief among these has been a failure to identify and select patient groups that are under the specific oxidative stress conditions that would best benefit from vitamin E antioxidant therapy.

It is important to identify groups of patients that will benefit from this cheap and accessible therapy. Aside from end-stage renal disease patients, one group that might benefit from antioxidant therapy are diabetic patients that are homozygous for a specific polymorphism in the haptoglobin (Hp) gene, Hp2. There is evidence suggesting that the Hp2-2 haplotype is a risk factor for CVD [33–35]. There is also retrospective data from the WHS and HOPE clinical trials showing that diabetic patients homozygous for Hp2 benefited from vitamin E therapy [13, 25, 36, 37]. Based on the response of these patients, the prospective Israel Cardiovascular Vitamin E (ICARE) study was designed to directly test this patient group and showed that cardiovascular events were significantly lower in those treated with the placebo [38]. A larger-scale, long-term prospective clinical trial has been designed to overcome the barriers associated with funding a trial for vitamin E, a non-patentable and inexpensive therapy, which hopes to provide strong evidence for the benefit of vitamin E in Hp2-2 diabetic patients [39]. The next chapter will expand on the potential protective effect of vitamin E in this patient group.

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Chapter 21
Haptoglobin Genotype and a Promising Pharmacogenomic Approach to Prevent Diabetic Atherothrombosis with Vitamin E Supplementation

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Keywords Vitamin E · Cardiovascular disease · Diabetes · Haptoglobin · Polymorphism · Genotype Clinical trials · Review · Precision medicine

Key Points

- Cardiovascular disease remains the most important chronic disease in the world, especially for those with diabetes mellitus.
- A polymorphism in the haptoglobin (Hp) gene strongly defines individuals with diabetes who are at greater risk of cardiovascular disease.
- A viable approach lies in the intersection of complementary and precision medicine. One highly tenable pharmacogenomic approach is the use of vitamin E supplementation to target high-risk diabetes patients defined by their Hp genotype.
- Independent clinical studies show that vitamin E supplementation may reduce the increased risk of cardiovascular disease associated with the Hp 2-2 genotype in diabetes. These clinical studies are supported by mechanistic data and animal studies.

Introduction

Despite identification of modifiable risk factors, cardiovascular disease (CVD) remains the most important chronic disease in the world, especially for those with diabetes mellitus (DM) [1–3]. Macro- and microvascular complications are major contributors to the morbidity and mortality of DM [1]. Diabetic complications of CVD cost over $174 billion annually in the United States alone and account for 80% of all deaths in individuals with DM [1, 4, 5], and DM is the major cause of

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_21

blindness and renal failure [1]. Importantly, neither conventional cardiac risk factors nor the degree of glycemic control adequately predicts which individuals with DM will develop vascular complications, suggesting the existence of genetic or nongenetic susceptibility factors. A polymorphism in the haptoglobin (Hp) gene appears to strongly define individuals with DM who are at greater risk of vascular disease [6–16].

The public health and economic burden of diabetic CVD is staggering, and the prevalence of DM is increasing at an alarming rate, particularly among individuals of lower socioeconomic status in the developing world [1–5, 17]. Tragically, the standard-of-care medications (e.g., statins and ACE inhibitors) to prevent diabetes-related CVD complications are neither affordable nor accessible to these populations [1]. Therefore, there is an urgent need for effective and inexpensive interventions to reduce the public health and economic burden of diabetic complications of CVD [1]. A viable approach lies in the intersection of complementary and precision medicine. One highly tenable pharmacogenomic approach is the use of vitamin E supplementation to target high-risk diabetes patients defined by their Hp genotype. This chapter reviews the clinical evidence and mechanistic basis as to why individuals with diabetes who carry a specific Hp genotype are at higher risk for vascular disease and appear to derive cardiovascular benefit with vitamin E supplementation.

Vitamin E Supplementation Trials

Several CVD primary and secondary prevention trials testing vitamin E supplementation have been conducted based on the hypothesis that vitamin E reduces oxidative stress [18]. Results from these trials in both diabetes and general populations primarily show a null effect of vitamin E on CVD risk [18–20]. Meta-analyses of these trials have also shown the possibility of increased CVD mortality with vitamin E [19]. Consistent with these findings are results from atherosclerosis progression trials where vitamin E effects range from null to pro-atherogenic [21].

Despite support from observational studies, there are likely many reasons as to why clinical trials failed to show benefit of vitamin E supplementation on CVD prevention. One potential explanation may be that antioxidant therapy is only beneficial under high oxidative stress. In the Vitamin E Atherosclerosis Prevention Study (VEAPS), a well-nourished, low-risk, general population of men and women, vitamin E supplementation appeared to act as a prooxidant increasing the progression of atherosclerosis relative to placebo [21]. On the other hand, the Secondary Prevention with Antioxidants of Cardiovascular Disease in End-Stage Renal Disease (SPACE) trial showed that in a highly oxidatively stressed cohort of hemodialysis patients with pre-existing CVD, vitamin E significantly reduced the primary trial endpoint of myocardial infarction, ischemic stroke, peripheral vascular disease, and unstable angina by 50% relative to placebo [22]. Comparison of VEAPS and SPACE suggests that the effect of vitamin E on CVD appears to range from a prooxidant effect to a protective effect depending upon the underlying level of oxidative stress. Additional health states, including diabetes mellitus, contribute to high levels of oxidative stress.

Haptoglobin Genotype and Protein

Hp is an abundant plasma α2-sialoglycoprotein synthesized by hepatocytes. Hp is secreted in response to IL-1, IL-6, and tumor necrosis factor [23]. The best known function of Hp is to bind free hemoglobin released from red blood cells [24]. Daily, 6–7 grams of hemoglobin is released into the bloodstream due to turnover of red blood cells. This free hemoglobin is capable of causing considerable oxidative tissue damage by releasing heme iron. However, whenever hemoglobin is released into the

circulation, it immediately binds to Hp with extremely high affinity (Kd ~10^{-15}) to form an Hp-hemoglobin complex. The binding of Hp to hemoglobin serves to inhibit the oxidative potential of hemoglobin from causing cellular damage and damaging organs such as blood vessels and the kidneys by preventing the release of heme iron [25–27]. Hp is normally found in the blood in a more than 400-fold molar excess to free hemoglobin (10 μM vs. 25 nM), and therefore all hemoglobin that is released after intravascular hemolysis is rapidly bound by Hp [28, 29]. Once the hemoglobin is bound to Hp, it is rapidly cleared from the bloodstream predominantly by the monocyte/macrophage CD163 Hp-hemoglobin scavenger receptor expressed on Kupffer cells of the liver [30–32].

Two classes of alleles (1 and 2) have been identified at the Hp locus on chromosome 16q22, with homozygous (1-1 or 2-2) and heterozygous (2-1) genotypes possible [28, 33]. The Hp polymorphism is an extremely common polymorphism. The frequency of the three genotypes in Western populations is approximately 16% Hp 1-1, 48% Hp 2-1, and 36% Hp 2-2 [28, 33] and is the same in individuals with and without DM [33]. The Hp 2 allele appears to have arisen from the Hp 1 allele early in human evolution (~100,000 years ago) by a duplication of exons 3 and 4 of the Hp 1 allele [28, 33]. The protein product of the Hp gene (Hp monomer) is found in serum as a polymer of between 2 and 10 covalently linked monomers. The stoichiometry of the Hp polymer is Hp-genotype-dependent due to differences in the valences of the Hp 1 (monovalent) and Hp 2 (bivalent) allelic protein products. The net result of these differences, as confirmed by electron microscopy, is that Hp is found as a dimer in Hp 1-1 individuals, a linear polymer in Hp 2-1 individuals, and a cyclic polymer in Hp 2-2 individuals [34].

Haptoglobin Genotype and Macrovascular Disease Risk

Multiple independent prospective longitudinal studies from diverse ethnic groups and geographic areas have shown that after accounting for all cardiac risk factors, the Hp 2-2 genotype is an independent risk factor for incident atherosclerotic cardiovascular disease (CVD) in type 1 and type 2 diabetes patients. These studies include 6 prospective longitudinal studies, in which a total of 1735 patients with diabetes and the Hp 2-2 genotype and 3038 patients with diabetes and the Hp 1-1 and Hp 2-1 genotypes were followed for 30 days to 18 years for CVD events (Table 21.1). Controlling for conventional CVD risk factors and diabetes characteristics, these studies reported a consistent 1.5- to 5-fold increased CVD risk in diabetes patients with the Hp 2-2 genotype (36% of all diabetes patients in these cohorts) compared to diabetes patients without the Hp 2-2 genotype [6–12].

In the Strong Heart Study, a population-based longitudinal study of CVD in American Indians ($n = 4549$), Hp genotype was determined in 206 CVD cases (incident cases of fatal and nonfatal CVD) and 206 matched controls aged 45–74 years with median follow-up of 6 years [6]. Controlling for diabetes characteristics and CVD risk factors, diabetes participants who carried the Hp 2-2 genotype

Table 21.1 Cardiovascular disease risk in diabetes patients who carry the Hp 2-2 genotype

Study	Outcome in Hp 2-2
Strong Heart Study	3–5-fold ↑ CVD
Heart Outcomes Prevention Evaluation	2.2-fold ↑ CVD
Munich Stent Study	2.3-fold ↑ MI
Rambam Myocardial Infarction Outcomes in Diabetes Study	2.5-fold ↑ death/heart failure
Israel Cardiovascular Atherosclerosis Risk and Vitamin E	2.3-fold ↑ CVD
Epidemiology of Diabetes Complications	2.2-fold ↑ CVD
Women's Health Study	1.4-fold ↑ CVD

had a fivefold (odds ratio (OR), 4.96; 95% confidence interval (CI), 1.85–13.33) increased risk of CVD relative to diabetes participants who carried the Hp 1-1 genotype and a threefold (OR, 3.04; 95% CI, 1.30–7.09) increased risk of CVD relative to diabetes participants who carried the Hp 2-1 genotype [6].

In the Heart Outcomes Prevention Evaluation (HOPE) trial, 9541 men and women 55 years and older at high risk for CVD with established CVD or diabetes in addition to one other CVD risk factor were randomly assigned in a 2 × 2 factorial design to either vitamin E 400 IU daily or placebo and either Ramipril or placebo for a mean 4.5 years [35]. From the 3654 diabetes patients randomized in HOPE, 30% were genotyped for Hp (n = 1078) of which 278 patients did not receive vitamin E supplementation or Ramipril [7]. Diabetes patients who carried the Hp 2-2 genotype and did not receive either vitamin E supplementation or Ramipril (n = 91) had a 2.2-fold (OR, 2.18; 95% CI, 1.22–3.90) increased risk for CVD as compared with diabetes patients who were non-Hp 2-2 genotype carriers and did not receive either vitamin E supplementation or Ramipril (n = 187) [7]. CVD was defined as a composite of myocardial infarction (MI), stroke, and CVD death.

In the Women's Health Study (WHS), 39,876 healthy women at least 45 years old were randomly assigned in a 2 × 2 factorial design to either vitamin E 600 IU every other day or placebo and either aspirin or placebo for a mean of 10.1 years [36]. From the 1027 diabetes patients randomized in WHS, 721 White women (representative of 95% of the WHS cohort) were genotyped for Hp. The incident CVD (nonfatal MI, nonfatal stroke, CVD death, and coronary revascularization) event rate was increased 35% (RR, 1.35; 95% CI, 0.89–2.06) in diabetes women who carried the Hp 2-2 genotype and did not receive vitamin E supplementation (31 events/131 women with Hp 2-2 genotype) compared with diabetes women who were non-Hp 2-2 genotype carriers and did not receive vitamin E supplementation (40 events/229 women with non-Hp 2-2 genotype [Hp 1-1, n = 56; Hp 2-1, n = 173]) [8].

In the Munich Stent Study, a consecutive series of 935 diabetes patients followed for 1 year after percutaneous transluminal coronary angioplasty (PTCA), Hp 2-2 genotype carriers (n = 382) had increased risk of major adverse cardiac events defined as restenosis, death, acute MI, target vessel revascularization, and repeat PTCA (OR, 1.73; 95% CI, 1.13–2.65) compared with Hp 1-1 genotype carriers (n = 129) [9]. Diabetes patients who carried the Hp 2-2 genotype had a 2.3-fold ($p < 0.0001$) increased risk of acute MI compared with diabetes patients with non-Hp 2-2 genotype [9]. The risk of adverse events was dependent on the number of Hp 2 alleles as demonstrated by a graded event risk across the Hp 1-1, Hp 2-1 (n = 424), and Hp 2-2 genotypes, with correlation to the number of Hp 2 alleles for major cardiac events ($p = 0.015$), repeat PTCA ($p = 0.029$), and acute MI ($p < 0.0001$) [9].

In the Rambam Myocardial Infarction Outcomes in Diabetes Study, the relationship between Hp genotype and mortality and heart failure 30 days post-MI were prospectively assessed in 1437 patients presenting with MI, including 506 participants with diabetes. Diabetes patients with the Hp 2-2 genotype (n = 209) had significantly larger myocardial infarctions (determined from echocardiographic examination of wall motion) and a 2.5-fold ($p = 0.02$) increased incidence of 30-day mortality and heart failure compared with diabetes patients with the Hp 1-1 genotype (n = 53) [10]. Diabetes patients with the Hp 2-1 genotype (n = 244) also had a 2.9-fold increased incidence of 30-day mortality and heart failure compared with diabetes patients with the Hp 1-1 genotype [10]. Among the 931 patients without diabetes, the Hp genotype was not related to these outcomes [10].

The Israel Cardiovascular Atherosclerosis Risk and Vitamin E (ICARE) study was the first randomized, double-blinded, placebo-controlled trial to determine the effect of vitamin E 400 IU/day relative to placebo on CVD in 1434 type 2 diabetes mellitus (T2DM) patients >55 years of age who carried the Hp 2-2 genotype [11]. In ICARE, diabetes patients who carried the Hp 2-2 genotype and were randomized to placebo (n = 708), as well as those who were not randomized (Hp 1-1 genotype (n = 285) and Hp 2-1 genotype (n = 1248)), were followed observationally for CVD outcomes for 18 months [11]. The CVD event rate (unadjusted or adjusted) was increased in the Hp 2-2 diabetes

patients (unadjusted hazard ratio [HR], 2.4; 95% CI, 1.4–4.0; adjusted HR, 2.3; 95% CI, 1.4–3.9) who received placebo (events = 4.7%) as compared with diabetes patients who carried the Hp 1-1 genotype (events = 2.1%) or the Hp 2-1 genotype (events = 2.0%) [11].

Although the association between diabetic complications and the Hp 2-2 genotype has been established predominantly in T2DM, the Hp genotype has also been shown to be a major determinant of CVD risk in T1DM. The Epidemiology of Diabetes Complications (EDC) study was a prospective cohort selected for childhood-onset (<17 years of age) T1DM and followed long-term for diabetes complications [37]. The Hp genotype was determined from EDC participants who had DNA available and were free from CVD at study entry ($n = 453$) [12]. At study entry, the mean age of the 453 participants was 27.1 years, and the mean duration of diabetes was 18.8 years. During 18 years of follow-up, the proportion of incident CVD (MI, revascularization, stenosis >50%, CVD death, angina, and ischemia) significantly increased across the Hp genotypes 1-1 ($n = 52$), 2-1 ($n = 187$), and 2-2 ($n = 214$) from 15.4% to 28.3% and 34.6%, respectively (p-trend = 0.007) [12]. Adjusting for CVD risk factors, the Hp 2-2 genotype was associated with a 2.2-fold (HR, 2.21; 95% CI, 1.05–4.65) and a 1.8-fold (HR, 1.78; 95% CI, 0.84–3.79) increased incidence of CVD compared with the Hp 1-1 genotype and with the Hp 2-1 genotype, respectively [12].

Consistent with the substantial CVD event risk associated with the Hp 2-2 genotype, the level of atherosclerosis has also been shown to be increased in carriers of the Hp 2-2 genotype [13–15]. In the Diabetes Heart Study of the genetic and epidemiological causes of CVD in T2DM, Hp genotype was determined in 1208 individuals (Hp 1-1, $n = 168$; Hp 2-1, $n = 505$; Hp 2-2, $n = 535$) who were on average 61.5 years of age [13]. The Hp 2-2 genotype was strongly associated with increased carotid artery intima-media thickness (CIMT) ($p = 0.001$) [13], a measure of subclinical atherosclerosis [38]. In a cross-sectional CIMT study of 160 T2DM and 40 healthy individuals on average 50 years of age, the Hp 2-2 genotype ($n = 115$ participants; 85 participants with non-Hp 2-2 genotype) was significantly correlated with greater CIMT ($p = 0.013$) [14]. In the Diabetes, Impaired glucose tolerance in Women and Atherosclerosis (DIWA) study, a cohort of 64-year-old women, women with established diabetes (Hp 2-2, $n = 44$, and non-Hp 2-2, $n = 72$) and newly detected diabetes at screening (Hp 2-2, $n = 87$, and non-Hp 2-2, $n = 139$), were genotyped for Hp [15]. Among all the participants, there was no significant difference in subclinical carotid atherosclerosis between the Hp 2-2 and non-Hp 2-2 genotypes [15]. However, in the participants with established diabetes (median duration of 6.3 years), the prevalence of carotid artery plaques ($p = 0.047$) and CIMT ($p = 0.024$) were greater in the diabetes patients who carried the Hp 2-2 genotype compared with diabetes patients who carried a non-Hp 2-2 genotype [15]. In the Coronary Artery Calcification in Type 1 Diabetes (CACTI) prospective study, coronary artery calcification (CAC) was assessed by serial computed tomography over 6 years in 1416 persons with and without T1DM aged 19 to 56 years without history of coronary artery disease [16]. The Hp genotype was determined in 436 participants with T1DM and in 526 persons without diabetes [16]. Diabetes participants free of CAC at baseline who carried the Hp 2-2 genotype had a twofold (OR, 1.95; 95% CI, 1.07–3.56) greater risk of development and progression of CAC compared with diabetes participants who were non-Hp 2-2 genotype carriers [16]. Among persons with T1DM, the Hp 2 allele had an allele-dose effect on development and progression of CAC compared with the Hp 1-1 genotype: Hp 2-1 (OR, 1.72; 95% CI, 1.09–2.71) and Hp 2-2 (OR, 2.94; 95% CI, 1.87–4.65) [16]. The Hp polymorphism was not associated with CAC progression in individuals without diabetes [16]. Further studies show increased iron deposition, increased oxidative stress and inflammation, increased heme oxygenase-1 expression, and increased apoptotic cells in atherosclerotic plaques from carriers of the Hp 2-2 genotype compared to non-Hp 2-2 carriers, consistent with the findings that the protein product of the Hp 2 allele is defective in its ability to block oxidative reactions mediated by iron derived from hemoglobin [39–41]. CIMT and CAC results validate those from studies of clinical endpoints demonstrating that the Hp genotype is a robust correlate for atherosclerosis in diabetes patients.

Haptoglobin Genotype and Microvascular Disease Risk

In addition to CVD risk, the Hp 2-2 genotype is also associated with greater microvascular complications of diabetes including nephropathy [42–44]. The Hp 2-2 genotype has been associated with increased rate of decline of renal function and end-stage renal disease (ESRD) in several independent cohorts [42–44]. Diabetic nephropathy is the leading cause of ESRD accounting for approximately 40% of all patients who require renal replacement therapy [45]. Traditional risk factors and glycemic control are inadequate for predicting incidence and severity of diabetic nephropathy. As reactive oxygen species (ROS) have been implicated in diabetic nephropathy progression, polymorphic genetic loci encoding functional variants in enzymes protecting against oxidative stress serve as potential susceptibility determinants for development of diabetic nephropathy [46].

In longitudinal analyses of renal function in EDC during 18 years of follow-up, risk of decline in renal function (estimated glomerular filtration rate (eGFR) >30 ml/min/1.73 m^2 from baseline eGFR) and development of ESRD (renal dialysis or transplantation) were increased approximately twofold in T1DM patients who carried the Hp 2-2 genotype compared with T1DM patients who carried the Hp 1-1 genotype: HR = 1.79 (95% CI, 1.06–3.00) for decline in GFR and HR = 2.45 (95% CI, 1.05–5.73) for ESRD [42]. The decline in renal function and development of ESRD were increased approximately 30% in TIDM patients who carried the Hp 2-2 compared with TIDM patients who carried the Hp 2-1 genotype, HR = 1.38 (95% CI, 0.82–2.31) for decline in GFR and HR = 1.32 (95% CI, 0.55–3.16) for ESRD [42].

The relationship between Hp genotype and decline in renal function and progression to ESRD were assessed in the Diabetes Control and Complications Trial (DCCT)-Epidemiology of Diabetes Interventions and Complications (EDIC) study over a mean follow-up of 22 years [43]. An increasing risk for developing sustained eGFR <60 ml/min/1.73 m^2 according to number of Hp alleles ($p = 0.037$ for trend) was observed: >2-fold increased risk for developing sustained eGFR <60 ml/min/1.73 m^2 in T1DM patients carrying the Hp 2-2 genotype compared with T1DM patients carrying the Hp 1-1 genotype and an intermediate risk for T1DM patients carrying the Hp 2-1 genotype [43]. Furthermore, DCCT/EDIC participants carrying the Hp 2-2 genotype had a significantly higher ESRD incidence (2.3% for Hp 2-2, 1.5% for Hp 2-1, and 0% for Hp 1-1, $p = 0.036$) [43].

A case-control study of Taiwan nationals showed in 213 patients with chronic kidney disease (defined as persistent proteinuria or eGFR <60 ml/min/1.73 m^2 for at least 3 months) and 213 matched controls that the frequency of the Hp 2-2 genotype and the Hp 2 allele was significantly higher in the chronic disease group than in controls ($p = 0.032$ and 0.024, respectively) [44]. After adjustment for covariates, the Hp 2-2 genotype relative to the Hp 1-1 genotype remained an independent risk factor significantly associated with the development of chronic kidney disease (OR, 3.84; 95% CI, 1.39–10.58) [44].

As further support for a direct connection between Hp genotype and diabetic nephropathy, it has been shown in diabetic mice that replacement by homologous recombination of the wild-type Hp 1 allele with the Hp 2 allele converted wild-type C57Bl/6 mice from a nephropathy-resistant to nephropathy-prone state [47]. Hp 2-2 diabetic mice developed histological and functional changes characteristic of early human diabetic nephropathy; vitamin E provided significant protection against development of diabetic nephropathy features in Hp 2-2 diabetic mice but not in Hp 1-1 diabetic mice [47]. Mechanistic studies suggest that diabetic nephropathy susceptibility to Hp 2-2 is due to renal proximal tubular cell damage from accumulation of Hp 2-2-Hb uptake [47]. Renal proximal tubular cells (via megalin and cubilin) act as a default mechanism (after CD163) for clearance of the Hp-Hb complex [48]. Since CD163-mediated clearance is impaired in diabetes patients who carry the Hp 2-2 genotype, there is a dramatic increase in iron deposition, oxidative stress, and hypertrophy in renal proximal tubular cells [47, 49].

Taken together, the data strongly indicate that the Hp genotype is a major determinant of development and progression of diabetic nephropathy. Although the data suggest that a pharmacogenomic interaction may exist between Hp genotype and vitamin E in the ability to modulate development and progression of diabetic nephropathy, no study has specifically investigated whether vitamin E supplementation reduces renal decline in diabetes patients who carry the Hp 2-2 genotype.

Vitamin E Supplementation, Haptoglobin Genotype, and Cardiovascular Disease

Vitamin E supplementation has not been shown to reduce CVD incidence in genetically unselected populations with and without diabetes; meta-analyses suggest mortality may be increased when given indiscriminately [18–20]. These findings may reflect a differential response of individuals to vitamin E effects on CVD risk, a reduction of CVD in some individuals and an increased risk in others, resulting in an overall null effect.

In the HOPE trial, the primary trial outcome, a composite of MI, stroke, and CVD death, and the secondary outcomes were not significantly different in participants randomized to vitamin E supplementation relative to placebo in the overall HOPE cohort or among participants with diabetes (n = 3654) [35]. In a subgroup analysis of 1078 participants with diabetes and genotyped for Hp, vitamin E supplementation reduced the primary CVD outcome by 30% (RR, 0.70; 95% CI, 0.45–1.10) and significantly reduced CVD death by 55% (RR, 0.45; 95% CI, 0.23–0.90) and nonfatal MI by 43% (RR, 0.57; 95% CI, 0.33–0.97) relative to placebo in those who carried the Hp 2-2 genotype (n = 399) [7]. In the diabetes non-Hp 2-2 carriers (n = 679), there was no difference in the CVD outcome in those randomized to vitamin E supplementation and to placebo [7]. There was a nonsignificant 11% increased CVD event rate in Hp 2-1 diabetes patients who received vitamin E supplementation compared with placebo [50].

In the WHS, the primary trial outcome, a composite of nonfatal MI, nonfatal stroke, and CVD death, was not significantly different in participants randomized to vitamin E supplementation relative to placebo in the overall WHS cohort or among participants with diabetes (n = 1027) [36]. In a subgroup analysis of 721 White participants with diabetes and genotyped for Hp, vitamin E supplementation reduced total CVD, a composite of nonfatal MI, nonfatal stroke, CVD death, and coronary revascularization by 14% (RR, 0.86; 95% CI, 0.51–1.44) relative to placebo in those who carried the Hp 2-2 genotype (n = 277) [8]. There was a nonsignificant 20–25% increased CVD event rate in the diabetes patients who carried the Hp 1-1 and Hp 2-1 genotypes (non-Hp 2-2 genotype, n = 444) and received vitamin E supplementation compared with placebo [8]. The subgroup analyses from HOPE and WHS support the interaction between Hp type and vitamin E supplementation on CVD with consistent directional effects on CVD, reduced hazard ratios in those who carry the Hp 2-2 genotype and increased hazard ratios in those who are non-Hp 2-2 carriers. The smaller reduction in CVD events in the Hp 2-2 participants who received vitamin E supplementation in WHS relative to HOPE and ICARE (summarized below) may be a result of differences between trials including vitamin E dose (600 IU every other day in WHS vs. 400 IU/day in HOPE and ICARE) and age (>60% of cohort was <55 years old in WHS vs. all >55 years old in HOPE and ICARE).

The subgroup analyses from HOPE [7] and WHS [8] were validated in ICARE [11], a randomized, double-blinded, placebo-controlled trial. In ICARE, 726 T2DM patients who carried the Hp 2-2 genotype were randomized to vitamin E 400 IU/day, and 708 T2DM patients who carried the Hp 2-2 genotype were randomized to placebo. Due to limited resources, only 505 participants received vitamin E supplementation, and 479 participants received placebo. In the intention-to-treat analyses, the primary

composite CVD outcome of CVD death, MI, and stroke was significantly reduced 53% in the participants randomized to vitamin E supplementation compared with placebo (event rate 2.2% for vitamin E supplementation vs. 4.7% for placebo; HR, 0.47; 95% CI, 0.27–0.82) after 18 months of randomization; the trial was terminated early [11]. Patients were also followed observationally, and the event rate in the Hp 2-2 diabetes patients randomized to placebo (events = 4.7%) was more than twofold greater than diabetes patients who carried the Hp 1-1 genotype (events = 2.1%) and who carried the Hp 2-1 genotype (events = 2.0%). However, in Hp 2-2 patients randomized to vitamin E supplementation (events = 2.2%), the event rate was reduced to an event rate very similar to that of patients who carried the Hp 1-1 and Hp 2-1 genotypes [11]. About half of the Hp 2-2 diabetes patients in ICARE used statin therapy [51]. Of the 801 Hp 2-2 participants using statins in ICARE, 386 participants were randomized to vitamin E supplementation, and 415 participants were randomized to placebo. Vitamin E supplementation along with statin use dramatically reduced the CVD event rate by 69% compared with statin use alone (1.3% for vitamin E supplementation + statin use vs. 4.1% for placebo + statin use; HR, 0.31; 95% CI, 0.15–0.83) [51]. Since lipid-lowering therapy is currently recommended for diabetes patients for CVD prevention, it is important that the effects of antioxidant therapy provide benefit to Hp 2-2 diabetes patients beyond statin therapy.

Mechanism for a Pharmacogenomic Effect of Vitamin E Supplementation in Diabetes

The Hp polymorphism differs from nearly all polymorphisms assessed in genome-wide association studies since the Hp polymorphism is a functional polymorphism [33]. Understanding functional differences between the Hp 1 and Hp 2 allelic protein products particularly in diabetes provides insight into why diabetes patients who carry the Hp 2-2 genotype have an increased risk of CVD and microvascular complications and how this increased burden of disease may be reduced with vitamin E antioxidant therapy [52].

The main reason why Hp 2-2 diabetes patients appear to uniquely derive benefit from vitamin E is that redox active hemoglobin (Hb) binds with high-density lipoprotein (HDL) only in Hp 2-2 diabetes patients [53]. HDL plays a central protective role in CVD primarily through its reverse cholesterol transport (measured as cellular cholesterol efflux) and antioxidant functions. Redox active Hb does not materially associate with HDL in diabetes patients with the Hp 1-1 genotype or in nondiabetes patients who carry Hp 1-1 and Hp 2-2 genotypes.

The key structural difference between HDL in Hp 1-1 and Hp 2-2 diabetes patients is the result of (1) an impairment in the CD163-mediated clearance of the Hp-Hb complex in diabetes patients who carry the Hp 2-2 genotype resulting in a higher steady-state concentration of Hp-Hb in Hp 2-2 diabetes patients [53–55] and (2) impairment in the ability of the Hp 2-2 protein to neutralize the redox activity of Hb when it binds to Hb to form a Hp-Hb complex [26, 27, 56]. Hp or Hp-Hb can bind to helix 6 (amino acid residues 141–164) on apolipoprotein A1 (ApoA1) in HDL [57]. This binding site overlaps with the binding site on ApoA1 (amino acid residues 159–170) for the lecithin-cholesterol acyltransferase (LCAT) enzyme that is essential for reverse cholesterol transport (cellular cholesterol efflux) and maturation of HDL [58]. Hp directly binds to HDL and serves to tether Hb to HDL. As noted above, due to the impaired clearance of the Hp 2-2-Hb complex in diabetes, there exist more Hp-Hb complexes bound to HDL in Hp 2-2 diabetes patients [53–55]. Furthermore, when bound to HDL, the Hp 2-2-Hb complex produces reactive oxygen species due to the impaired ability of the Hp 2-2 protein to block the oxidation by the Hb-associated iron tethered to HDL by the Hp protein [26, 27, 56]. As opposed to the Hp 1-1 protein, the Hp 2-2 protein is unable to fully stabilize iron in the heme pocket of Hb, and therefore, the heme moiety in the Hp 2-2-Hb complex but not in the Hp 1-1-Hb complex can mediate oxidative reactions [23]. The reactive oxygen species generated by the redox active Hb bound

to HDL oxidize HDL-associated proteins (ApoA1, glutathione peroxidase, paraoxonase, and LCAT) and HDL lipid components (cholesterol) rendering the HDL dysfunctional (decreased reverse cholesterol transport and antioxidant activity), proinflammatory, and pro-atherogenic [53, 59]. The binding of Hb to HDL in Hp 2-2 diabetes patients explains why the HDL of Hp 2-2 diabetes patients is more oxidized and dysfunctional and why these individuals uniquely derive benefit from vitamin E antioxidant therapy. Supportive of this proposed mechanism are studies that show that vitamin E supplementation improves dysfunctional HDL in diabetes patients who carry the Hp 2-2 genotype.

Clinical studies show that peroxidation levels in HDL are greater in diabetes patients who carry the Hp 2-2 genotype compared with diabetes patients who carry the Hp 1-1 genotype [53]. Additionally, compared with diabetes patients who carry the Hp 1-1 genotype, serum from diabetes patients who carry the Hp 2-2 genotype has a reduced ability to mediate reverse cholesterol transport from macrophages and monocytes in vitro [53, 59, 60]. Increased peroxidation levels in HDL and impaired ability to mediate reverse cholesterol transport characterize dysfunctional HDL. In the absence of diabetes mellitus, there is no difference in reverse cholesterol transport across the Hp genotypes. In clinical studies conducted in T2DM and T1DM patients who carry the Hp 2-2 genotype, vitamin E supplementation dramatically improved HDL function with the improved ability of serum from these patients to mediate efflux of cholesterol in vitro [53, 60]. Withdrawal of vitamin E supplementation for as little as 2 months resulted in a loss of the beneficial effect of vitamin E with HDL reverting to its baseline dysfunctional state of impaired cholesterol efflux capability [53, 60]. The beneficial effect of vitamin E supplementation on HDL function in diabetes patients who carry the Hp 2-2 genotype was also associated with a reduction in HDL lipid peroxidation, which also reverted to elevated baseline levels with withdrawal of vitamin E supplementation [53]. The beneficial effect of vitamin E supplementation on cholesterol efflux in diabetes patients who carry the Hp 2-2 genotype was associated with a reduction in HDL lipid peroxidation [53]. On the other hand, vitamin E supplementation had no effect or worsened cholesterol efflux and lipid peroxidation in diabetes patients who carry the Hp 1-1 genotype [53, 60]. These findings in human studies have been replicated in Hp 2-2 and Hp 1-1 diabetic mice [53, 59].

Conclusions

Diabetic complications of CVD account for 80% of all deaths in diabetes patients, and diabetes is the major cause of blindness and renal failure. Although modifiable risk factors have been identified, CVD remains the most important chronic disease in diabetes. The Hp 2-2 genotype appears to account for a large portion of the increased burden of CVD associated with diabetes that heretofore has been unexplained. The Hp 2-2 genotype is associated with a 1.5–5-fold increase in the incidence of CVD in diabetes patients, an association consistently shown in multiple independent studies examining CVD in diabetes patients from different geographic areas with diverse ethnic backgrounds. These data show that the Hp 2-2 genotype increases the risk for CVD in diabetes patients with and without established coronary artery disease. The Hp 2-2 genotype also appears to be an independent risk factor for development of microvascular disease complications of diabetes including nephropathy. The Hp 2-2 genotype is associated with a twofold to threefold increased incidence of decline in renal function, chronic kidney disease, and ESRD. Independent clinical studies show that vitamin E supplementation may reduce the increased risk of CVD and renal failure associated with the Hp 2-2 genotype in diabetes. These clinical studies are supported by mechanistic data and animal studies.

Pharmacogenomics is a key component of precision medicine. Therapy targeted to a specific individual based on his or her genetically determined pathophysiology responsible for disease offers the possibility of significantly improving patient care and reducing costs. Despite clear public health and economic benefits attained from such an approach, pharmacogenomics has yet to be deployed in the

optimization of drug therapy especially in relation to chronic disease. As outlined in this chapter, a potentially viable approach includes the intersection of complementary and precision medicine that deploys vitamin E supplementation to target high-risk diabetes patients defined by their Hp genotype.

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Chapter 22
Vitamin E and Metabolic Syndrome

Richard S. Bruno

Keywords Metabolic syndrome · Nonalcoholic fatty liver disease · α-Tocopherol · Vitamin E status

Key Points

- Obesity-related disorders, such as the metabolic syndrome, type 2 diabetes mellitus, and nonalcoholic fatty liver disease (NAFLD), are on the rise, largely due to the consumption of energy-dense and micronutrient-poor diets and physical inactivity.
- Vitamin E consumption in many populations is below the recommended dietary allowance.
- Supplementation with pharmacological doses of α-tocopherol has shown benefit in metabolic syndrome-related diseases, including NAFLD.
- Scientific focus on metabolic aberrations in relation to vitamin E intake, pharmacokinetics, and status may be a more appropriate approach to evaluate α-tocopherol requirements in relation to its health benefits.

Introduction

The growing prevalence of obesity and associated insulin resistance over the past half-century has given significant rise to numerous cardiometabolic disorders that were previously observed at low rates in the general population. In particular, metabolic syndrome (MetS) now affects a significant proportion of the worldwide population, especially in developed countries where obesity is more prevalent. The high prevalence of obesity-related disorders (e.g., MetS, type 2 diabetes mellitus, nonalcoholic fatty liver disease (NAFLD)) is largely attributed to poor lifestyle, such that diets often contain excess energy in association with limited intakes of essential nutrients, including the antioxidant α-tocopherol. Beyond poor α-tocopherol intakes, evidence indicates that individuals with MetS have compromised α-tocopherol status that is accompanied by a physiological inability to efficiently achieve adequacy of α-tocopherol, which likely creates a vicious cycle of increasing inflammatory damage that further provokes the depletion of α-tocopherol. This chapter will therefore emphasize the growing problem of MetS along with related morbidities because their pathologies likely drive

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_22

impairments along the gut-liver axis that limit α-tocopherol bioavailability and alter its metabolism. These dysregulated physiological responses contribute to inadequate α-tocopherol status, provide rationale for increased attention to establish specialized dietary recommendations, and help to explain why pharmacological administration of α-tocopherol functions to manage NAFLD in humans, a condition that is recognized to be the hepatic manifestation of MetS [1].

Metabolic Syndrome

MetS is at epidemic proportions worldwide [2]. For example, national survey data from the United States indicate that approximately 35% of the total adult population is afflicted with MetS. However, its prevalence occurs in an age-dependent manner such that ~18% of young adults (20–39 years old), ~34% of middle-aged adults (40–59 years old), and ~47% of older adults >60 years old are afflicted. Prevalence rates are also marginally higher among women regardless of age, and minority groups (i.e., Hispanics, Blacks) compared with Caucasians appear to be more susceptible to MetS particularly among the older adult population. These trends are of significant concern because complications in MetS are driven by systemic inflammation in association with cardiometabolic abnormalities that provoke premature mortality by increasing the risk of more advanced metabolic disorders and chronic diseases (e.g., nonalcoholic fatty liver disease (NAFLD), type II diabetes, heart disease) [3, 4].

MetS is defined by the presence of a cluster of conditions that includes at least three of five risk factors (Table 22.1): hypertension, hyperglycemia, central obesity, hypertriglyceridemia, or depressed high-density lipoprotein (HDL) [5]. In this regard, most patients with type 2 diabetes mellitus will be classified as having MetS. Classification of MetS, or at least specific criteria, can also be established on the basis of select drug therapy. For example, fibrates and nicotinic acid are the most commonly prescribed drugs for hypertriglyceridemia and low HDL-C, respectively. Thus, patients using these drugs are presumed to have these MetS criteria. Similarly, the use of ω-3 fatty acids at high doses presumes the presence of hypertriglyceridemia and can be used to establish MetS criteria.

Given that metabolic abnormalities in MetS are closely aligned with the presence of obesity, which is known to affect a considerable proportion of the worldwide population, it is not surprising that the incidence of MetS has dramatically increased in association with the underway

Table 22.1 Criteria for the diagnosis of MetS.[a] Individuals classified with MetS must meet at least three of the following five specified criteria

Criteria	Cutoff point
Elevated waist circumference[b]	≥102 cm (male) or ≥88cm (female)
Elevated blood triglycerides	≥150 mg/dL or pharmacological treatment for hypertriglyceridemia
Reduced HDL-C	<40 mg/dL (males) or <50 mg/dL (females) or use of pharmacological treatment for reduced HDL-C
Elevated blood pressure	Systolic ≥130 mmHg and/or diastolic ≥85 mmHg or use of antihypertensive medications
Elevated fasting blood glucose	≥100 mg/dL or pharmacological treatment to manage hyperglycemia

[a]Obtained from a joint statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity [5]

[b]Cutoff point for waist circumference is specific to the United States

obesity epidemic. Nonetheless, identification of individuals specifically meeting MetS criteria is of critical concern because their underlying morbidities are often subclinical and/or go unmanaged for years. Consequently, compared with healthy individuals, they often have increased oxidative stress that is evidenced by circulating biomarkers of lipid peroxidation (e.g., F_2-isoprostanes, oxidized LDL) and low antioxidant capacity. Similarly, inflammatory responses including circulating pro-inflammatory interleukins (IL), tumor necrosis factor-α (TNFα), C-reactive protein, myeloperoxidase, and other inflammatory mediators are typically heightened in individuals with MetS. These abnormalities in association with classic symptoms of dyslipidemia (i.e., hypercholesterolemia, hypertriglyceridemia) not only provoke chronic disease risk but likely alter the metabolism and requirements of essential antioxidants including α-tocopherol. Thus, despite current dietary requirements being defined for only healthy individuals [6], it has been recognized that individuals with underlying morbidities like MetS may have specialized dietary requirements that are higher than those of healthy individuals. This is important in the context that overweightness and obesity, a central component of MetS, now afflicts a significant proportion of the population. In the United States alone, surveys by the Centers for Disease Control and Prevention indicate that more than one-third of adults are obese (body mass index (BMI) ≥30 kg/m^2) and nearly an equivalent proportion of Americans is considered overweight (BMI = 25–30 kg/m^2). This indicates that "healthy" individuals are no longer the societal norm and that focus is needed to establish dietary requirements that address the needs of *all* individuals, with consideration for potential alterations occurring in a health status-dependent manner. Specific population-level estimates are not available for α-tocopherol intakes in MetS individuals. However, data indicate that obese individuals compared with normal weight adults have 5–12% lower intakes of micronutrients (vitamins A, C, E, calcium, magnesium) and a higher prevalence of nutrient inadequacy [7].

Diet, Requirements, and Adequacy of Vitamin E

Dietary Requirements of α-Tocopherol

The term "vitamin E" is used to describe eight structurally related tocopherols (α-, β, γ-, δ-) and tocotrienols (α-, β, γ-, δ-). Most research tends to focus specifically on α-tocopherol because this is the only vitamin E form that is essential for humans, and none of the seven other vitamin E forms are interconverted to α-tocopherol in humans; thus, α-tocopherol must be specifically obtained from the diet [6]. In the United States, the Estimated Average Requirement (EAR) of α-tocopherol for adults regardless of gender is 12 mg/d, a dietary level that is used to assess adequate intakes for populations. When evaluating dietary intakes for individuals, 15 mg/d of α-tocopherol represents the Recommended Dietary Allowance (RDA) and is derived by extrapolation from the EAR to meet the needs of 97–98% of the population.

There are currently no additional dietary requirements for α-tocopherol for any specialized conditions, including for any health disorders or smoking status. The latter is despite the antioxidant vitamin C having an additional requirement for smoking status [6]. Indeed, a similar recommendation was not considered for α-tocopherol although evidence indicates that oxidative stress sufficient to increase lipid peroxidation levels in smokers accelerates α-tocopherol depletion compared with nonsmokers [8]. Similarly, when the most recent dietary requirements for α-tocopherol were established in 2000, there was insufficient evidence to support higher recommendations in relation to cardiometabolic risk factors (e.g., obesity, hyperlipidemia, insulin resistance, MetS) [6]. This is despite reports providing evidence that these populations have increased oxidative stress.

Dietary Intake of α-Tocopherol

Regardless of MetS, α-tocopherol is one of the major shortfall nutrients in the diet with estimates indicating that only 2.4% of American women and 8% of men meet the EAR for α-tocopherol from food alone [9]. There is limited evidence evaluating α-tocopherol intakes in MetS individuals, but on the basis that obese individuals consume less α-tocopherol than healthy individuals [7], α-tocopherol intakes in MetS are expected to be similarly poor compared with non-obese Americans.

The development and progression of MetS could be alleviated by improved diet quality and a focus on nutrient-dense foods [10], especially increased intake of whole grains, fruits and vegetables, nuts, and seeds and decreased intake of refined sugars, white flour, and saturated fats [11]. However, these public health recommendations have had little impact [12] consistent with the growing trends of obesity and MetS during the past several decades [13, 14]. This is especially concerning in children and adolescents where MetS is being detected at a younger age [13]. Even in controlled settings, energy restriction to help promote weight loss actually results in substantially lowering α-T intakes [15, 16]. Thus, considerable effort for nutrition education is warranted to promote higher intakes of α-tocopherol-rich foods while limiting excess energy intake (Fig. 22.1). However, the complete meal pattern must be considered beyond the conventional focus on individual foods. For example, nuts are an energy-dense food that are especially rich in vitamin E, but they have been predicted erroneously to increase cardiometabolic risk. Their higher intakes are actually associated with reduced weight gain, a lower risk of becoming obese [17], improving vascular endothelial function [18], and reducing CVD-related morbidity and mortality [19, 20].

Nuts, especially almonds, are an α-tocopherol-rich food source (Fig. 22.1). A controlled weight loss study that implemented an almond-enriched diet (15% of energy) as part of an energy-restricted diet (−500 kcal/d) was shown to outperform energy restriction alone to reduce trunk fat [21]. In addition to improved blood pressure [21], substitution of a high-carbohydrate snack with almonds

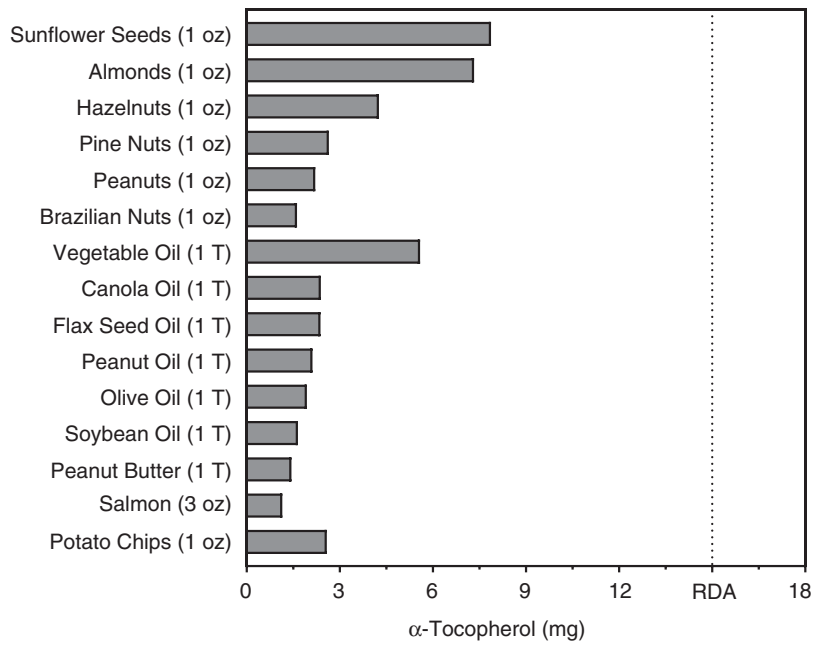

Fig. 22.1 Nuts and oils are α-tocopherol-rich foods [22]. Food portions are expressed on a per serving basis to visualize their potential contribution to meeting the recommended dietary allowance (RDA) for male and female American adults, which is currently set at 15 mg/d [6]

(1.5 oz/d) as part of a cholesterol-lowering diet more substantially decreased abdominal fat without affecting total body mass in association with a greater lowering of LDL-C and non-HDL-C [23]. Dietary incorporation of almonds (56 g/d) also reduces inflammation (IL-6, TNFα, C-reactive protein) and increases the resistance of LDL to oxidation [24]. When consumed as a snack during a 4-week intervention, almonds decrease postprandial glycemic excursions during an oral glucose tolerance test in association with acute decreases in hunger and desire to eat [25]. Thus, it is clear that, despite being energy-dense, nuts have the potential to substantially improve α-tocopherol intakes without undesirable effects that preclude effective weight management.

Assessment of α-Tocopherol Adequacy

It is obvious from dietary pattern studies that most individuals, regardless of MetS or obesity status, fail to meet dietary recommendations for α-tocopherol. However, overt clinical deficiency of α-tocopherol is rarely observed and is generally restricted to those individuals having a mutation in the α-tocopherol transfer protein (α-TTP) or secondary to gastrointestinal- or hepatobiliary-related diseases that affect lipid absorption. This has placed increased emphasis on defining adequate α-tocopherol status on the basis of circulating α-tocopherol itself and/or the development of novel approaches and biomarkers.

Current α-tocopherol requirements are based on limited data from the 1950s from only seven institutionalized men and do not consider assessments relevant to MetS morbidities (e.g., body mass, liver fat, hyperlipidemia, inflammation), sex, or potential interactions with other circulating antioxidants like vitamin C [6]. From these deletion-repletion studies in which overt clinical vitamin E deficiency did not manifest, it was determined ex vivo that hydroperoxide-induced hemolysis of erythrocytes was minimized in individuals having circulating α-tocopherol of 12 μmol/L. This "biomarker" was utilized in modern day recommendations to establish the EAR for α-tocopherol. This approach has considerable limitations because (1) it lacks physiological relevance and (2) few individuals have circulating α-tocopherol concentrations that fall below 12 μmol/L. In fact, it is estimated that fewer than 1% of adults have circulating levels less than 12 μmol/L, suggesting that vitamin E deficiency is not a concern [26, 27]. To the contrary, if plasma α-tocopherol concentrations of 20 μmol/L or 30 μmol/L are used as the cutoff threshold for adequacy, based on outcomes of observational studies, then ~30% or ~80% of adults would be classified as having inadequate α-tocopherol status, respectively (see also Chaps. 12–15).

The challenge with utilizing any of these circulating α-tocopherol cutoffs is that α-tocopherol status is difficult to assess in hyperlipidemic adults, such as those with MetS. Specifically, elevated blood lipids "trap" α-tocopherol in the circulation. This yields apparently "normal" plasma α-tocopherol concentrations that mask the fact that α-tocopherol concentrations in target tissues are insufficient and permit oxidative injury [28, 29]. This paradigm in hyperlipidemic cohorts, including MetS, is best described by the concept of "physiological inadequacy" and is driven by impaired trafficking of α-tocopherol along the gut-liver axis [30]. Consistent with these concepts, national survey data that compared circulating α-tocopherol between healthy and MetS adults found no difference in concentrations by health status even after adjusting for age, sex, race or ethnicity, education, smoking status, cotinine concentration, physical activity, fruit and vegetable intake, and vitamin or mineral use [31]. However, differences by health status became apparent after additional adjustment for circulating cholesterol and triglyceride concentrations such that levels were lower in individuals with MetS compared with healthy individuals. While this supports poorer α-tocopherol status in MetS, this approach remains limited because (1) overt α-tocopherol deficiency or even inadequacy is not readily observed in the clinical setting and (2) there are no well-established lipid-normalized cutoffs for classifying α-tocopherol status. For these reasons, the antioxidant function of α-tocopherol has been considered

in relation to mitigating oxidative stress, which is readily observed in MetS and related cardiometabolic disorders [32]. In a well-controlled intervention in hypercholesterolemic adults with high oxidative stress, α-tocopherol supplementation (0–3200 IU/d) was shown to dose-dependently decrease plasma F_2-isoprostane concentrations, with statistical significance observed at a minimal dose of 1600 IU/d [33]. It was further determined in these participants who completed a time-dependent study that a minimum of 16 weeks was needed to decrease plasma F_2-isoprostane concentrations. While improving α-tocopherol intakes exhibits favorable benefits on lipid peroxidation, it should be noted that the dose needed is pharmacological and greatly exceeds dietary α-tocopherol recommendations. For this reason, investigators have employed modern-day pharmacokinetic approaches using isotopically labeled α-tocopherol to define altered physiological responses (e.g., MetS, hyperlipidemia, insulin resistance) on the differential requirements of α-tocopherol by health status (Fig. 22.2). This will be discussed in the context of α-tocopherol bioavailability and metabolism in the subsequent sections of this chapter.

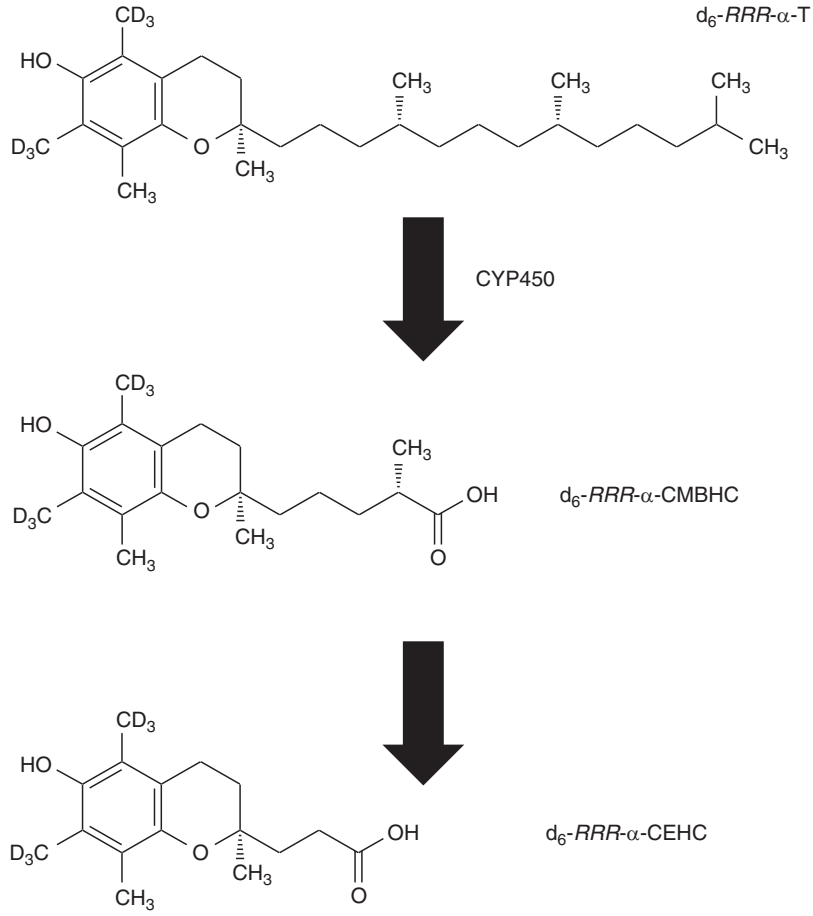

Fig. 22.2 Deuterium-labeled α-tocopherol is a tool to evaluate α-tocopherol trafficking, bioavailability, and metabolism in humans. Strategic placement of deuterium (d) atoms on the chromanol head of α-tocopherol permits detailed studies of cytochrome P-450 (CYP450)-mediated metabolism of α-tocopherol to its intermediate metabolite α-carboxymethylbutyl-hydroxychromanol (α-CMBHC) and its terminal metabolite α-carboxyethyl-hydroxychromanol (α-CEHC). Deuterium-labeled α-tocopherol and associated metabolites can be assessed from biological fluids using liquid chromatography-mass spectrometry

Bioavailability and Metabolism of α-Tocopherol

Benefits of α-Tocopherol Isotopes

Early studies using oral administration of isotopically labeled α-tocopherol have provided clear evidence that dietary fat and the food matrix regulate the bioavailability of α-tocopherol [34–36]. Similarly, this approach has been used to demonstrate faster α-tocopherol depletion in smokers [8, 37, 38], and more recent efforts have focused on assessing α-tocopherol bioavailability and metabolism in more contemporary cardiometabolic disorders including hyperlipidemia and MetS [29, 30, 39].

The benefit of α-tocopherol isotopes (Fig. 22.2) is that their uptake, metabolism, and disposition can be measured in a sensitive manner independent of endogenous α-tocopherol status. α-Tocopherol bioavailability is regulated at the level of the liver through the selectivity of the α-TTP that preferentially secretes hepatic α-tocopherol into the systemic circulation. However, "bioavailability" encompasses all the intestinal and hepatic-level processes that influence dietary lipid absorption and secretion. These include intestinal micellarization, enterocyte packaging and secretion of α-tocopherol as part of chylomicrons (Fig. 22.3), receptor-mediated uptake of chylomicrons at the liver, and repackaging of hepatic α-tocopherol for its secretion as part of very-low-density lipoprotein (VLDL; Fig. 22.4). In addition, the liver is regarded as the primary site for α-tocopherol metabolism, which occurs in a cytochrome P450-mediated manner (see also Chap. 4 [Vitamin E metabolism and bioavailability]). At the liver, excess α-tocopherol that is not directed for VLDL secretion via the actions of the α-TTP is either disposed of in the bile and/or catabolized to a water-soluble metabolite known as α-carboxyethyl-hydroxychromanol (α-CEHC; Fig. 22.2). Clearly, there are many checkpoints along the gut-liver axis in which lipid processing can be dysregulated in obesity-related disorders.

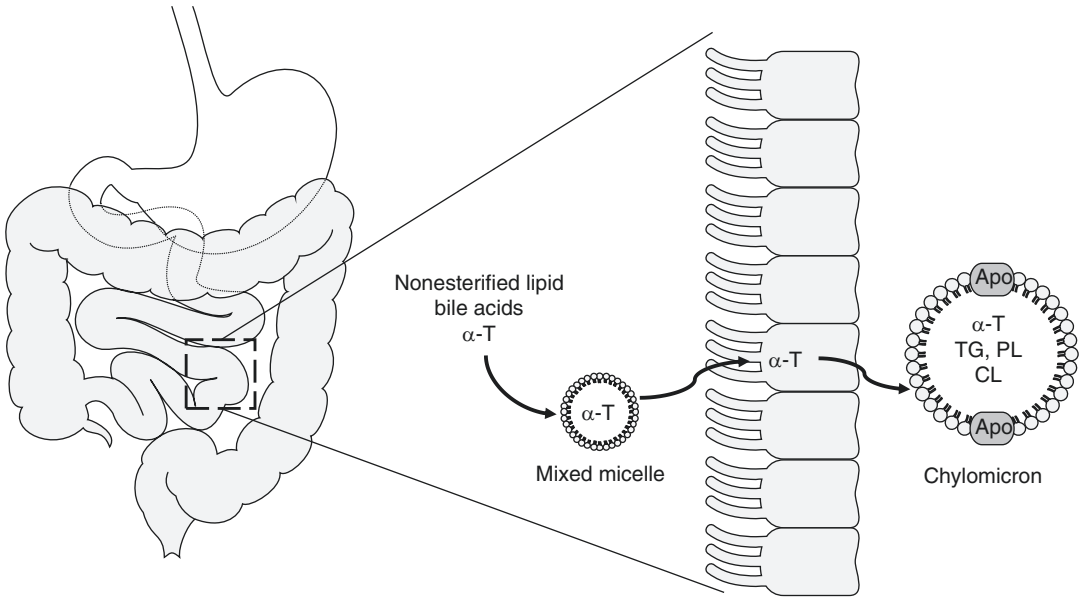

Fig. 22.3 Intestinal uptake, packaging, and secretion of α-tocopherol in enterocyte-derived chylomicrons. In order for α-tocopherol to be absorbed, dietary α-tocopherol is initially emulsified into mixed micelles through the coordinated activities of bile acids and nonesterified fatty acids. α-Tocopherol is then transferred from the micelle to the enterocyte where α-tocopherol and esterified lipids are packaged into apolipoprotein-containing chylomicrons prior to secretion. Abbreviations: Apo apolipoprotein (e.g., apoB48), α-T α-tocopherol, CL cholesterol, PL phospholipid, TG triglyceride

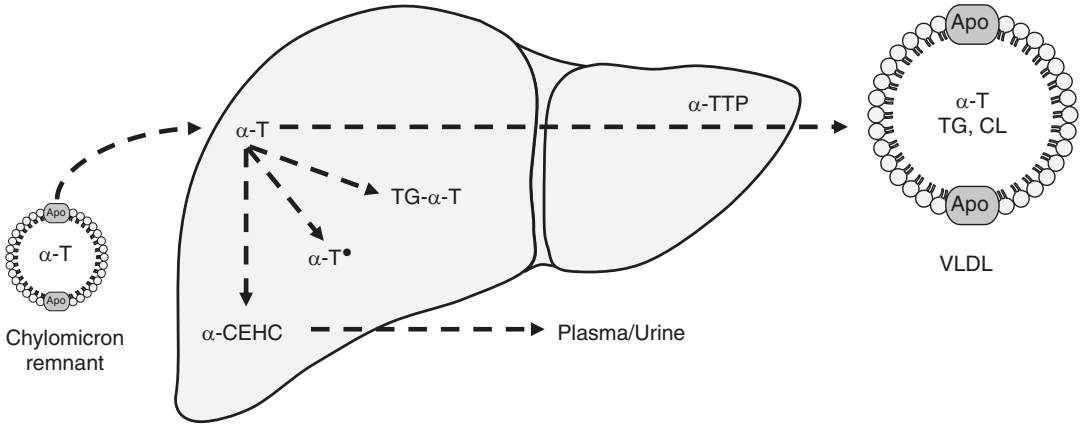

Fig. 22.4 α-Tocopherol trafficking and metabolism at the liver. α-Tocopherol is delivered to the liver as part of chylomicron remnants, which are taken up in a receptor-dependent manner. Under normal physiological conditions, α-tocopherol is packaged and secreted from the liver as part of VLDL in an α-tocopherol transfer protein-dependent manner. Excess hepatic α-tocopherol is catabolized in a cytochrome P450-dependent manner to α-CEHC that gets excreted. Under conditions of metabolic stress, α-tocopherol can be oxidized to α-tocopheroxyl radicals or potentially sequestered in triglyceride-rich hepatocytes resulting from liver steatosis. Abbreviations: Apo apolipoprotein, CL cholesterol, α-CEHC α-carboxyethyl-hydroxychromanol, TG triglyceride, TG-α-T, triglyceride-associated α-tocopherol (such as that in steatotic hepatocytes), α-T α-tocopherol, α-T$^{\bullet}$ α-tocopheroxyl radical, α-TTP α-tocopherol transfer protein

Thus, isotopically labeled α-tocopherol is an important tool to define physiological responses that hinder its bioavailability, contribute to inadequate α-tocopherol status, and hence, drive recommendations for increased dietary requirements in disorders such as MetS that are characterized by insulin resistance, oxidative stress, inflammation, and dyslipidemia.

Hyperlipidemia

Consistent with the gap in knowledge regarding the role of hyperlipidemia on α-tocopherol homeostasis and requirements, a pharmacokinetics study was conducted to compare responses of normolipidemic individuals to those having hypercholesterolemia alone (HC; total cholesterol >6.5 mmol/L and triglyceride <1.5 mmol/L) or combined hypercholesterolemia and hypertriglyceridemia (HCT; total cholesterol >6.5 mmol/L and triglycerides >2.5 mmol/L) [40]. For 48 h following the ingestion of 150 mg deuterium-labeled *RRR*-α-tocopherol, labeled α-tocopherol was measured in plasma and isolated lipoproteins from these middle-age and otherwise healthy individuals (43–50 years old; BMI = 25–26 kg/m^2). Plasma α-tocopherol half-life was greater in HC individuals compared with normolipidemic individuals (68 h vs. 52 h) and even greater in individuals with HCT (136 h), likely due to slower lipoprotein turnover in these individuals. However, this occurred without affecting area under the curve ($AUC_{0–48\,h}$) for plasma α-tocopherol concentrations, suggesting that hyperlipidemia "traps" α-tocopherol in the circulation without affecting its bioavailability. However, evaluation of labeled α-tocopherol in chylomicrons indicated a greater $AUC_{0–48\,h}$ for HC individuals compared with both normolipidemic individuals and those with HCT. $AUC_{0–48\,h}$ for labeled α-tocopherol in VLDL was also lower in HC individuals compared with both normolipidemic individuals and those with HCT. Based on these observations in relation to separate data showing reduced α-tocopherol in blood components (platelets, lymphocytes, and erythrocytes), the investigators concluded that hyperlipidemia affects the uptake of newly absorbed α-tocopherol, which may be relevant to the pathogenesis of

atherosclerosis. Indeed, studies in transgenic mice lacking expression of both α-TTP and apolipoprotein E, which induces α-tocopherol deficiency combined with hypercholesterolemia, accelerate the progression of atherosclerotic lesions mediated by increased aorta lipid peroxidation [41].

Influence of MetS on Intestinal Absorption and Hepatic Trafficking of α-Tocopherol

Hyperlipidemia is a significant component of cardiometabolic risk, but it rarely occurs in the absence of other risk factors. Not only is dyslipidemia a hallmark feature of MetS, but central obesity, hyperglycemia, and hypertension are also often present and in association with poor antioxidant status, systemic inflammation, and oxidative stress. In MetS and healthy adults, plasma and lipoprotein pharmacokinetics of α-tocopherol were evaluated over 72 h following co-ingestion of deuterium-labeled α-tocopherol with dairy milk beverages containing 0–8 g of fat [30]. At baseline, individuals met established criteria for MetS and also had increased fasting insulin and calculated insulin resistance that was accompanied by increased plasma concentrations of C-reactive protein, IL-10, IL-6, oxidized LDL concentrations, and lower circulating ascorbic acid. Plasma α-tocopherol concentrations (μmol/L) were not different between cohorts, but lipid-normalized α-tocopherol concentrations (μmol/mmol total lipid) were lower in MetS, suggesting suboptimal vitamin E status at study onset. Healthy and MetS adults then completed a randomized crossover study in which they ingested deuterium-labeled α-tocopherol (15 mg unesterified *RRR*-α-tocopherol) with fluid dairy milk beverages that contained 0, 2.4, or 8 g fat to examine whether α-tocopherol bioavailability would improve in a fat-dependent manner. Surprisingly, dairy milk fat had no influence on plasma or lipoprotein pharmacokinetic responses in healthy or MetS participants, nor did estimated absorption of α-tocopherol differ in response to dairy milk fat. This was contrary to earlier studies in healthy humans showing fat-dependent increases in α-tocopherol bioavailability following the oral ingestion of α-tocopheryl acetate [34]. The discrepancy between studies is potentially attributed to the form of α-tocopherol administered (esterified vs. unesterified) and/or physiochemical properties of milk (e.g. phospholipids, dairy proteins) independent of its fat content that promote α-tocopherol bioavailability [30]. The latter is in agreement with studies by another group of investigators who reported that the absorption of unesterified α-tocopherol in healthy adults was higher than previously observed in humans at 81%, which occurred in response to ingesting 1.81 nmol of C^{14}-labeled α-tocopherol with 60 g of 2% fat milk [42].

When evaluating α-tocopherol pharmacokinetics irrespective of dairy fat ingestion, MetS adults had a lower $AUC_{0–72\ h}$ for plasma α-tocopherol compared with healthy adults (Fig. 22.5) [30]. Their overall poor bioavailability occurred without affecting time to maximal concentrations (T_{max}) of α-tocopherol but was explained by lower maximal plasma α-tocohperol concentrations (C_{max}) that corresponded with MetS adults absorbing an estimated ~26% of the α-tocopherol dose compared with ~30% in healthy adults. Similar to prior studies in hyperlipidemic adults, MetS adults also had slower rates of plasma α-tocopherol disappearance that remained slower even after normalizing α-tocopherol concentrations for circulating lipids. Thus, the half-life of α-tocopherol in MetS was ~37 h compared with ~31 h in healthy adults.

Because estimated absorption was reduced in MetS and occurred without accelerated plasma α-tocopherol disappearance, this indicated that α-tocopherol trafficking along the gut-liver axis in MetS was dysregulated. Consistent with this notion, α-tocopherol enrichment in isolated chylomicrons and VLDL was decreased in MetS compared with healthy adults (Fig. 22.6). One potential explanation for reduced chylomicron enrichment is that obese adults tend to have excess lipid accumulation in enterocytes, and its mobilization is inversely related to adiposity [43, 44]. This suggests

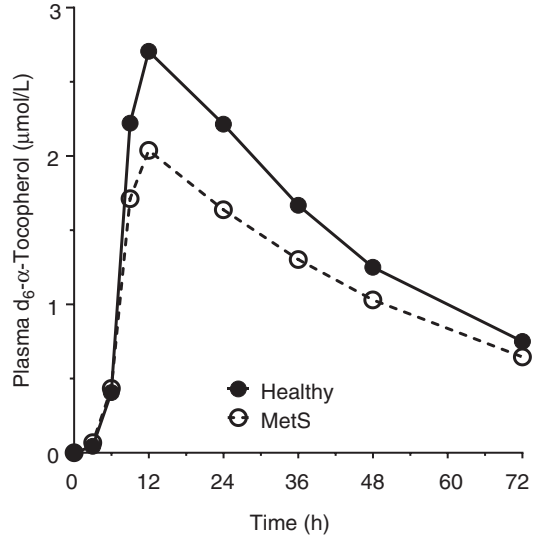

Fig. 22.5 Pharmacokinetics of plasma α-tocopherol in healthy adults and those with MetS who ingested 15 mg of hexadeuterium-labeled *RRR*-α-tocopherol. Data adapted from a published reported [30]

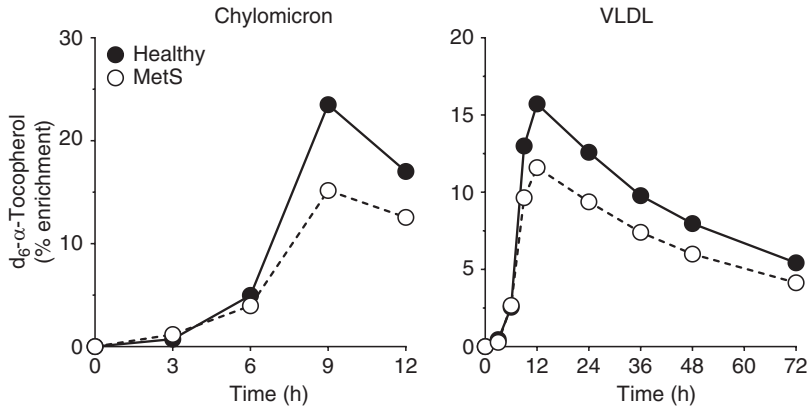

Fig. 22.6 Enrichment of d_6-α-tocopherol in isolated chylomicron and VLDL lipoprotein fractions from MetS and healthy adults who ingested d_6-α-tocopherol. Data adapted from a published report [30]

that α-tocopherol may get "trapped" in enterocytes and go unabsorbed consistent with the short life-span of enterocytes that are sloughed off at frequent intervals prior to their elimination in feces. Regardless of the mechanism, less chylomicron enrichment of α-tocopherol (Fig. 22.6) results in less delivery of α-tocopherol to the liver for packaging and hepatic secretion as part of VLDL. This may directly explain why MetS adults have less α-tocopherol enrichment in VLDL. However, individuals with MetS likely have nonalcoholic steatohepatitis (NASH), which is considered the hepatic manifestation of MetS [1]. In this regard, α-tocopherol may also get sequestered in steatotic livers thereby rendering it unavailable for packaging in VLDL. Separate from this, participants with MetS had increased inflammatory responses (C-reactive protein, IL-6, IL-10) that were inversely correlated with α-tocopherol enrichment in both chylomicrons and VLDL. This indicates the possibility that intestinal and/or hepatic level oxidative stress responses may have depleted α-tocopherol consistent with its antioxidant function that terminates the cyclic progression of lipid peroxidation [45].

Consistent with this possibility, plasma α-tocopherol C_{max} was also inversely correlated with defined criteria of MetS (waist circumference, systolic blood pressure, and glucose and triglyceride concentrations), and calculated insulin resistance, and positively related with HDL-C concentrations.

Overall, these findings collectively suggest that MetS drives increased dietary requirements for α-tocopherol to compensate for metabolic and/or oxidative stress responses that impair α-tocopherol trafficking along the gut-liver axis. However, it should be noted that prior studies have suggested that rates of plasma α-tocopherol disappearance could be used to calculate α-tocopherol requirements using a balance approach [8, 34, 46]. This perspective is likely confounded in MetS and other populations where hyperlipidemia adversely influences α-tocopherol pharmacokinetics [47]. Thus, in hyperlipidemic populations, consideration may be needed to determine the quantity of α-tocopherol that achieves a similar C_{max} compared with healthy individuals, and/or future study is warranted to establish complementary biomarkers of α-tocopherol status having direct physiological relevance.

α-CEHC: A Complementary Tool for Assessing α-Tocopherol Status

α-CEHC is thought to be produced primarily at the liver when excess α-tocopherol is present and may serve as a physiologically relevant biomarker of α-tocopherol status that circumvents the long-standing reliance on ex vivo hydrogen peroxide-induced hemolysis of erythrocytes [6]. In this regard, the liver is theoretically protected from α-tocopherol toxicity by upregulating cytochrome P450-mediated metabolism (see also Chap. 4 [Vitamin E metabolism and bioavailability]) to generate a water-soluble catabolite (i.e., α-CEHC) that can be more readily excreted (Fig. 22.2). Studies in smokers showed that α-tocopherol depletion is accelerated by oxidative metabolism and provided early evidence that reduced α-CEHC concentrations reflect less α-tocopherol availability for this catabolic pathway [37]. Findings from a cohort study demonstrated that 24-h urinary α-CEHC concentrations increased in response to increasing dietary intakes of α-tocopherol in the preceding 24 h [48]. Interestingly, the inflection point in which urinary α-CEHC increased occurred at an estimated dietary intake of 12.8 mg, which is close to the EAR for α-tocopherol (12 mg). With this approach, urinary α-CEHC has been suggested to be a valid biomarker that predicts α-tocopherol status.

A similar approach using α-CEHC to corroborate α-tocopherol status was undertaken in adults with MetS and healthy individuals [29]. In these individuals, urinary deuterium-labeled α-CEHC was evaluated from 24-h urine collections following the oral ingestion of 15 mg deuterium-labeled α-tocopherol. Urinary concentrations of d_6-α-CMBHC and d_6-α-CEHC were highly correlated with each other, suggesting that the flux of α-tocopherol through this catabolic pathway is unrestricted. From urine samples collected from 0–8 h, 8–16 h, and 16–24 h, individuals with MetS had significantly lower urinary α-CEHC and α-CMBHC at each time point compared with healthy adults. This was consistent with their lower α-tocopherol bioavailability that was observed previously [30]. Interestingly, urinary d_6-α-CEHC in MetS individuals failed to increase in a time-dependent manner, suggesting that underlying hepatic α-tocopherol status may have been depleted at study onset and that the 15 mg oral dose was insufficient to replete the liver. Contrasting this, d_6-α-CMBHC increased in healthy adults at 8–16 h and was reduced at 16–24 h, whereas d_6-α-CEHC was only lowered at 16–24 h compared with 8–16 h. These findings were corroborated by measuring plasma d_6-α-CEHC. Consistent with the investigators' hypothesis, overall plasma concentrations of d_6-α-CEHC ($AUC_{0–24\,h}$) were lower in MetS adults compared with healthy individuals. An interesting observation in this study was that peak plasma d_6-α-CEHC occurred at 6 h for both groups, whereas peak plasma d_6-α-tocopherol occurred at 12 h. The early appearance of d_6-α-CEHC relative to d_6-α-tocopherol was unexpected and raised the question of whether the liver is the exclusive site of α-tocopherol catabolism despite the overall evidence supporting the utility of α-CEHC as a biomarker of α-tocopherol status.

Vitamin E Therapy for Nonalcoholic Fatty Liver Disease

The outcomes of large-scale interventions investigating α-tocopherol on chronic diseases, especially cardiovascular disease, are mostly equivocal with some studies supporting the use of α-tocopherol, whereas others found no benefit. The close association of MetS with NAFLD has reinvigorated interest in α-tocopherol to potentially alleviate a contemporary metabolic disorder that afflicts 80–100 million Americans [49] (see also Chap. 23). Indeed, patients with simple steatosis or overt NASH have lower circulating cholesterol-normalized concentrations of α-tocopherol compared with healthy individuals [50]; there was no difference between patients with steatosis versus NASH. Using regression analysis, it was observed that serum α-tocopherol normalized to cholesterol was an independent predictor of simple steatosis (odds ratio = 0.76). This suggests that α-tocopherol insufficiency occurs as a result of steatosis or potentially contributes to its manifestation. Based on these findings and knowledge that NASH is driven by oxidative stress that increases hepatic lipid peroxidation [51], α-tocopherol is an attractive potential therapy to manage liver injury.

Numerous pilot studies have examined α-tocopherol alone, or in combination with other antioxidants or lifestyle modifications, to manage NAFLD. The PIVENS trial is perhaps the most rigorous randomized clinical study to date that has examined α-tocopherol to alleviate NASH in liver biopsy-confirmed patients [52]. In this study, 247 adults with NASH but without diabetes were randomized to receive an insulin-sensitizing agent (pioglitazone), α-tocopherol (800 IU/d), or placebo for 96 weeks. The primary outcome of the study was an improvement in histologic evidence of NASH, as determined using a composite scoring scheme that considers the magnitude of liver steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis. Dietary supplementation of α-tocopherol compared with placebo was associated with a higher rate of histologic improvement. In contrast, pharmacological use of pioglitazone compared with placebo improved histologic features of NASH, but this occurred without achieving statistical significance. Despite these disparate outcomes, serum liver injury biomarkers (alanine and aspartate aminotransferase) were significantly lowered in response to either treatment compared with placebo, and both were associated with improvements in liver steatosis and lobular inflammation, but not fibrosis.

The TONIC trial was another rigorously performed intervention that was conducted in children with biopsy-confirmed NAFLD (8–17 years old) who were randomized to receive α-tocopherol (800 IU), metformin, or placebo for 96 weeks [53]. For this study, a sustained reduction in serum alanine aminotransferase was the primary outcome variable and was specifically defined as 50% or less of the baseline value or 40 U/L or less from 48 to 96 weeks during the intervention period. Relative to placebo, a sustained reduction in serum alanine aminotransferase in response to α-tocopherol or metformin was not achieved. However, despite not being the primary outcome, there was a statistical tendency for a mean change in serum alanine aminotransferase in response to placebo (−35.2 U/L) compared with α-tocopherol (−48.3 U/L; $P = 0.07$), but not compared with metformin ($P = 0.40$). α-Tocopherol and metformin each improved hepatocellular ballooning compared with placebo, whereas α-tocopherol but not metformin improved the NAFLD activity score compared with placebo. Overall, NASH was shown to be resolved in 58% of children randomized to receive α-tocopherol compared with 28% receiving placebo ($P = 0.006$), whereas there was no significant difference for NASH resolution for children randomized to metformin compared with placebo. Despite several improvements in several secondary outcomes, the authors concluded that neither α-tocopherol nor metformin was superior compared with placebo based on the null outcome of the primary outcome variable to sustain a reduction of serum alanine aminotransferase.

Consistent with the generally supportive outcomes of PIVENS and TONIC, the benefits of α-tocopherol for NAFLD have also been demonstrated through a meta-analysis [54]. Using the outcomes of randomized controlled trials ($n = 5$), dietary α-tocopherol supplementation was associated with significantly lowering aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase,

steatosis, inflammation, and hepatocellular ballooning compared with the control group. Nonetheless, the high dose of α-tocopherol used in each of these clinical studies greatly exceeds that of conventional dietary recommendations (i.e., RDA). This suggests that the RDA is limited in scope to prevent α-tocopherol deficiency, whereas higher, pharmacological doses may be warranted to achieve optimal intakes that effectively manage morbidity and mortality risk. It also emphasizes the need for studies to define the lowest dietary intake level that functions to ameliorate NAFLD-related morbidity and to establish biomarkers of α-tocopherol status that can effectively monitor therapy while minimizing the possibility of any adverse effects.

Conclusions

Similar to healthy individuals, those with MetS have poor dietary α-tocopherol intakes that are substantially lower than the RDA. Their intakes may actually be lower than those of healthy individuals, and it is clear that they have compromised α-tocopherol status based on their lipid-normalized concentrations of circulating α-tocopherol. This is corroborated by sophisticated measures of deuterium-labeled α-tocopherol pharmacokinetics showing that MetS status impairs intestinal absorption and hepatic trafficking of α-tocopherol while also demonstrating insufficient hepatic α-tocopherol status that is evidenced by limited catabolism of α-tocopherol to α-CEHC. Although circulating α-tocopherol concentrations are within physiological limits despite ongoing controversy over appropriate cutoff values [26, 27], individuals with MetS are likely to have "physiological inadequacy" of α-tocopherol. This helps to explain how circulating α-tocopherol is apparently normal despite biochemical indices indicating heighted inflammation and lipid peroxidation. Nonetheless, the field would benefit substantially from advances in approaches and/or technologies that can reliably assess α-tocopherol status. This would be expected to improve health outcomes for a substantial proportion of the population while also advancing an appreciation for effective therapeutic management in metabolic conditions (e.g., NAFLD) that are closely linked to MetS [52, 53]. This is especially important because pharmacological intakes of α-tocopherol show promise in MetS-related metabolic disease, whereas findings of meta-analyses are discrepant in that some suggest that pharmacological intakes increase all-cause mortality, whereas others report no relationship on all-cause mortality [55, 56]. Thus, scientific focus on disease-specific morbidity and mortality may be a more appropriate approach to evaluate the benefits of α-tocopherol on health. In addition, a hierarchy of research evidence could be utilized to formulate evidence-based recommendations, especially when conducting randomized controlled trials may not be practical or would pose significant limitations in data interpretation [57].

Acknowledgments A sincere thanks to Katie England, MS (The Ohio State University, Columbus, OH), for providing editorial assistance. This work was supported by the Ohio Agricultural Research and Development Center at the Ohio State University (Columbus, OH) and a grant from the National Dairy Council.

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Chapter 23
Vitamin E in Nonalcoholic Fatty Liver Disease

Bubu A. Banini and Arun J. Sanyal

Keywords α-tocopherol · Metabolic syndrome · Nonalcoholic fatty liver disease · Nonalcoholic steatohepatitis · Oxidative stress · PIVENS trial · TONIC trial · Vitamin E

Abbreviations

4-HNE	4-Hydroxy-2-nonenal
8-OHdG	8-Hydroxy-2′-deoxyguanosine
ALT	Alanine aminotransferase
CRN	Clinical Research Network
CYP2E1	Cytochrome P450 2E1
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide
FFA	Free fatty acids
GPx	Glutathione peroxidase
GSH	Glutathione
IR	Insulin resistance
JNK	Janus kinase
MCD	Methionine-choline-deficient
MDA	Malondialdehyde
MRC	Mitochondrial respiratory complex
NAD	Nicotinamide adenine dinucleotide
NAFL	Nonalcoholic fatty liver
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
PIVENS	Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients with Nonalcoholic Steatohepatitis
PPAR-α	Peroxisome proliferator-activated receptor-α
PUFAs	Polyunsaturated fatty acids
RCT	Randomized control trial

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_23

ROS	Reactive oxygen species
SELECT	Selenium and Vitamin E Cancer Prevention Trial
SH3BP5	SH3 domain-binding protein 5
TNF	Tumor necrosis factor
TONIC	Treatment of nonalcoholic fatty liver disease in Children

Key Points

- Nonalcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver disease worldwide.
- Nonalcoholic steatohepatitis (NASH) is a subtype of NAFLD that can progress to cirrhosis and liver cancer.
- There are several drugs in the pipeline of testing for NASH; however, none has been approved to date.
- The multiple parallel hypothesis suggests that NASH results from various processes occurring in tandem, including abnormal lipid metabolism, lipotoxicity, oxidative stress, mitochondrial dysfunction, altered cytokine and adipokine production, gut dysbiosis, and endoplasmic reticulum stress.
- Vitamin E in an antioxidant that improves the histological features of NASH.
- Large randomized control trials with long follow-up periods are needed to define the long-term benefits and potential side effects of vitamin E.
- Based on currently available data, vitamin E is recommended by both European and North American practice guidelines for the treatment of NASH.

Nonalcoholic Fatty Liver Disease

Over the past few decades, nonalcoholic fatty liver disease (NAFLD) has become one of the leading causes of chronic liver disease worldwide, especially in Western countries where obesity and metabolic syndrome have been on the rise. NAFLD is broadly subdivided into nonalcoholic fatty liver (NAFL) also known as simple fatty liver and nonalcoholic steatohepatitis (NASH) [1]. NASH, characterized by histological hallmarks of steatosis, lobular inflammation, and hepatocellular ballooning, can progress to fibrosis and liver cancer [2] (Fig. 23.1). Although there are many drugs in the pipeline of testing for NASH, none has been approved to date [3] (Fig. 23.2).

The pathogenesis of NASH is complex. Day and James initially proposed the two-hit hypothesis of NASH, the first hit being the appearance of steatosis, with a subsequent second hit resulting in inflammation, hepatocyte damage, and fibrosis [4]. However, with the expanding wealth of information on NASH over the past two decades, it is not clear that steatosis is necessarily a prerequisite for the development of NASH. A recently proposed multiple parallel hypothesis [5] suggests that NASH results from various processes occurring in tandem, including abnormal lipid metabolism, lipotoxicity, oxidative stress, mitochondrial dysfunction, altered production of cytokines and adipokines, gut dysbiosis, and endoplasmic reticulum (ER) stress, with genetic susceptibility to NASH serving as an underlying contributing factor.

Oxidative Stress and Free Radicals in NAFLD

Oxidative stress, resulting from an imbalance between prooxidant and antioxidant mechanisms in favor of prooxidation, has been recognized as a key mechanism in the progression of steatosis to steatohepatitis [6, 7]. Several studies have shown an association between NAFLD and biomarkers of oxidative stress or lipid oxidation [8–12]. Oxidative stress is mediated largely by reactive oxygen

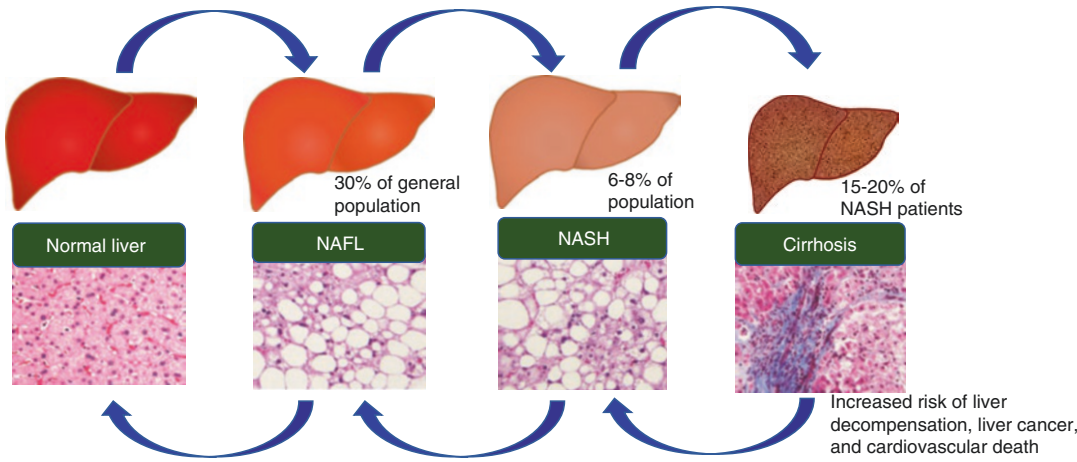

Fig. 23.1 Nonalcoholic fatty liver disease (NAFLD): spectrum of disease. Lifestyle habits (diet and exercise) and environmental factors can lead to the development of nonalcoholic fatty liver (NAFL). About 6–8% of the population develop nonalcoholic steatohepatitis (NASH), characterized histologically by steatosis, inflammation, and hepatocyte ballooning with or without fibrosis. About 15–20% of NASH patients develop cirrhosis, which significantly increases the risk of hepatic decompensation, liver cancer, and cardiovascular-related mortality. Abbreviations: NAFL nonalcoholic fatty liver, NASH nonalcoholic steatohepatitis

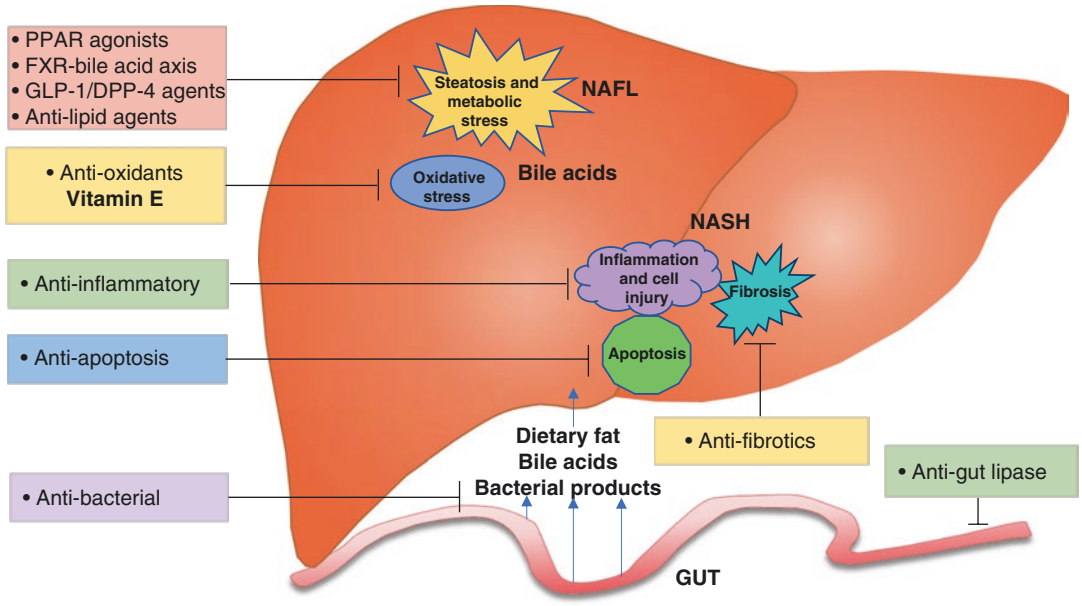

Fig. 23.2 Drug categories in clinical trials for nonalcoholic fatty liver disease (NAFLD). The pathogenesis of NAFLD is complex, involving a large number of pathways that serve as targets for therapeutic development. Vitamin E is a naturally occurring compound with antioxidant properties shown to improve liver enzymes and liver histology in patients with NAFLD. Abbreviations: FXR farnesoid X receptor, GLP-1/DDP-4 glucagon-like peptide-1/dipeptidyl peptidase-4, NAFLD nonalcoholic fatty liver disease, NASH nonalcoholic steatohepatitis, PPAR peroxisome proliferator-activated receptor

species (ROS). Molecular oxygen readily accepts electrons from exogenous substances and during intracellular metabolic processes to form various free radicals including hydroxyl radical, nitric oxide radical, and superoxide anion. These free radicals react with proteins, free fatty acids (FFA), and DNA to mediate oxidative injury [13].

The production of ROS occurs mainly in mitochondria, endoplasmic reticulum (ER), and peroxisome, and to a lesser extent through various enzymatic processes including xanthine oxidase and cytochrome P450 [11, 14]. Models of NAFLD have been shown to have altered mitochondrial morphology, impaired mitochondrial bioenergetics, increased mitochondrial lipid peroxides, and increased mRNA levels of peroxisome proliferator-activated receptor-α (PPAR-α), involved in regulating the expression of mitochondrial and peroxisomal β-oxidation enzymes [11, 15–17].

Oxidative Stress and Organelle Dysfunction in NAFLD

Mitochondrial Dysfunction

In mitochondria, the catabolic process of β-oxidation which breaks down FFA occurs through three main steps [7]. During the first step, long-chain FFA enter into the mitochondria. In the second step, the long-chain FFA are oxidized into medium- and short-chain fatty acids, a process that involves conversion of oxidized nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) into their deoxidized version, NADH and FADH2. In the third step, NADH and FADH2 are re-oxidized into NAD and FAD through the transfer of electrons along the mitochondrial respiratory complex (MRC) enzymatic chain. Most of these electrons combine with oxygen and protons to form water; however, a few electrons react with oxygen to form ROS. Impairment of the MRC complex results in disruption of β-oxygenation and increased production of ROS, including superoxide, hydrogen peroxide, and hydroxyl radicals [11, 18–22] that increase steatosis and resultant inflammation.

Mitochondrial dysfunction facilitates NASH pathogenesis at several levels, including impaired lipid metabolism, increased ROS, and cytokine production, triggering cell death and promoting inflammation [7, 11]. Accumulation of lipid in the mitochondria leads to influx of water and calcium, causing release of cytochrome C and cell death [23]. Lysosomal permeabilization, associated with caspase activation and ROS generation via cytochrome P450 2E1 (CYP2E1) [24], can also mediate mitochondrial injury. Mitochondrial injury can subsequently lead to apoptosis via mechanisms involving Sab, SH3 domain-binding protein 5 (SH3BP5), a substrate of Janus kinase (JNK) located in the mitochondrial outer membrane [25]. Continuous mitochondrial injury further results in hepatocyte injury and progression to NASH.

Other Organelles

Although the mitochondria are the main source of hepatocyte ROS, other organelles have also been shown to participate in this process [7]. Peroxisomes are able to rapidly oxidize long-chain FFA through β-oxidation, resulting in hydrogen peroxide that is converted into the highly reactive hydroxyl (OH) radical [23]. The generation of ROS in a lipid-rich environment induces lipid peroxidation of polyunsaturated fatty acids (PUFAs), leading to the formation of 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) that have longer half-lives than ROS and amplify the effects of ROS via diffusion into the extracellular space to affect distant cells. The increase in lipid peroxidation and protein oxidation impair the hepatocyte mitochondrial respiratory chain, increasing ROS production with resultant detrimental effects on fat metabolism in the liver. Moreover, chronic ER stress may contribute to oxidative stress by promoting toxic accumulation of ROS which triggers other signaling pathways within the cell; ROS generated through inflammation of mitochondria and other organelles may also accelerate ER dysfunction [26, 27]. In addition, lipo-oxygenation of long-chain fatty acids via microsomal cytochromes, CYP2E1 and cytochrome P450 4A (CYP4A), leads to release of ROS.

Role of Inflammation and Oxidative Stress in NAFLD

The generation of intracellular ROS via mitochondria, ER, peroxisomes, or enzymatic pathways all contribute to disease progression in NAFLD. In addition to excessive production of ROS within organelles, oxidative stress also occurs as a result of reduced antioxidant defense. NASH is associated with decreased levels of various antioxidant pathways and enzymes, including hepatic glutathione (GSH), glutathione transferase activity, SOD, glutathione peroxidase (GPx), and catalase [11, 28]. The process of oxidative injury is augmented by obesity and insulin resistance (IR), which lead to increased production of ROS in the liver, increased lipid peroxidation, and decreased plasma antioxidant capacity [29, 30].

Mitochondrial dysfunction facilitates the production of ROS and contributes to NAFLD progression through induction of hepatic inflammatory cytokines. It has been proposed that obesity, IR, and adipokine/cytokine pathways mediate liver fat accumulation and NASH development [7, 31]. This results in additional lipid peroxidation of mitochondrial membranes, augmenting mitochondrial dysfunction and ROS generation [32]. The resultant damage to nuclear and mitochondrial DNA results in necro-inflammation especially in the hepaltocyte nucleus and cytoplasm and in sinusoidal cells [33, 34]. Hepatic necro-inflammation is evidenced by increased levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a modified DNA base product generated by ROS [33]. Hepatic inflammation and fibrogenic progression are key features in the pathogenesis of NASH.

Vitamin E Mechanisms in NASH

Given the crucial role of oxidative stress and free radical injury in the pathogenesis of NASH, it is not surprising that free radical-scavenging agents have shown ameliorating effects on disease progression [35, 36]. Vitamin E is an antioxidant that has been studied in several animal models as well as in clinic trials of NAFLD. There are 8 naturally occurring forms of vitamin E, including 4 tocopherol homologs (α-, β-, γ-, δ-) with a saturated 16-carbon phytol side chain and 4 tocotrienols (α-, β-, γ-, δ-) that have 3 double bonds on the side chain [37]. The term vitamin E describes any combination of tocopherol and tocotrienols. However, α-tocopherol is the most abundant form of vitamin E found in human plasma and in nature. Furthermore, it is the most widely used vitamin E in supplements and in biomedical research.

Vitamin E is efficient in scavenging peroxyl radicals in vivo and preventing lipid peroxidation [38, 39]. It also inhibits the production of isoprostanes which are crucial to peroxidation of lipids [40, 41]. Vitamin E is rapidly consumed and converted back to its reduced form by the cytoplasmic antioxidant cycling network including vitamin C, glutathione, and lipoic acid [42]. The antioxidant role of vitamin E is important in maintaining membrane stability for cell signaling events.

In addition, α-tocopherol regulates gene expression and cell signaling independent of its antioxidant properties; other tocopherols and tocotrienols which possess equal antioxidant potency do not have the ability to mediate these signaling processes [43]. α-tocopherol activates protein phosphatase 2A, inhibiting protein kinase C in vascular smooth muscle cells. It has also been shown to activate mitogen-activated protein kinase and phosphatidylinositol 3-kinase and inhibit phospholipase A2, cyclooxygenase, lipooxygenase, and NADPH-oxidase [44, 45], leading to the inhibition of cell proliferation, platelet and adhesion and aggregation, monocyte-endothelial adhesions, and cytokine release [42, 44, 46]. It is currently unclear if the beneficial effects of vitamin E on NASH are due to its antioxidant role versus its effect on signal transduction versus both.

Vitamin E in Animal Models of NAFLD

Vitamin E has been studied in several experimental models of NAFLD. Vitamin E supplementation reduced the levels of liver enzymes, hepatic steatosis, and necro-inflammation in a methionine-choline-deficient (MCD) diet-induced animal model of NASH [47]. Superoxide dismutase activity was enhanced in these animals, and levels of MDA and genes related to inflammation, apoptosis, and fibrosis were reduced. In another MCD diet-induced model of NASH, hepatic glutathione stores were restored, while oxidative stress markers, hepatic stellate cell activation, and histologic fibrosis were reduced in mice on vitamin E [48].

In a diet-induced obesity model in rats, vitamin E ameliorated oxidative stress [49]. In obese (ob/ob) mice, α- as well as γ-tocopherol protected against lipopolysaccharide-induced liver injury, decreasing hepatic MDA and tumor necrosis factor (TNF)-α levels [50]. In leptin receptor-deficient rats, vitamin E protected against bile acid-induced hepatocyte injury, improving portal inflammation, lobular inflammation, and hepatocellular necrosis [51]. These animal studies laid the groundwork for clinical trials in NAFLD.

Clinical Trials of Vitamin E in NAFLD

Plasma levels of α-tocopherol are decreased in NASH patients compared to healthy controls, providing a rationale for vitamin E supplementation [52]. Among the many agents that have been tested for NASH, vitamin E has had the most substantial effect on disease activity [53]. The impact of vitamin E on clinically meaningful outcomes and progression to cirrhosis is yet to be demonstrated; however, current recommendations for therapeutics focus on decreasing disease activity. Vitamin E is recommended by both European and North American practice guidelines for the treatment of NASH [1, 54]. Although some studies have raised concerns about the long-term safety of vitamin E [55, 56], other studies have challenged these concerns [57, 58]. Earlier studies assessing the efficacy of Vitamin E in NAFLD as monotherapy (Table 23.1) and combination therapy (Table 23.2) gave conflicting results likely due to small sample sizes, differences in primary end points, and differences in vitamin E formulations.

Two separate multicenter randomized-control trials conducted by the NASH Clinical Research Network (CRN) showed that vitamin E was effective in improving steatohepatitis. In Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients with Nonalcoholic Steatohepatitis (PIVENS) trial, 247 nondiabetic and non-cirrhotic adults with NASH received vitamin E (800 IU/day), pioglitazone (30 mg/day), or placebo for a period of 96 weeks [38]. The primary outcome measured was improvement in histological features, based on standardized scores for steatosis, inflammation, and hepatocellular ballooning. A p-value less than 0.025 between the treatment group (vitamin E or pioglitazone) versus the placebo group was considered significant. Vitamin E therapy demonstrated significant histologic improvement in NASH (43% vs 19%, $P = 0.001$), while pioglitazone did not reach statistical significance (34% vs 19%, $P = 0.04$). Significant reduction in hepatic steatosis, lobular inflammation, and hepatocellular ballooning was observed; however, no significant improvement in fibrosis was seen in both treatment groups compared to placebo. It is noteworthy that in a prior small pilot study, combination therapy with vitamin E and pioglitazone was shown to be superior to vitamin E alone in reducing the histological features of NASH and improving pericellular fibrosis [59].

In the treatment of nonalcoholic fatty liver disease in children (TONIC) trial, 173 children received vitamin E (400 IU twice daily), metformin (500 mg twice daily), or placebo for a 96-week period [60]. Resolution of NASH, attributed mainly to improved hepatocellular ballooning, was significantly

Table 23.1 Vitamin E monotherapy in patients with NAFLD

Author and year	Population and design	N	Vitamin E dosage	Duration of study	ALT	Steatosis	Inflammation	Ballooning	Fibrosis
Lavine et al. [70]	Children with US-diagnosed NAFLD and elevated transaminases given vitamin E	11	400 IU–1200 IU daily	4–10 months	↓	No change(based on US)	NA	NA	NA
Hasegawa et al. [71]	Adults with NASH and NAFLD given dietary instruction for 6 months, followed by vitamin E	22	300 mg daily	1 year	↓(NASH group)	↓(6/9 NASH patients)	↓(5/9 NASH patients)	NA	↓(5/9 NASH patients)
Kugelmas et al. [72]	Adults with NASH randomized to vitamin E plus diet/exercise versus diet/exercise alone	16	800 IU daily	12 weeks	↓	NA	NA	NA	NA
Vajro et al. [73]	Children with US-diagnosed NAFLD and elevated transaminases randomized to vitamin E plus low calorie diet versus low calorie diet alone	28	400 mg daily for 2 months, then 100 mg daily for 3 months	5 months	↓	↓(based on US)	NA	NA	NA
Bugianesi et al. [74]	Adults with NAFLD or NASH randomized to vitamin E versus metformin versus placebo	110	400 IU twice daily	1 year	↓	NA	NA	NA	NA
Yakaryilmaz et al. [75]	Adults with NASH given vitamin E	9	800 mg daily	24 weeks	↓	↓	No change	NA	No change
Sanyal et al. [38] (PIVENS)	Nondiabetic, non-cirrhotic adults with NASH randomized to vitamin E versus pioglitazone versus placebo	247	800 IU daily	96 weeks	↓	↓	↓	↓	No change
Lavine et al. [60] (TONIC)	Children with NAFLD and ALT elevation randomized to vitamin E versus metformin versus placebo	173	400 IU twice daily	96 weeks	No change	No change	No change	↓	No change

Abbreviations: *ALT* alanine aminotransferase, *NA* not available, *NAFLD* nonalcoholic fatty liver disease, *NASH* nonalcoholic steatohepatitis, *US* ultrasound

Table 23.2 Vitamin E combination therapy in patients with NAFLD

Author and year	Population and design	N	Vitamin E dosage	Duration of study	ALT	Steatosis	Inflammation	Ballooning	Fibrosis
Harrison et al. [76]	Adults with NASH randomized to vitamin E plus vitamin C versus placebo	49	1000 IU daily	6 months	No change	NA	No change	NA	↓
Sanyal et al. [59]	Adults with NASH randomized to vitamin E plus pioglitazone versus vitamin E alone	20	400 IU daily	6 months	↓	No change	↓	↓	No change
Dufour et al. [77]	Adults with NASH randomized to vitamin E plus UDCA versus UDCA plus placebo versus placebo	48	400 IU twice daily	2 years	↓	↓	No change	NA	No change
Nobili et al. [78]	Children with NAFLD randomized to vitamin E plus vitamin C plus diet/exercise versus placebo plus diet/exercise	90	600 IU daily	1 year	No change	↓(based on US)	NA	NA	NA
Foster et al. [79]	Adults randomized to vitamin E plus vitamin C plus atorvastatin versus placebo	1005 (80 with NAFLD on CT and transaminitis < 1.5 times ULN)	1000 IU daily	4 years	NA	↓(based on CT)	NA	NA	NA
Pietu et al. [80]	Adults with NASH given vitamin E plus UDCA	110	500 IU daily	4 years	↓	↓(3/10 patients)	↓(3/10 patients)	↓(3/10 patients)	↓(4/10 patients)

Abbreviations: *ALT* alanine aminotransferase, *CT* computed tomography scan, *NA* not available, *NAFLD* nonalcoholic fatty liver disease, *NASH* nonalcoholic steatohepatitis, *UCDA* ursodeoxycholic acid, *ULN* upper limit of normal, *US* ultrasound

greater for vitamin E treatment group compared to placebo; however, neither vitamin E nor metformin was superior to placebo in attaining the primary outcome of reduction in alanine aminotransferase (ALT) level by 50% or more compared to baseline or a level of 40 U/L or less from 48 to 96 weeks.

Recently, two meta-analyses reported significant improvement in liver histology in NASH patients treated with vitamin E [61, 62]. A meta-analysis of five studies showed significant improvement in liver enzymes as well as the individual histological features of NASH, steatosis, lobular inflammation, and hepatocellular ballooning. Among adult patients, there was also an improvement in fibrosis [61]. Another meta-analysis performed by a different group showed improvement in all the histological parameters of NASH and also in fibrosis [62].

Vitamin E in Other Forms of Chronic Liver Disease

Oxidative injury and decreased levels of vitamin E have been implicated in various etiologies of chronic liver diseases other than NAFLD, including alcoholic liver disease and viral hepatitis. However, the role of vitamin E in these conditions remains to be defined. In a randomized, placebo-controlled study of alcoholic hepatitis, vitamin E did not confer any benefit on liver function or survival during the 1-year study period [63]. In chronic hepatitis C patients, high-dose vitamin E significantly reduced oxidative stress with no effect on liver enzymes or the degree of hepatocellular inflammation or fibrosis [64]. In a small pilot study of 32 patients with chronic hepatitis B, treatment with vitamin E was associated with improved ALT levels and complete response (normal ALT and negative hepatitis B virus DNA) in 47% of the patients treated [65]. These findings are yet to be replicated in larger studies. At present, vitamin E supplementation can be proposed only for patients with NASH, with the role of vitamin E supplementation in other etiologies of chronic liver disease remaining controversial.

Safety of Vitamin E

Some studies have raised concerns about the long-term safety of vitamin E [55, 56], while other studies have challenged these concerns [57, 58]. A meta-analyses published in 2005 suggested that high-dose vitamin E increased all-cause mortality in a dose-dependent manner and cautioned against the use of any high-dose vitamin supplementation until appropriately designed trials were available [55]. However, this meta-analysis has been criticized because it excluded several studies with low mortality. Moreover, concomitant use of vitamin A and other drugs, as well as factors such as smoking, was not taken into consideration during the analyses. Subsequently, a meta-analysis of those trials, together with more studies investigating vitamin E, suggested that the observed differences were due to a disproportionately higher number of male patients in the trials, compared to other trials, thus casting doubt on the causal relationship of vitamin E supplementation and increased mortality [66] (please see Chap. 17 “Vitamin E and Mortality: A Critical Perspective of the Conflicting Meta-analysis Outcomes” from Wolfgang Köpcke).

A large meta-analysis of 57 studies involving 246,371 subjects studied for 1–10 years did not demonstrate an association between vitamin E supplementation and all-cause mortality [57]. A RCT in 2011, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), showed modestly increased incidence of prostate cancer in healthy men on vitamin E over a 7-year period, translating to an absolute increase of 1.6 per 1000 person-years of vitamin E 400 IU/day (Klein 2011). However, based on subsequent analysis, it appears that this risk may be modified by baseline selenium concentration [67] or genetic variants involved in the metabolism of vitamins [68].

Based on the efficacy of vitamin E in improving the histological features of NASH, it is recommended as first-line off-label therapy for NASH. Current practice guidance from the American Association for the Study of Liver Disease (AASLD) recommends consideration of vitamin E in treating nondiabetic, non-cirrhotic NASH patients with biopsy-proven NASH [1, 54, 69]. It is suggested that risks and benefits should be discussed with the patient prior to initiating therapy. Guidance from a joint task force including the European Association for the Study of the Liver (EASL)/European Association for the Study of Diabetes (EASD)/European Association for the Study of Obesity (EASO) suggests the use of vitamin E for NASH, with a plan to stop treatment if there is no significant improvement in liver enzymes after 6 months of therapy [54].

Conclusion

Vitamin E is beneficial in improving the biochemical and histological features of NAFLD, a major cause of chronic liver disease which increases the risk of cirrhosis and liver cancer. Although the mechanisms by which vitamin E improves NAFLD, more specifically NASH, are unclear, vitamin E is known to restore antioxidant activity in NASH patients and regulate the expression of several genes and cell signaling pathways that are involved in cell proliferation, platelet and monocyte function, and in cytokine release. The beneficial effects of vitamin E on NAFLD and NASH have been demonstrated both in animal studies and in human clinical trials. Larger studies and longer follow-up RCTs are crucial in defining long-term beneficial effects of vitamin E as well as potential side effects. Nonetheless, current findings substantiate an important role of vitamin E in ameliorating chronic liver disease including NASH.

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Chapter 24
The Role of Vitamin E in Aging and Alzheimer's Disease

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Keywords Alzheimer's disease · Clinical trials · Mild cognitive impairment · Reactive species Tocopherols · Tocotrienols · Vitamin E

Abbreviations

ADAS-cog	Alzheimer's Disease Assessment Scale-cognitive subscale
BPT	Brief Praxis Test
BSD	Blessed dementia scale
CDR	Clinical dementia rating
CDT	Clock drawing test
CERAD	Consortium to Establish a Registry in Alzheimer's Disease
Clox-1	Spontaneous clock drawing task
CS	Cohort study
DMR SOC	The Dementia Questionnaire for Mentally Retarded Persons Sum of Cognitive Scores
DRS	Dementia Rating Scale
DS	Down syndrome
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders-IV diagnosis
GSSG	Oxidized glutathione (glutathione disulfide)
LS	Longitudinal study
MCI	Mild cognitive impairment
MIS	Memory Impairment Screen
MMSE	Mini-Mental State Examination
nc	Not calculated
n.s.	Not specified
OLS	Open-label study

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_24

RCT	Randomized clinical trial
SIB	Severe Impairment Battery
TICS-m	Modified Telephone Interview for Cognitive Status

Key Points

- The global rise in life expectancy leads to increasing numbers of patients suffering from age-related diseases, including Alzheimer's disease.
- Increased concentrations of reactive species and oxidatively damaged macromolecules in the brain are hallmarks of Alzheimer's disease.
- Animal studies provide evidence that vitamin E treatment may lower oxidative damage in the brain and improve cognitive function in rodent models of Alzheimer's disease.
- Clinical trials with patients suffering from mild cognitive impairment or Alzheimer's disease show conflicting results, with some trials suggesting benefits and other null effects on disease progression when high doses of α-tocopherol are given.
- Additional and better designed clinical trials, with carefully selected study cohorts and relevant outcomes (e.g. quality of life), are therefore required.

Introduction

Population aging is a key policy issue and is subject of the recently released *World Report on Ageing and Health* of the World Health Organization [117]. There is a dramatic increase in both, the proportion and the absolute number of older people in populations worldwide. One key factor for this development is the increasing life expectancy which is, on average, observed around the world. However, not only living longer but also living healthier lives is one goal that is strongly wished to be achieved.

Epidemiology of Alzheimer's Disease

Dementia contributes greatly to the burden of disability experienced at old age, particularly in high-income countries. To date, more than 47 million people worldwide suffer from dementia, and this number is estimated to triple by 2050, with dramatic personal, social, and economic consequences [117]. Alzheimer's disease (AD) is the most common cause for dementia, responsible for more than 60% of all dementia cases [92]. Only a small number of AD cases are genetically determined. More than 95% of all cases are sporadic and occur in the elderly. The incidence rate of dementia increases exponentially with age and is quite similar across regions [90]. The disease has an estimated prevalence of 10–30% in the population >65 years of age with an incidence of 1–3% [70]. The risk to suffer from AD also grows exponentially with age, doubling approximately every 5–6 years [123]. Thus, AD can be characterized as an age-related neurodegenerative disease that represents the primary cause for dementia in the elderly and a growing public health issue [32].

Clinical Manifestation of Alzheimer's Disease

After a long prodromal phase, AD manifests itself clinically by a progressive cognitive decline followed by gradual personality changes [91]. The transition is often accompanied by a stage of mild cognitive impairment (MCI). Ten to fifteen percent of patients suffering from MCI develop AD within

1 year. In the early phase of the disease, patients show deterioration of memory, difficulties in finding the right words for everyday objects, and/or mood swings. As AD progresses, patients forget recent events, names, and faces, have difficulties in understanding the context of a conversation, and become confused when handling money or driving a car. They undergo personality changes, appearing not to care anymore about themselves and those around them. Patients sometimes burst into tears for no apparent reason or become convinced that someone is trying to harm them. As the disease progresses, people may also adopt unsettling behavior, such as getting up in the middle of the night or wandering off and getting lost. Moreover, they lose their capability and sense for a suitable behavior, undress in public, or make inappropriate sexual advances [48, 49, 87].

Currently, there is no proven disease-modifying treatment available [14]. Interventions with current approved drugs (Ginkgo biloba, acetylcholine esterase inhibitor, and NMDA receptor modulators), if started early enough, may at best temporarily slow down the progression, but cannot impede dementia [109]. Thus, new therapeutic strategies are in the focus of drug discovery programs [16, 39].

Etiology of Alzheimer's Disease

The cause of the sporadic forms of AD with late onset is not yet known but seems to be a result of multiple factors [5]. Aging and Apo E genotype are strongly associated with AD [72, 92, 100]. High blood pressure, high blood levels of homocysteine and cholesterol, diabetes mellitus, adipositas, depression, and smoking are other – modifiable – risk factors [6, 8, 54].

Neuropathological hallmarks of the disease are extracellular senile plaques containing beta-amyloid peptides (Aβ) and intracellular neurofibrillary tangles containing paired helical tau proteins, which have been associated with neuronal loss and atrophy of the cerebral cortex [97, 102]. Thus, misfolded proteins appear to contribute to the pathogenesis, but are not the only factors in the disease process. Especially, the role of amyloidosis as a causative agent in sporadic AD remains undefined. Numerous failures and discontinuations have further highlighted possible inconsistencies in the so-called amyloid hypothesis [60]. Thus, the concept that Aβ is causative for the disease was questioned, and its general rejection is currently debated [18, 45, 78]. Hence, new hypotheses about the causes of this disease are under investigation. For instance, loss of function of presenilins has been claimed to explain both familiar and sporadic forms of AD. The pathogenic contribution of the ε4 isoform of apolipoprotein E in late-onset forms is also actively studied. Other processes such as calcium dysregulation, disturbed autophagy, aberrant proteasome function, mitochondrial failure, and many other alterations are under investigation and will lead to new concepts for sporadic AD including new preclinical models [113].

Another important hallmark of the disease is elevated markers of oxidative stress, and oxidative damage has been widely reported at the levels of nucleic acids, proteins, and lipids in AD brains [2, 68, 108, 114]. MCI, which commonly develops into AD, and human aging are also characterized by higher concentrations of reactive species and oxidized macromolecules [34, 101, 106].

Oxidative Stress, Brain Aging, and Dementia

Oxidative stress is induced by an imbalanced redox state, involving either excessive generation of reactive oxygen species (ROS) or dysfunction of the endogenous antioxidant system. Endogenous production of ROS is mediated by mitochondrial and non-mitochondrial ROS-generating enzymes including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox), xanthine oxidase (XO), cytochrome P_{450} from endoplasmic reticulum (ER), and flavin oxidases from peroxisomes [34].

Mitochondria are the most important source of ROS in mammalian cells, since the reduction of oxygen during oxidative phosphorylation is not always proceeding flawlessly and inchoately reduced oxygen molecules can escape [65]. Mitochondrial electron carriers can inadvertently transfer one electron to an oxygen molecule; the same reaction can also take place at redox-active prosthetic groups within proteins. This one-electron reduction of oxygen leads to the formation of superoxide (O_2^-). Dismutation of superoxide leads to formation of hydrogen peroxide (H_2O_2), which can be further reduced to yield the hydroxyl radical ($OH^·$), a very strong oxidant [115]. Furthermore, superoxide can also react with radicals such as nitric oxide ($NO^·$) to form nitric peroxide, another strong oxidant and member of the reactive nitrogen species (RNS). ROS production in mitochondria is especially high when ATP production is low and the quinone pool in the inner mitochondrial membrane is reduced. Free radicals can attack polyunsaturated fatty acids in biological membranes, rendering them radicals themselves and thus starting a chain reaction that affects numerous other polyunsaturated fatty acids (see also Chap. 3) [1].

The "free radical theory of aging" was proposed in 1956 to explain the close relationship of the aging process and the production of ROS. This theory suggests that the rate of aging is controlled by accumulation of oxidative damage [40]. Recent results, on the other hand, indicate that the free radical theory of aging might not be entirely correct. Experiments failed to find a correlation between the amount of ROS production and the longevity of various species, and the modulation of antioxidant-related gene expression in genetically modified mice does not generally affect life span [42]. Thus, the original hypothesis has been revised, and ROS are regarded not only as harmful but also as important signal molecules [61, 71].

Modulation of antioxidant-related gene expression alters the development of age-related disease and pathology in preclinical mouse models [21]. For instance, a reduction in superoxide dismutase 2 (SOD2) has been shown to accelerate neurological disorders when introduced into a mouse model of AD [21]. Conversely, overexpression of antioxidant enzymes in mice has been shown to reduce or delay the development of several age-related diseases without significantly altering life span. Increased expression of SOD1 has been shown to be beneficial in mouse models of AD [11, 21].

ROS are endogenously detoxified by several antioxidative enzymes, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR), and peroxiredoxins (PRX). SOD catalyzes the conversion of superoxide to H_2O_2, which is converted to H_2O by either catalase or glutathione peroxidase [7]. Glutathione, a tripeptide with a thiol group in its active center, can reduce disulfide bonds formed within proteins. GR recycles oxidized glutathione. Besides these endogenous mechanisms, the antioxidative vitamins C and E are involved in the cellular defense against oxidative stress [4].

As mentioned above, elevated markers of oxidative stress are an important hallmark of AD [2, 68, 108, 114]. The brain is one of the organs especially vulnerable to the effects of ROS, because of its high oxygen demand and its abundance of peroxidation-susceptible lipids [55]. Thus, tocotrienols and tocopherols are supposed to have beneficial effects on neuronal and mitochondrial function and are promising molecules to reduce free radical species in AD [33, 96].

Preclinical Studies

To assess the effects of vitamin E administration or administration of vitamin E together with other antioxidants on brain cellular and mitochondrial function, various feeding studies have been conducted using mouse and rat models of brain aging. A multiplicity of studies found positive correlations between vitamin E administration and beneficial effects on brain cellular and mitochondrial function [38]. In aged rats, for example, supplementation with N-acetyl cysteine (500 mg/kg body weight), α-lipoic acid (30 mg/kg body weight), and α-tocopherol (15 mg/kg body weight) for

4 months decreased mitochondrial ROS production as well as the activity of proinflammatory cytokines [111]. Similarly, in aged rats supplemented with α-tocopherol (50 IU/kg body weight) for 30 days, protein carbonyl content as well as the content of other advanced protein oxidation products was decreased [52]. In young rats, vitamin E supplementation improved cognitive function and the ability of learning and memory in the water maze and the eight-arm radial maze test. When young rats were maintained at 100% oxygen for 48 h, oxidative stress and the content of oxidized products in brain cells increased. Subsequently the performance of the animals in the behavioral tests declined. Vitamin E supplementation of rats before induction of oxidative stress prevented the memory decline seen in non-supplemented rats [29]. Feeding vitamin E (5 g of α-tocopherol acetate/kg food) from 28 weeks of age in rats leads to an increase in lifespan as well as to improvements in neuromuscular function and exploratory activity. Moreover, ameliorated mitochondrial respiratory system function and antioxidant defense mechanisms in the brain were observed [79]. Dietary supplementation of rats from 9 to 12 months of age with vitamin E (2 or 5 g/kg of food) restored mitochondrial respiration and CI and CIV enzyme activities that were decreased with age. Additionally, the supplementation prevented ROS production and an accumulation of oxidation products in brain cells of aged animals [80].

Epidemiological Evidence

Vitamin E is important for the central nervous system (CNS) [77], and it is associated with brain disorders [37, 83, 95, 104]. Vitamin E deficiency especially affects the motor activity, which could be restored by the supply of vitamin E [37, 104]. Patients with a mutation in the α-tocopherol transfer protein (see Chap. 9) show low serum vitamin E and thus suffer from ataxia with vitamin E deficiency (AVED) [83]. Symptoms of this autosomal recessive cerebellar ataxia can be improved by vitamin E supplementation [30]. Moreover, low performance in memory tests was associated with low vitamin E serum concentrations in a population-based study [86]. Accordingly, worse memory performance was associated with low vitamin E intake in elderly people, and normal cognition was associated with high plasma vitamin E in centenarians [56, 82]. The positive correlation between cognition and vitamin E is supported by another study observing the intake of vitamins E and C in a large cohort for several years. People that used vitamin E and C supplements showed better cognitive performance which was best when the intake was longer than 10 years [35]. However, significant effects were only seen for the combination of vitamins E and C, pointing out that vitamin C is necessary to regenerate vitamin E (see Chap. 3). This point will be discussed later, especially in view to the study design of intervention studies.

Two large observational studies generally showed improved cognition after intake of food rich in antioxidants, and in both studies, this effect was related to vitamin E [74, 85]. This association was confirmed by another study that also showed that a high intake of vitamin E is related to a reduced risk for AD [73]. A lower risk for developing AD was also reported in a large cohort of people that consumed food containing high amounts of vitamins E and C [22]. In the Cache County Study, a cross-sectional and prospective study of dementia in elderly residents, the use of vitamin E and vitamin C supplements in combination was associated with reduced prevalence and incidence of AD [122]. Accordingly, high plasma concentrations of vitamin E were associated with a reduced risk for AD in subjects older than 80 years [66, 67]. On the other hand, low plasma vitamin E was associated with an increased risk to develop AD or MCI in a large cohort study that aimed to identify biomarkers for AD (AddNeuroMed cohort) [67]. A recent meta-analysis of 116 selected publications showed significantly lower plasma and CSF/brain concentrations of several nutrients, including vitamin E [118]. The authors concluded that brain nutrient status appears to parallel the lower circulatory nutrient status.

The Honolulu-Asia Aging Study on Japanese-American men living in Hawaii showed that subjects regularly consuming vitamin E and C supplements led to significantly decreased risks of developing vascular dementia. On the other hand, no positive correlation between vitamin E intake and decreased risk of developing AD was found [69]. The authors did not include a control group, and information about duration and amount of intake of vitamins E and C are missing.

A subset of 616 community-representative persons (age, 65–106 years) of the Epidemiologic Studies of the Elderly (ESPE) cohort, a longitudinal study for dementia, was followed for 10 years, and among other things, information on vitamin use was gathered during in-home interviews [26]. Diagnosis of dementia and AD was based on neuropsychological testing according to CERAD. Fillebaum et al. concluded that neither the use of any vitamin C and/or E nor high-dose use reduced the time to dementia or AD. However, only 8% of subjects used vitamin E and/or C at baseline. Because of the limited use of antioxidant vitamins in this sample, the investigators combined use of any vitamin C with use of any vitamin E, classifying use in a multivitamin preparation as low dose and single supplement use of either vitamin C or E as high dose [26]. Unfortunately, no information on dose and duration of vitamin intake were given.

In summary, the evidence from epidemiological studies suggests a role for vitamin E in the prevention of neurodegeneration and probably also the treatment of AD [89]. AD patients showed lower plasma, cerebrospinal fluid, and brain concentrations of vitamin E [13, 51], while higher plasma concentrations were associated with a reduced risk of AD [38, 66]. Accordingly, high vitamin E concentrations in ventricular cerebrospinal fluid isolated from postmortem samples taken from AD patients were related to better performance in perceptual speed and rarer neuropathological hallmarks [43]. However, cognition was not related to vitamin E concentrations in this study.

Apolipoprotein E gene (Apo E4) represents one of the most important risk factors for late-onset AD [17], and lower α-tocopherol concentrations were measured in brains of humans carrying the Apo E4 allele [47]. Accordingly, findings form the Cache County Study suggest an association between using vitamin E and C supplements in combination with NSAID and less cognitive decline in elderly harboring the Apo E4 allele [27]. On the other hand, a prospective study including 815 residents 65 years and older suggested that vitamin E from food may be associated with a reduced risk of AD [73]. This association was observed only among individuals without the APO E4 allele in this study.

Intervention Studies

Based on preclinical and epidemiological evidence, clinical trials have been carried out to investigate the preventive or therapeutic effects of vitamin E supplementation in humans at risk or suffering from MCI or AD.

Prevention

In a sub-study of the Women's Health Study, 6377 elderly healthy women were randomized and assigned to receive α-tocopherol acetate (600 IU/day) on alternate days for 4 years [53]. Vitamin E treatment was favorable in cognitive measures. However, vitamin E use did not lower the risk for cognitive decline. Cognitive performance was evaluated by telephone interviews which could be a limitation of the study.

The Prevention of Alzheimer Disease by Vitamin E and Selenium (PREADViSE) trial began as ancillary to a randomized controlled trial for prostate cancer prevention, which ended prematurely due to lack of efficacy. PREADViSE initially enrolled 7540 older men who were randomized to receive

selenium (200 μg/day), vitamin E (400 IU/daily), vitamin E and selenium, or placebo for an average of 5.4 years. A subset of 3786 men continued into the cohort study and were observed and evaluated with at least 1 memory screen for an additional 6 years without taking the supplements [59]. During the study, men were contacted by telephone and assessed using an enhanced two-stage cognitive screen. In both phases, men were encouraged to visit their physician if the screen results indicated possible cognitive impairment. Neither supplement prevented dementia; the incidence of dementia (4.4%) did not differ among the four study groups at the end of observation period [105]. The authors cautioned that the study had significant limitations, such as the loss of about half of the participants to long-term follow-up during the transition from a randomized clinical trial to a cohort study and the refusal of many participants to see clinicians for definitive testing for dementia. The relatively young age (mean 67.5 years) and the high level of education of participants at baseline likely contributed to the low incidence of dementia, which may have made it difficult to detect any effects of the interventions [105].

Treatment

Sano and coworkers reported a 2-year treatment of 341 patients with moderate AD (age, 65–82 years) in a randomized clinical trial using α-tocopherol (2.000 IU/day) and/or selegiline (10 mg/day) (Table 24.1). Death, institutionalization, the loss of ability to perform daily living activities, and the incidence of severe dementia were significantly reduced in the treatment group. However, treatment had no effect on the improvement of cognitive tests or on the delay of mental deterioration. Moreover, the combination of vitamin E with selegiline was not effective at all. No significant side effects were reported [98].

For a large RCT, 769 people suffering from MCI (age, 65–80 years) were recruited from the Alzheimer's Disease Cooperative Study. Subjects were randomly assigned to receive 2.000 IU of vitamin E (twice daily 1.000 IU), 10 mg of donepezil daily, or placebo for 3 years [88]. Of note, all groups received a daily multivitamin containing 15 IU vitamin E. There were no significant differences in the rate of progression to AD between the vitamin E and placebo groups at any point, either among all patients or among apolipoprotein E4 carriers. Although vitamin E treatment was not successful, the treatment was safe [88]. To investigate structural changes, magnetic resonance imaging (MRI) scans were performed, and size of hippocampus, entorhinal cortex, and whole brain and ventricular volumes were measured in a subset of 139 patients [50]. Although a nonsignificant trend toward slowing of hippocampal atrophy rates was seen in Apo ∈4 carriers treated with donepezil, no treatment effect was confirmed for any MRI measure in either treatment group [50]. Thus, even an early vitamin E treatment neither improved cognition nor reduced brain atrophy [50, 88].

These results prompted Loret et al. [62] to introduce the novel concept of stratifying AD patients into vitamin E responders and non-responders, based on measures of plasma glutathione disulfide (GSSG), the oxidized form of the antioxidant peptide glutathione (GSH) [12]. Fifty-seven AD patients (25 with mild, 26 with moderate, and 6 with severe dementia) and 18 age-matched controls were included in the RCT. The authors did not specify the age of the participants. The verum groups received 800 IU vitamin E for 6 months. About half of the patients failed to respond to vitamin E with lower plasma GSSG. In those non-responders, cognitive decline progressed during the study period. In responders, vitamin E prevented oxidative stress and cognitive decline [62]. However, a critical analysis of the study identified some points of weaknesses, including a short treatment period, a high dropout rate of 42%, and a small sample size. The latter probably hindered the detection of a decline in cognition (measured as MMSE) in the placebo group. Moreover, there was a strong negative correlation between GSSG and the outcome of the Mini-Mental State Examination, but most of the effect was due to 4 of 19 AD patients with 5–10% drop in GSSG in response to vitamin E [12].

Table 24.1 Clinical studies on vitamin E in aging and Alzheimer's disease

Type	Participants	Outcome measure (primary)	Study type	Dose	Form/ combination	Duration	Conclusions	Annotation	Reference
Treatment	341 moderate to severe AD patients (65–82 years)	BSD, CDR	RCT	2.000 IU	Alpha-tocopherol	24 months	Vitamin E slowed the progression of AD	Primary outcome parameters were related to functional scores Secondary outcome parameters on cognition were not changed Combination with selegiline was not effective	[98]
Treatment	20 AD patients (57–78 years)	Plasma and CSF vitamin levels; in vitro protein oxidation	OLS, no control	400 IU/400 IU + 1.000 mg	Vitamin E/ vitamins E and C	1 month	A superiority of the combination vitamin E + C was superior compared to vitamin E	Pure biomarker study, effects on cognition not tested Supplementation with vitamins E and C or vitamin E alone significantly increased vitamin levels in plasma and CSF; only the combination reduced protein oxidation in vitro (but effects were marginal)	[58]
Treatment	60 AD patients (58–73 years)	MMSE, ADAS-cog; latency of P300 potentials	RCT	2.000 IU	Vitamin E	6.5 months	In patients treated with vitamin E, latency increments correlated with worsening neuropsychological test scores	Small sample size	[112]
Prevention	769 MCI patients (65–80 years)	ADAS-cog/ CERAD	RCT	2.000 IU	Vitamin E	36 months	Vitamin E had no benefit in patients with MCI	Among all patients and among all ApoE4 carriers, there was no effect of vitamin E at any timepoint All study groups received a multivitamin daily	[88]
Prevention	6377 elderly healthy women	MMSE	RCT	600 IU	Alpha-tocopherol acetate	48 months	Cognitive decline was not substantial lowered	Women's Health Study	[53]

Prevention	57 AD patients (age n.s.)	MMSE, BSD, CDT, GSSG	RCT	800 IU	Vitamin E	6 months	In responders, vitamin E prevented oxidative stress and cognitive decline In non-responders, vitamin E did not prevent OS and even worsens cognitive decline Supplementation of vitamin E in general was not recommended	High rate of dropouts (42%) Short treatment time Small sample size, not able to detect a decline in MMSE for treatment with placebo	[62]
Treatment	53 individuals with DS and AD-type dementia (45–55 years)	DMR SOC SIB	RCT	900 IU + 200 mg + 600 mg/d	α-Tocopherol + ascorbic acid + α-lipoic acid	24 months	Antioxidant supplementation is safe, though ineffective		[64]
Treatment	78 mild to moderate AD	CSF biomarkers, MME, ADCS-ADL	RCT	800 IU + 500 mg + 900 mg/d	α-Tocopherol + ascorbic acid + α-lipoic acid	4 months	Combination did not influence CSF biomarkers related to AD pathology but reduced oxidative stress markers; caution: treatment resulted in faster cognitive decline	Adverse effects on cognition NCT00117403	[31]
Treatment	40 AD patients	Urine 8-iso-PGF2α, telomere length in PBMCs; DSM-IV	OLS	400 IU	Vitamin E	6 months	AD patients showed an elevated OS marker level, and vitamin E lowered the OS level. Compared to controls, AD patients showed shorter telomere lengths Vitamin E-associated telomeric changes could not be detected	Biomarker study showing antioxidative effects in urine	[36]

(continued)

Table 24.1 (continued)

Type	Participants	Outcome measure (primary)	Study type	Dose	Form/ combination	Duration	Conclusions	Annotation	Reference
Treatment	613 mild to moderate AD (53–96 years)	ADCS-ADL	RCT	2.000	DL-α-tocopheryl acetate	48 months	Vitamin E slowed the functional decline, but not in combination with memantine	TEAM-AD VA Significant, but fairly modest reduction of 19% in functional outcome but no effects on cognition NCT00235716	[19]
Treatment	106 AD patients (average age, 77,8 years)	Clox-1; DRS	RCT (II)	60	α-Tocopherol in complex formulation (MemoryXL)	3 or 6 months	Participants maintained or improved cognitive performance	Statistically significant, but small effects, functional relevance questionable NCT01320527	[93]
Prevention	337 individuals with DS (>50 years)	BPT	RCT	2.000 IU	Tocopherol + multivitamin daily	36 months	Vitamin E did not slow the progression of cognitive deterioration in older individuals with DS	NCT00056329	Mary [99]
Treatment	24 AD patients	Clox-1; DRS	OLS	60	α-Tocopherol in complex formulation (MemoryXL)	12 months	Participants maintained their baseline cognitive performance	Open-label continuation of an RCT (see [93]) NCT01320527	[94]
Prevention	7540 // 3786 Men (>60 years)	MIS/ CERAD // MIS/ TICS-m	RCT // CS	400 IU	*all-rac*-α-tocopheryl acetate	6 years // 6 years	Vitamin E supplementation did not prevent dementia. men	PREADViSE; first long-term study on the association of antioxidant supplement use and dementia incidence among asymptomatic; NCT00040378	[59]

The effects of vitamin E (400 IU daily) alone or in combination with vitamin C (1 g daily) were clinically tested in a small open-label study running for 1 month in 20 AD patients (age, 57–78 years) [58]. Supplementation with vitamin E or the combination with vitamin C significantly increased vitamin concentrations in plasma and liquor. Vitamin C concentrations were significantly lower in AD patients and returned to normal following treatment. The susceptibility of CSF and plasma lipoproteins to in vitro oxidation was significantly decreased. In contrast, the supplementation with vitamin E alone significantly increased its CSF and plasma concentrations but was unable to decrease the in vitro lipoprotein oxidizability. Although the effects of the in vitro study were marginal, the authors concluded that their findings document a superiority of a combined vitamin E and C supplementation over a vitamin E supplementation alone in AD [58]. The trial was designed as a pure biomarker study. Thus, cognitive parameters were not tested.

Another biomarker study tested the effect of vitamin E administration on the elevated oxygen stress and the telomeric and subtelomeric status in AD. This open-label study included 40 AD patients (mean age, 68,9 years) who were supplemented with 400 IU vitamin E for 6 months [36]. 8-Iso-PGF2α, a marker of lipid peroxidation, was measured in the urine, and methylated and non-methylated telomere lengths were examined in peripheral blood mononuclear cells. AD patients showed elevated levels of lipid peroxidation and shorter telomere lengths. Vitamin E lowered the levels of 8-iso-PGF2α but had no effect on telomere-associated changes [36]. In this study cognitive status was not measured.

In a randomized clinical trial with CSF biomarker measures, 78 patients with mild to moderate AD (age, 50–85) were randomly assigned to treatment for 4 months with α-tocopherol (800 IU/day) plus vitamin C (500 mg/day) and α-lipoic acid (LA, 900 mg/day), coenzyme Q (3 × 400 mg/day) (E/C/LA group), or placebo [31]. The combination of vitamin E + C and α-lipoic acid had no effect on CSF biomarkers related to AD pathology but reduced F2-isoprostane concentrations – a lipid peroxidation marker – significantly by 19%. Clinical measures of cognitive abilities (MMSE) and function (ADCS-ADL) did not differ between groups at baseline. The MMSE scores showed a greater decline in the E/C/LA group relative to placebo. There were no differences between groups in changes in ADCS-ADL total scores, or in scores for subscales of basic or instrumental ADL, although there were trends toward greater decline in the E/C/LA group [31]. Thus, the treatment with antioxidants, including vitamin E, raised the concern of a faster cognitive decline.

An earlier study including 60 AD patients with mild to moderately severe probable AD also raised concerns on the treatment with vitamin E based on electroencephalography recordings [112]. The P300 wave is an event-related potential that is triggered during decision-making. Forty patients were randomly assigned in an RCT to donepezil (5–10 mg/day) versus vitamin E (2.000 IU/day). Twenty patients were treated in an open trial with rivastigmine (1.5–12 mg/day). Patients were treated for 26 weeks. The authors observed latency increments (7.4 +/− 3.5 msec) correlated with worsening neuropsychologic test scores in patients treated with vitamin E. In patients treated with donepezil and rivastigmine, significant P300 latency reductions (15.3 +/− 3.2 msec and 22.0 +/− 3.3 msec) were found. Shorter P300 latencies were associated with higher Wechsler Adult Intelligence Scale scores and with lower AD Assessment Scale-cognitive subscale (ADAS-cog) scores ($R = 0.72$) [112].

The combination of α-tocopherol, ascorbic acid, and α-lipoic acid was also investigated in 53 individuals with Down syndrome (DS) and AD-type dementia (age, 45–55 years) [64]. DS is characterized by trisomy of chromosome 21 on which the gene for the amyloid precursor protein (APP) is located. This protein is the precursor of Aβ that has been related to AD (see above), and persons with DS have a high Aβ load and a high prevalence for AD [63]. DS patients often present with some congenital defects and chronic diseases, including early onset dementia, which affects 70% of DS patients over 55 years of age and has a clinical presentation similar to Alzheimer disease (AD) [57]. Therefore, DS has been proposed as a model to study predementia stages of AD [110]. The daily administration of a combination of α-tocopherol (900 IU), ascorbic acid (200 mg), and α-lipoic acid (600 mg) for 2 years was safe, though ineffective as a treatment for dementia in individuals with DS and AD-type dementia [64].

In a recently published clinical trial, Sano et al. determined whether vitamin E would slow the progression of cognitive deterioration and dementia in aging persons with DS [99]. For the multicenter randomized, double-blind controlled clinical trial, 337 individuals with DS (older than 50 years) were recruited. Participants were randomly assigned to receive vitamin E (1.000 IU twice daily) for 3 years or placebo. The primary outcome was change on the Brief Praxis Test (BPT). Secondary outcomes included incident dementia and measures of clinical global change, cognition, function, and behavior. Both groups demonstrated deterioration on the BPT with no difference between drug and placebo. Vitamin E did not slow the progression of cognitive deterioration in older individuals with DS [99].

The efficacy of α-tocopherol, memantine, or a combination in delaying progression of AD taking ACh-I was tested in the Vitamin E and Memantine in Alzheimer's Disease (TEAM-AD) study. In this RCT, 613 older veterans (53–96 years; 97% men) with mild to moderate AD who received acetylcholinesterase inhibitors were assigned to 4 treatment groups receiving either α-tocopherol (2.000 IU daily), memantine (20 mg daily), both agents (2.000 IU vitamin E, 20 mg memantine), or placebo for 48 months [19]. The primary trial outcome was scored on ADCS-ADL inventory, measuring activities of daily living. Secondary outcome parameters included scores on the MMSE and the ADAS-cog. Compared with individuals assigned to placebo, those assigned to vitamin E alone experienced a modest 19% reduction of decline on the ADCS-ADL inventory that was statistically significant ($P = 0.03$). This improvement of daily living may well be meaningful as the authors suggest [19, 23] and confirm earlier data from Sano et al. discussed above [98]. Treatment with memantine or the combination did not differ significantly from placebo with respect to the primary outcome. None of the groups assigned to active interventions differed from the placebo group on the cognitive outcomes (MMSE and ADAS-cog) [19, 23]. However, treatment with vitamin E was associated with favorable but not significant effects on memory and language properties [19].

Remington et al. determined whether a nutritional intervention including a low dose of vitamin E could positively impact cognitive performance and behavioral difficulties for individuals diagnosed with AD [93]. AD patients ($n = 100$, average age, 77.8 years) were included in a double-blind, multisite, phase II study and randomized to a nutraceutical formulation (NF) consisting of folate (400 μg), α-tocopherol (30 IU), B12 (6 μg), S-adenosyl methionine (200 mg), N-acetyl cysteine (600 mg), and acetyl-L-carnitine (500 mg) twice a day for 3 or 6 months. Improved cognition was measured in the NF versus the placebo cohort within 3 months. Activities of daily living did not change for either cohort. Following initial randomization, code was broken, and all participants received NF under open-label conditions for the remainder of the study, and participants maintained their baseline cognitive performance over 12 months [94].

A recent randomized, double-blind controlled trial investigated the efficacy of a specific multinutrient (Souvenaid) in people with prodromal Alzheimer's disease [107]. A total of 311 participants who fulfilled criteria for prodromal Alzheimer's disease (age, 55–85 years) was randomly assigned to the active group or control group. The primary endpoint was change in a neuropsychological test battery (NTB) score. The active component of Souvenaid is the multi-nutrient combination (Fortasyn Connect), which contains docosahexaenoic acid (DHA); eicosapentaenoic acid (EPA); uridine monophosphate; choline; vitamins B12, B6, C, and E, folic acid; phospholipids; and selenium. The amount of α-tocopherol is 40 IU per serving. The intervention had no significant effect on the NTB primary endpoint over 2 years in prodromal Alzheimer's disease. Group differences on secondary endpoints of disease progression measuring cognition and function and hippocampal atrophy were observed [107]. However, it is unclear to what proportion the low dose of vitamin E had an impact on the outcome of the study.

A recent meta-analysis included all double-blind, randomized trials in which treatment with any dose of vitamin E was compared with placebo in people with AD or MCI [24]. Farina et al. concluded that the α-tocopherol form of vitamin E did not prevent progression to dementia in people with MCI nor improved cognitive function in people with MCI or dementia due to AD. However, a single study by Dysken et al. was identified with moderate-quality evidence that it may slow functional decline in AD [19, 24].

Discussion

Vitamin E is an essential antioxidant and plays an important role in protecting cells from oxidative damage – especially against the harmful effects of free radicals that are involved in brain aging and AD. Animal studies showed sufficient evidence to consider a treatment with vitamin E for patients with AD [24, 38]. However, to date, the results of clinical studies on AD patients are not clear. Only two studies showed functional or cognitive benefits from vitamin E supplementation in MCI or AD patients (Table 24.1). An early study by Sano et al. observed that vitamin E (2.000 IU/day) supplementation for 2 years delayed AD progression in patients affected by moderately severe AD [98]. Similarly, another study using the same dose for 4 years reported reduced functional decline and caregiver burden in patients with mild to moderate AD [19]. However, in both studies no improvements in cognitive function were observed [19, 98]. Although, those two studies seem to be favorable, some methodological issues were identified [44]. In the study of Sano et al., the baseline sores for cognitive measures (evaluated with MMSE) varied considerably between groups [98]. In the study of Dysken et al., vitamin E improved disease development only when administered alone, but not in combination with memantine [19].

In a 3-year study, Petersen et al. showed that vitamin E administration did not exert beneficial actions in patients with MCI. In particular, vitamin E treatment did not influence the probability of progression from MCI to AD [88]. Lloret et al. conducted a small RCT in which they treated AD patients with 800 IU/day vitamin E for 6 months [62]. Interestingly, the study showed an adverse effect on cognition in those patients whose oxidative stress markers did not respond to vitamin E intake [62]. However, the small sample size and a high dropout rate are limiting factors in this study [38]. Other double-blind, randomized vitamin E trials did not prevent the progression to dementia in people with MCI, nor did it improve cognitive function in people with MCI or dementia due to AD, as a recent meta-analysis concludes [24]. Moreover, a revised consensus statement from the British Association for Psychopharmacology concluded that vitamin E besides other drugs including statins, and anti-inflammatory agents, can neither be recommended for the treatment nor the prevention of AD [81].

In a comment to the TEAM-AD study, Evans et al. noted a high death rate, a moderated medication adherence, and loss to follow-up that is evident in almost all AD therapy trials, reflecting the practical challenges in conducting randomized trials among people of older age with this disease [23]. Moreover, the total number of patients recruited in the studies was small (2.084 patients; Table 24.1). Besides these general hurdles, other key issues why vitamin E therapies fail for treatment of AD, including dosing, timing, unbalanced monotherapy, and target selection, can be identified [12].

The recommended daily dietary intake for vitamin E varies between 6 and 30 IU in different countries and varies depending on age [119]. The daily vitamin E doses in RCT studies range from 60 to 2.000 IU (Table 24.1) and are many fold higher than dietary doses and may exceed the recommended upper intake [15]. Particularly in both studies showing beneficial effects, 2.000 IU vitamin E was applied (M Sano et al. 1997; [19]). An epidemiological study investigating a cohort of people aged ≥65 years found the use of vitamin E supplements to be correlated with a higher mortality risk in patients with a history of severe cardiovascular disease, but not in other subgroups [41]. It should be noted that AD patients often have vascular comorbidities, especially cerebral microangiopathy [44]. However, treatment with vitamin E in RCT studies, even in high doses up to 2.000 IE, was generally reported as safe [25], with only few exceptions [31, 62, 112] (see also Chap. 16).

It seems that the duration of treatment (1 and 48 months; Table 24.1) of MCI or AD patients with vitamin E and clinical outcomes were not related. In the case of clinical AD or possibly even MCI, neurodegeneration and the loss of synapses and neurons occur. Until today, there is no possibility to stop this process and to restore cognition. Even novel drugs that were developed as disease-modifying agents failed in clinical phase III trials [3]. This may explain in part why vitamin E trials failed to improve cognition in patients with MCI [12, 88].

Epidemiological studies suggested a protective effect for vitamin E in combination with vitamin C, while either vitamin alone had no significant effects [122]. A small open-label study with 20 AD patients showed a superiority of a combined vitamins E and C on oxidative biomarkers over vitamin E alone [58]. The combination of vitamin E, vitamin C, and α-lipoic acid was either safe, though ineffective, in individuals with DS suffering from AD-type dementia or even showed adverse effects on cognition in patients with mild to moderate AD [31, 64]. However, several trials of this combination and other antioxidants have failed to show efficacy in the prevention of various diseases [10, 12]. Remington et al. reported that vitamin E as part of a complex nutrient mixture given to AD patients preserved or even improved cognitive performance [93, 94]. Another multi-nutrient (Souvenaid) failed to improve cognition in people with prodromal Alzheimer's disease [107]. Thus, it is unclear how and to which extent vitamin E contributes to the effects when applied in complex mixtures.

The most common form of natural vitamin E is γ-tocopherol followed by α-tocopherol [28]. All eight forms of vitamin E are present in food, and α- and γ-tocopherols are the most abundant forms found in the body, whereas tocotrienol concentrations are usually very low [9, 28]. While most food supplements contain only α-tocopherol, the major form of vitamin E in US diets is γ-tocopherol [116]. The majority of randomized clinical trials on cognitive decline, which for the most part failed, used chemically synthesized *all-rac*-α-tocopherol (Table 24.1). Studies on dietary tocopherols, on the other hand, have shown benefits [76].

In a postmortem study on 115 deceased participants of the prospective Rush Memory and Aging Project, high γ-tocopherol concentrations were correlated with less severe neuropathology, while α-tocopherol concentrations were not associated with amyloid load or the number of neurofibrillary tangles [76]. Thus, brain concentrations of γ- and α-tocopherols may be associated with AD neuropathology in interrelated and complex ways [76]. It has been reported that α-tocopherol supplements significantly reduce serum γ-tocopherol, which might impede the biological effects of α-tocopherol [116]. Accordingly, γ-tocopherol was found to be more effective to combat oxidative stress, which is associated with neurodegenerative diseases [116].

Tocotrienols (T3) seem to be more potent antioxidants than tocopherols (see also Chap. 3), probably due to their faster recycling and better membrane distribution [28, 103]. To act as antioxidants, tocotrienols are required inside the cell (membrane) at micromolar concentrations. T3 concentrations necessary for free radical scavenging may not be reached in plasma and brain tissue after of oral administration [28]. However, at nanomolar concentrations, which are reached in human plasma after tocotrienol supplementation, T3 have been found to modify various enzymes and cellular signaling pathways in the brain including the mevalonate pathway [28, 120]. T3, but not tocopherols, block the processing and nuclear localization of SREB-2 the transcriptional factor for HMG-CoA reductase and farnesyl pyrophosphate synthase. Moreover, T3 enhance the degradation of HMG-Co reductase and thereby deplete cellular farnesyl pyrophosphate and geranylgeranyl pyrophosphate which may affect AD pathogenesis [121]. Prenylation of proteins regulating neuronal function requires mevalonate-derived farnesyl pyrophosphate and geranylgeranyl pyrophosphate [46, 121]. Farnesyl pyrophosphate and geranylgeranyl pyrophosphate, but not cholesterol, have been reported to be significantly elevated in brains of AD patients [20, 84]. The role of these isoprenoid intermediates of the HMG-CoA pathway and the impact of T3 on AD etiology represent a challenge for upcoming research [46]. Thus, various tocopherol forms, rather than α- tocopherol alone, may be important in the protective association of vitamin E with Alzheimer's disease [75], and future studies should therefore also investigate other tocopherol and tocotrienol forms [24].

Conclusion

To date there is no convincing evidence from meta-analyses on randomized, placebo-controlled trials that vitamin E alone or in combination with other antioxidants prevents the progression of MCI or AD or improves cognitive function in subjects suffering from these conditions. Additional research is

needed to explore the emerging role of vitamin E in the treatment of AD or other clinically related outcomes, including quality of life. Moreover, there is a need for more uniform methodology and outcome measures across trials. Besides practical challenges in conducting randomized trials among older people with AD, key issues for further vitamin E therapy trials include dosing, timing, genotype, mono- or multimodal therapy, and selection of additional vitamers, including γ-tocopherol and tocotrienols.

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Chapter 25
The Impact of Vitamin E Isoforms on Asthma and Allergy

Joan M. Cook-Mills

Keywords Allergy · Asthma · Vitamin E · α-Tocopherol · γ-Tocopherol · Human · Animal models

Key Points

- Asthma and allergic disease result from complex environmental and genetic interactions.
- Vitamin E is one of the environmental factors that can regulate responses to challenge with allergens.
- However, outcomes of clinical studies on benefits of vitamin E regarding asthma and allergic inflammation vary.
- The differential and partially opposing functions of the vitamin E isoforms α-tocopherol and γ-tocopherol as demonstrated in mechanistic studies appear to be an important variable in these inconsistent results reported in clinical studies.
- A better understanding of the differential regulation of inflammation by isoforms of vitamin E is considered an important factor to improve quality of clinical studies when investigating the role of vitamin E to improve lung function in disease in adults and during development.

Asthma/Allergy Prevalence and Changes in Consumption of Forms of Vitamin E

Non-allergic asthma, allergic asthma, and allergy are heterogeneous diseases, resulting from complex interactions of environmental and genetic factors [1]. Allergic and asthmatic responses include bronchoconstriction, itch, pain, inflammation, and tissue remodeling. In humans, measurements of lung function for monitoring asthma include forced expiratory volume in 1 s (FEV1, forced volume blown

Sources of Support: This study was supported by National Institutes of Health Grants R01AI127695, U01AI131337, and R01 AT004837.

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_25

out in 1 s) and forced vital capacity (FVC, forced volume when all air is blown out). Current therapies for asthma and allergies, including corticosteroids, have serious side effects. Therefore, it is critical to determine mechanisms for regulation of inflammation in allergy/asthma in order to identify novel approaches for interventions. Asthma is characterized by inflammatory processes with T-helper (Th) cell responses of the Th2 phenotype being considered crucial for the initiation and perpetuation of the inflammatory responses [2]. Important mediators of asthma and allergic inflammation are cytokines such as interleukin IL-4, IL-5, and IL-13. Asthmatic and allergic inflammation is characterized by elevated immunoglobulin E, mast cell degranulation, and eosinophilic inflammation [3]. Recruitment of eosinophils into the tissue is a consistent feature of allergic inflammation and allergic asthma [4–6]. A crucial component of recruitment of eosinophils during inflammation is leukocyte migration from the blood, across the endothelium, and into the tissue (transendothelial migration) [7–9]. Mechanisms for this eosinophil recruitment involve the coordinate actions of adhesion molecules, chemokines, and cytokines [10, 11]. Tocopherol isoform regulation of leukocyte recruitment and allergic inflammation has been studied in humans, animal models, and cell systems.

The World Health Organization reported a worldwide increase in asthma and allergies from 1950 to the present [12–15]. In 2012–2013, the US Centers for Disease Control and Prevention reported asthma and allergy prevalence as 10–20%, affecting about 26 million people, costing $56 million/year and 9 deaths/day [16–18]. The rapid rise in rates of asthma and the differences in rates among countries and in migrating populations indicate a role of the local environment, including the diet. In this same timespan, there has been an increase in the γ-tocopherol isoform in the diet, in infant formulas containing soybean oil that is rich in γ-tocopherol, and in vitamin supplements [19, 20]. The variation in global prevalence of asthma and allergies may be influenced, at least in part, by country-specific plasma γ-tocopherol concentrations. Moreover, it has been suggested that early life exposures to environmental factors increase risk of allergic disease [21]. Maternal exposure to environmental factors can alter neonatal hematopoietic or metabolic function [22–29]. In reports examining human maternal and paternal asthma associations with development of allergies in offspring, most associations are with maternal asthma [30]. Furthermore, there is higher prevalence of early-onset persistent asthma if the mother has uncontrolled asthma or moderate-to-severe controlled asthma as compared to mothers with mild controlled asthma [31].

In animal studies, offspring of allergic mothers have increased responses to suboptimal doses of allergens [30], and tocopherol isoforms regulate development of offspring cells of the immune system and immune responses [32–40]. Therefore, studies of the regulation of adult allergies and asthma as well as the development of allergic disease and asthma early in life are critical to generating approaches to limit these diseases.

Vitamin E Isoforms and Function During Asthma

During allergic responses and asthma, isoforms of vitamin E regulate cell signaling and scavenging of reactive oxygen species. The isoforms of vitamin E consumed and in human plasma vary among countries. The natural isoforms of vitamin E are synthesized by plants from tyrosine and chlorophyll [41]. Thus, although mammals do not synthesize vitamin E, mammals acquire vitamin E isoforms from food, cooking oils, and vitamin supplements. The natural isoforms of vitamin E are d-α-tocopherol, d-β-tocopherol, d-γ-tocopherol, and d-δ-tocopherol and the d-α-, d-β-, d-γ-, and d-δ-tocotrienol (Fig. 25.1). The level of these isoforms in cooking oils and supplements varies widely among products. Upon consumption of tocopherols, the tocopherols, which are lipids, are transported along with other lipids from the intestine by the lymph to the blood and then to the liver. These tocopherol isoforms are not interconverted by mammals. The isoform with the highest concentration in tissues is α-tocopherol. There are tenfold higher tissue concentrations of α-tocopherol than

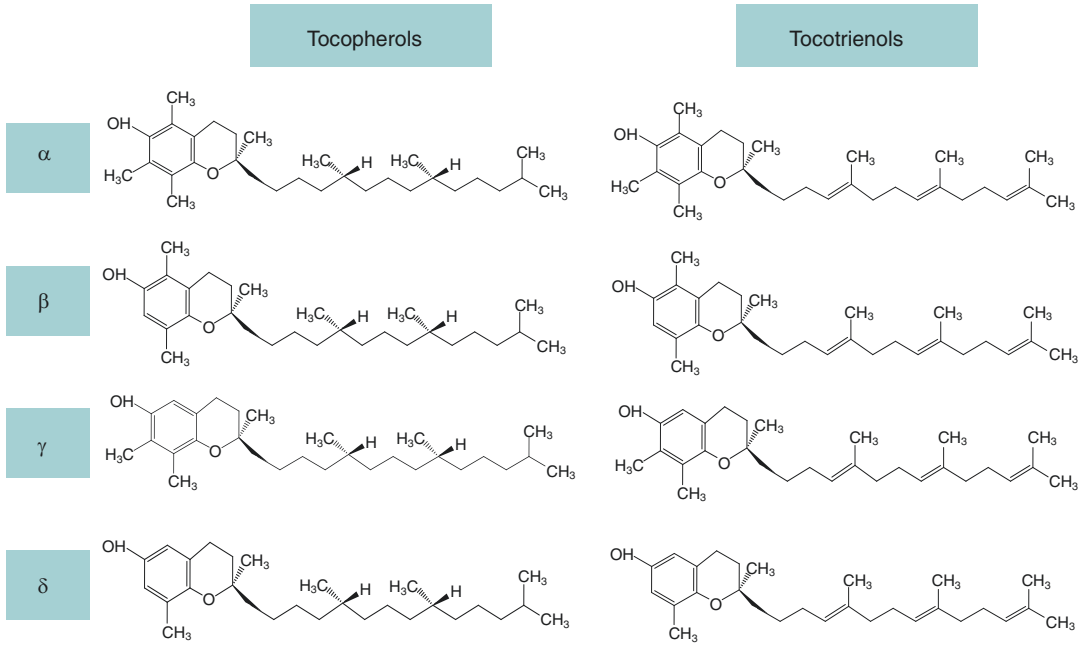

Fig. 25.1 Natural vitamin E isoforms. The most abundant isoforms in human plasma and tissues are α-tocopherol and γ-tocopherol

γ-tocopherol [42] because there is preferential loading of α-tocopherol on lipid particles in the liver by α-tocopherol transfer protein (αTTP) and because there is a higher rate of degradation of γ-tocopherol into its metabolites for excretion [43, 44]. The plasma concentrations of α-tocopherol are relatively similar across many countries [45]. αTTP is also expressed by trophoblasts, fetal endothelium, and amnion epithelium of the placenta, consistent with the critical role for α-tocopherol in placenta development [46, 47]. Tocopherols on plasma lipid particles are transferred to cells by plasma phospholipid transfer protein, scavenger receptors, or the lipoprotein lipase pathway [23]. In cells, tocopherols, which are lipids, are located in cell membranes and associated with lipophilic domains of proteins. On lipid particles and in cells, tocopherol isoforms have both antioxidant and non-antioxidant functions. The antioxidant activity of α-tocopherol and γ-tocopherol, at equal molar concentrations, is relatively similar with regard to scavenging reactive oxygen species (ROS) during lipid peroxidation [48, 49]. Because α-tocopherol is at tenfold higher concentrations in tissues than γ-tocopherol, α-tocopherol has tenfold more capacity for scavenging of ROS in vivo.

In addition to antioxidant functions of tocopherols, non-antioxidant functions of tocopherol regulate allergic inflammation. Important for interpretation of tocopherol regulatory effects is that although γ-tocopherol is at tenfold lower concentrations in vivo, γ-tocopherol is very potent and at these tenfold lower concentrations, γ-tocopherol can block the benefit of α-tocopherol during allergic inflammation and asthma. This potent opposing effect of γ-tocopherol occurs because, at least, γ-tocopherol is an agonist of cell signals, whereas α-tocopherol is an antagonist of cell signals involved in the recruitment of inflammatory cells [50]. During allergic inflammation, leukocytes are recruited from the blood into the tissues by migrating across the vascular endothelial cells. The migration of leukocytes across endothelial cells is inhibited by pretreatment of the endothelial cells with α-tocopherol and elevated by pretreatment of the endothelial cells with γ-tocopherol [51]. Endothelial cells pretreated with α-tocopherol plus γ-tocopherol result in a phenotype that is not different from the vehicle-treated control endothelial cells [51]. Thus, α-tocopherol and γ-tocopherol have opposing regulatory functions during leukocyte recruitment. These opposing functions of tocopherol isoforms occur through

direct regulation of mediators of signal transduction. Briefly, the recruitment of eosinophils to sites of allergic inflammation requires eosinophil binding to adhesion molecules on endothelial cells such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) [6]. These adhesion molecules signal through protein kinase C-α (PKCα) for the recruitment of eosinophils, dendritic cells, lymphocytes, and mast cells [51–53]. Upon loading endothelial cells with physiological tocopherol concentrations, which are at the same concentrations of tocopherols as in lung tissues in mice, α-tocopherol inhibits and γ-tocopherol elevates VCAM-1 and ICAM-1 activation of PKCα in endothelial cells [51–53]. PKCα is regulated by the tocopherols because, upon binding to the C1A regulatory domain of PKCα, α-tocopherol is an antagonist and γ-tocopherol is an agonist of PKCα activity [50]. α-Tocopherol has been also reported to inhibit PKCα activation in other cell systems or cell extracts, but the mechanisms for inhibition in these systems were not demonstrated [54]. In summary, PKCα is differentially regulated by tocopherol isoforms in endothelial cells, which is critical for leukocyte recruitment in allergic lung inflammation and airway hyper-responsiveness. Thus, α-tocopherol and γ-tocopherol have opposing functions during regulation of allergic responses that utilize PKCα signaling.

Another difference in function of α-tocopherol and γ-tocopherol is that γ-tocopherol, but not α-tocopherol, scavenges reactive nitrogen species (RNS) with peroxynitrite forming 5-nitro-γ-tocopherol [55]. Although α-tocopherol has benefit in allergic eosinophilic inflammation, γ-tocopherol scavenging of RNS may be beneficial by short-term administration for acute inflammation in the lungs with increased RNS, such as neutrophil inflammation induced by ozone or endotoxin [56]. In sheep, nebulized γ-tocopherol reduced neutrophilia, IL-8, and IL-6 in burn and smoke inhalation injury [57]. In other studies, supplementation with mixed tocopherol isoforms containing γ-tocopherol blocks acute endotoxin-stimulated or ozone-stimulated neutrophil inflammation in the rat and human lung [58–61]. Although γ-tocopherol has been demonstrated to limit responses to ozone, a recent study also indicates that α-tocopherol can also inhibit ozone responses. Acute administration of α-tocopherol at the time of ozone/OVA challenge blocked ozone-induced exacerbation of OVA-stimulated lung neutrophil and eosinophil inflammation [62]. It is reported that γ-tocopherol supplementation reduces antigen induction of rat lung inflammation in which there was severalfold more neutrophils than eosinophils [63] and that high doses of another isoform γ-tocotrienol reduce house dust mite-induced asthma [64]. In asthmatic children exposed to ozone, vitamin E (isoforms not reported) and C supplementation reduced IL-6 in nasal lavages [65]. Therefore, γ-tocopherol may be of benefit for acute neutrophilic inflammation. In a study with ex vivo treatment of human macrophages, 300 μM γ-tocopherol decreased macrophage phagocytosis via modulation of surface receptor activity [57]. However, such highly elevated γ-tocopherol levels are not achievable in vivo. In contrast to reports of γ-tocopherol benefits in acute inflammation or neutrophilic inflammation, it is reported that plasma γ-tocopherol associates with lower lung function in humans [66] and with increased lung eosinophilia and airway hyper-responsiveness in mouse models of allergic asthma [66]. Therefore, chronic consumption of γ-tocopherol may be unfavorable during lung development or during chronic inflammatory diseases such as allergies and asthma. Further clinical studies for mechanisms of tocopherol isoform regulation of allergic lung inflammation are much needed.

Further substantiation of opposing functions of α-tocopherol and γ-tocopherol exists with other chronic inflammatory diseases, including cardiovascular disease and osteoarthritis. In osteoarthritis, plasma γ-tocopherol positively associates with osteoarthritis, whereas plasma α-tocopherol negatively associates with osteoarthritis [67]. In coronary heart disease and stroke, plasma γ-tocopherol is either not associated with heart disease or is associated with an increase in risk for myocardial infarction [68]. In contrast, for α-tocopherol, it is either not associated with heart disease or is associated with reduced death from heart disease [69–72]. Therefore, for those reports with an effect on heart disease, γ-tocopherol associates with an increase and α-tocopherol associates with a decrease in heart disease.

Asthma and Tocopherol Isoforms in Clinical Studies

Studies on vitamin E and disease outcomes often have focused on one isoform of tocopherol, but the subjects in these clinical studies in fact consume multiple isoforms of tocopherols in differing quantities in the diet, in over-the-counter multivitamins, and in vehicles used in studies with tocopherol supplements. Moreover, there are differences in outcomes of the clinical studies. These differences in clinical effects are consistent when taking into account regional diets, regional plasma tocopherol isoform concentration, multivitamin supplement use, and isoforms administered [20, 73, 74] and when taking into account the mechanistic studies of opposing functions of γ-tocopherol and α-tocopherol [50–52, 66, 73, 75]. Therefore, it is essential in clinical studies to determine the plasma or tissue concentration of tocopherol isoforms for adequate interpretation of study results. The tocopherol concentrations in plasma often correlate with those in the lung tissue in humans and in mice [51, 75, 76].

Intriguingly, countries with the highest prevalence rate of asthma tend to have the highest plasma levels of γ-tocopherol [45, 77]. In contrast, the average plasma levels of α-tocopherol are similar among all countries, likely because the liver regulates α-tocopherol uptake by the α-tocopherol transfer protein [45, 77]. The United States has one of the highest rates of asthma, and, in the United States, the average human plasma γ-tocopherol levels are 2–5 times higher than those of most European and Asian countries [45, 77]. These high plasma γ-tocopherol levels in the United States reflect the diet consumption of soybean oil, corn oil, and canola oil, all of which are high in γ-tocopherol per gram of oil (Table 25.1) [45, 77–85]. The administration of soybean oil does increase plasma γ-tocopherol levels two- to fivefold in humans and in hamsters [86, 87]. Similar fivefold changes in γ-tocopherol in mice elevate allergen-induced lung inflammation as well as suppress the anti-inflammatory functions of α-tocopherol [51]. In contrast to the United States, most European countries regularly use sunflower oil, safflower oil, and olive oil that are relatively low in γ-tocopherol per gram of oil (Table 25.1) [45, 51, 77, 80]. When administering dietary olive oil to preterm human infants starting 24 h after birth, there is a significant 1.5-fold increase in plasma α-tocopherol as compared to feeding with soybean oil [88]; unfortunately, in this study, plasma concentrations of γ-tocopherol were not reported.

Outcomes for clinical studies of α-tocopherol and asthma differ among countries that consume different dietary oils. In Italy and Finland which preferentially consume safflower oil or olive oil, α-tocopherol supplementation of asthmatic patients resulted in reduced incidence of physician-diagnosed asthma and better lung function as measured by forced expiratory volume in 1 s (FEV1) or

Table 25.1 Isoforms of tocopherols in dietary oils

Tocopherol (mg/100 g of oil)				
Oils		α-T	γ-T	δ-T
Oils with low γ-T	Sunflower	56.27 ± 2.95[a]	1.22 ± 0.10	0.22 ± 0.02
	Safflower	49.33 ± 6.88	3.85 ± 0.45	ND
	Olive	6.13 ± 0.61	0.08 ± 0.004	ND
	Grape seed	7.58 ± 0.54	2.07 ± 0.12	0.11 ± 0.01
Oils with high γ-T	Soybean	7.82 ± 0.20	53.87 ± 0.09	15.99 ± 0.25
	Corn	29.01 ± 3.21	46.69 ± 0.07	5.07 ± 1.38
	Canola	16.45 ± 0.19	21.92 ± 0.05	0.13 ± 0.01
	Peanut	14.41 ± 0.19	9.66 ± 0.27	0.80 ± 0.23
	Sesame	77.68 ± 6.07	75.41 ± 4.82	56.27 ± 0.45

α-T α-tocopherol; *γ-T* γ-tocopherol; *δ-T* δ-tocopherol
ND not detected
[a]mean ± standard deviation
Adapted from Cook-Mills et al. [45]

wheeze [89–93]. Consistent with this, in two Scottish cohorts, it was reported that reduced maternal intake of vitamin E (likely referring to α-tocopherol) was associated with increased asthma and wheezing in children up to 5 years old [94, 95]. Unfortunately, in the United States or the Netherlands where there is preferential consumption of soybean oil, corn oil, and canola oil, α-tocopherol was not beneficial for adult asthmatic patients [89–93]. This is consistent with opposing functions of γ-tocopherol because the United States and the Netherlands have high plasma levels of γ-tocopherol and high intake of soybean oil (Table 25.1). However, in the United States, some benefit is shown with consumption of very high levels of the acetate form of α-tocopherol. Briefly, oral supplementation with acetate-conjugated α-tocopherol at a very high dose (1500 IU which is 1006 mg) to mild atopic asthmatics in the United States for 16 weeks resulted in increased plasma α-tocopherol, decreased plasma γ-tocopherol, and improved airway responsiveness to methacholine challenge [96]. The studies on vitamin E and lung function have been also examined by meta-analysis, but unfortunately, these analyses have not taken into account the opposing functions of tocopherol isoforms [97]. As would be expected, meta-analyses of data with multiple forms of tocopherols that have opposing functions indicate no association with lung function and wheeze [97]. Thus, we interpret the meta-analysis results [98] as a combination of data from studies with marked variation of vitamin E isoforms that have opposing functions and that were present in the diets, supplements, and supplement vehicles. In contrast to allergic asthma studies in the United States, a study of exercise-induced asthma demonstrated that α-tocopherol supplementation for 3 weeks blocks the exercise-induced transient drop in lung function in humans [99].

As countries adopt Western-like lifestyles and diets, there is increased consumption of soybean oil [100]. In a review by Devereaux et al. of increasing prevalence of asthma and changes in the environment in Scotland [94] from 1967 to 2004, the vegetable oil intake by Scottish significantly increased. We suggest that this would at least result in an increase in γ-tocopherol since vegetable oil (soybean oil) is rich in γ-tocopherol. In England, dietary supplementation with α-tocopherol in soybean oil vehicle to asthmatics had no impact on FEV1, asthma symptom scores, or bronchodilator use [101], which is consistent with γ-tocopherol in soybean oil opposing the function of α-tocopherol. In another study in the United Kingdom, α-tocopherol administration in soybean oil to asthmatics also did not have benefit [102]. Although a consideration is that the clinical studies have differences in asthma prevalence among racial and ethnic groups [103], the studies examining tocopherol association with clinical outcomes generally adjust for several known confounding factors such as gender, age, body mass index, race, and smoking. Even though other differences may occur regarding the environment and genetics of people in different countries, tocopherol isoforms and outcomes for asthma in clinical studies are consistent with the mechanistic studies demonstrating inhibitory functions of α-tocopherol and agonist functions of γ-tocopherol [51]. An interpretation is that differences in outcomes from clinical reports on the associations of vitamin E and asthma may, in part, reflect the opposing regulatory effects of α-tocopherol and γ-tocopherol in the supplements, in the vehicles for the supplements, and in the diets of the individuals.

Opposing regulatory functions of tocopherol isoforms in humans are demonstrated in studies of human lung function in the United States and in China. In these studies, since tocopherol isoforms have opposing functions, the analysis of associations of tocopherol isoforms in clinical studies includes quartiles of plasma tocopherols and the determination of whether there is an association of the tocopherol isoform with the clinical outcome when the concentration of the opposing tocopherol isoform is low and causing the least competing opposing effects. Taking the ratio of α-tocopherol to γ-tocopherol is an unsuitable approach because a high α-tocopherol to high γ-tocopherol ratio that is the same as a low α-tocopherol to low γ-tocopherol ratio is functionally quite different. In a study of individuals in China, high plasma γ-T with low α-T resulted in a fourfold increase in odds ratio for adult asthma in 8 years [104]. In 4526 adults in the United States in the Coronary Artery Risk Development in Young Adults (CARDIA) multicenter cohort, α-tocopherol and γ-tocopherol had opposing associations with lung spirometry [66]. In this study, there were equal numbers of blacks and whites and equal numbers

of females and males by study design. Interestingly, increasing serum levels of γ-tocopherol associated with lower FEV1 or FVC, whereas increasing serum levels of α-tocopherol associated with higher FEV1 or FVC [66], with adjustments for several known confounding factors such as gender, age, body mass index, race, and smoking. In the CARDIA cohort, fivefold higher human plasma γ-tocopherol (>10 μM γ-tocopherol) associated with reduced FEV_1 and FVC in all participants (asthmatics and non-asthmatics) by age 21–27 years old. This association with decreased FEV_1 and FVC before age 21 may occur during development and lung responses to environmental pollutants, allergens, or infections because tocopherol isoforms can directly regulate PKCα during leukocyte recruitment and cell activation [20, 51, 73, 75]. For asthmatics with plasma γ-tocopherol >10 μM, there was 350–570 mL lower FEV_1 or FVC as compared to the low to moderate γ-tocopherol concentrations (<10 μM γ-tocopherol) at ages 21–27 [66]. This 10–17% decrease in FEV_1 with >10 μM plasma γ-tocopherol in asthmatics is comparable to the 5–10% reduction in FEV_1 reported for other environmental factors. In illustration, in asthmatics with occupational allergen exposure, there is a 5–8% decrease in FEV_1 compared to non-asthmatics, and this decrease is associated with dyspnea, chest tightness, chronic bronchitis, and chronic cough [105]. Responders to particulate matter have a 2–6% decrease in FEV_1 [106], responders to cold or exercise have a 5–11% decrease in FEV_1 [107], and responders to house dust mite or dog/cat dander have a 2–8% decrease in FEV_1 [108]. With a 2% prevalence of serum γ-tocopherol >10 μM in adults in CARDIA and the adult US population in the 2011 census, 4.5 million adults in the US population may have >10 μM serum γ-tocopherol and have lower FEV_1 and FVC. These results are consistent with the preclinical studies demonstrating opposing functions for α-tocopherol and γ-tocopherol in cell signaling, in cell recruitment in allergic responses, and in lung function of allergic mice [32, 33, 50–52, 75, 109]. Also in a study in Finland, lower serum α-tocopherol is associated with self-reported asthma [110], and the highest quartile of γ-tocopherol in early childhood (age 1–4) increased the risk of developing asthma [111].

In tissues of asthmatics, tocopherols are reduced. Low plasma α-tocopherol levels are present in adults or children with asthma [90, 91, 112–115]. It is reported that patients with asthma have reduced α-tocopherol and ascorbic acid in airway fluid but the average plasma concentration of α-tocopherol and ascorbic acid in these patients is normal [112, 113], despite reports in normal individuals that plasma and tissue tocopherols correlate [51, 75, 76]. As in humans, in allergic guinea pigs, α-tocopherol and ascorbic acid levels are decreased in bronchoalveolar lavage [116], and in allergic mice, tocopherol isoforms are reduced in the plasma and lungs [32, 33]. Therefore, since α-tocopherol levels are low in asthmatics and since α-tocopherol can reduce allergic inflammation, supplementation with natural α-tocopherol and maintenance of low dietary levels of γ-tocopherol in combination with other treatments may be an ideal strategy to either prevent or improve control of allergic disease/asthma. A potential target for balance of tocopherol isoforms during allergic disease and asthma may be about

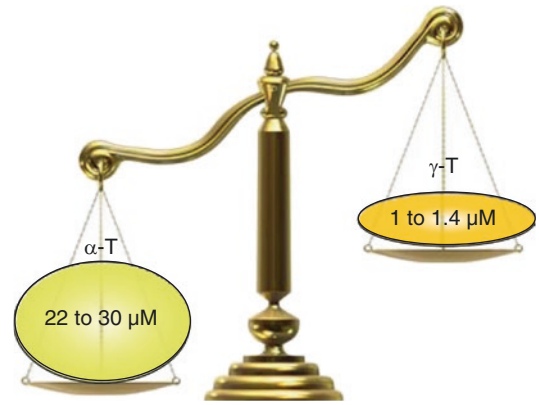

Fig. 25.2 A potential balance in human plasma for α-tocopherol (α-T) and γ-tocopherol (γ-T) concentrations during lung development and allergic disease. Additional clinical and mechanistic preclinical studies are needed

1–1.4 μM plasma γ-tocopherol and 22–30 μM plasma α-tocopherol (Fig. 25.2) as based on average human plasma tocopherol isoforms in countries [45], prevalence of asthma in countries [45], and low α-tocopherol in asthma [90, 91, 112–115]. Additional intervention studies in humans with analysis of the tocopherol isoforms in plasma and tissues are necessary to assess tocopherol isoform regulation of allergic inflammation and asthma.

Tocopherol Doses in Humans Versus Preclinical Mouse Studies

The tocopherol levels in preclinical animal models of human disease need to be relevant to tocopherol levels in humans. Direct dose comparisons per kg body weight are not sufficient because of large differences in rates of metabolism between mice and humans. Thus, calculations for mouse tocopherol doses that are relevant to humans need to include adjustments for the large differences in lipid metabolic rates and in body weight. Standard mouse basal diet contains about 45 mg α-tocopherol/kg of the diet. The calculation for humans is translated as follows: [(45 mg α-tocopherol/kg of diet) × (1 kg/1000 g) × (6 g diet eaten/mouse/day)/(28 g body weight for an adult mouse)] × 65,000 g human adult = 627 mg α-tocopherol/day for human adult. However, mouse metabolism is about eightfold less efficient, and mice have a higher metabolic turnover rate/kg of body weight than humans [117, 118]. Thus, mice require about eightfold higher intake/g body weight. Furthermore, mice eat 1/6 their body weight in food/day [119] which is considerably higher than the average amount of food/day for adult humans. Thus, to adjust for metabolic rate: (627 mg/day for adult human)/(8 for metabolic rate difference) = 78 mg α-tocopherol/day for human adults. For supplementation levels during disease, a three- to fivefold increase in α-tocopherol for supplementation of mice during studies of lung inflammation (150 or 250 mg α-T/kg of diet for mice) is then 235–392 mg α-tocopherol/day for human adults (calculation: 78 mg/day × (3 or 5)). The 235–392 mg α-tocopherol supplemental doses are well below upper limits suggested for human safety of 1000 mg α-tocopherol/day in pregnancy. Moreover, the supplemental level is near clinical levels in preeclampsia pregnancy trials of 268 mg (400 IU) α-tocopherol [120–125]. Also, a supplemented mouse diet with 150 or 250 mg α-tocopherol/kg of diet is reasonable in mice as it is 30–60 times lower than the rodent maternal α-tocopherol diet dose that reduces rodent hippocampus function [126]. Importantly, the doses of 150 mg or 250 mg α-tocopherol/kg of diet for mice achieve two- to threefold increases in tissue concentrations of α-tocopherol, which is similar to fold tissue changes achievable in humans [20, 50–52, 73, 75].

Physiological, nontoxic doses of tocopherol isoforms for studies in mice are doses that achieve fold changes in mouse tissues similar to fold changes in human tissues. For healthy adult humans, the recommended daily allowance of α-tocopherol is 15 mg/day. However, because during asthma the levels of tocopherol isoforms are decreased in tissues in humans and mice, it is imperative that studies be done to determine recommended daily doses of tocopherol isoforms during disease. Recommended daily doses of γ-tocopherol have not been addressed. For mice, the standard basal mouse chow diet contains about 45 mg α-tocopherol/kg of diet and 45 mg γ-tocopherol/kg of diet [32, 33]. With equal levels of α-tocopherol and γ-tocopherol in the diet, it results in a tenfold higher tissue α-tocopherol concentration than γ-tocopherol concentration [32, 33] because of the preferential transfer of α-tocopherol by α-TTP in the liver. This is also relevant to studies in humans with mixed tocopherol isoforms. It is important to be aware that the α-tocopherol isoform is preferentially transferred in the liver but nevertheless, γ-tocopherol is potent and, at tenfold lower levels than α-tocopherol in tissues, γ-tocopherol can ablate the benefit of α-tocopherol in allergic responses. Moreover, in humans and mice, a basal rather than deficient α-tocopherol dose is relevant as a baseline, especially in studies of development because α-tocopherol is necessary for mouse and human placental development [46, 47].

In Preclinical Mechanistic Studies in Adult Mice, α-Tocopherol and γ-Tocopherol Have Opposing Functions in the Regulation of Allergic Inflammation and Airway Hyper-responsiveness

As in the design of human studies, in preclinical animal studies, there are differences in the intake of tocopherol isoforms and doses of tocopherols present in the supplementation and in the oil vehicles used for supplementation. For example, Suchankova et al. reported that the administration of purified α-tocopherol in soybean oil by gavage had no major effect on immune parameters or lung airway responsiveness in mice challenged with OVA [127], but tissue levels of tocopherols were not reported. Moreover, an interpretation of this study is that high γ-tocopherol in the soybean oil vehicle opposed the effect of the α-tocopherol. Okamoto et al. [128] found that feeding mice α-tocopherol starting 2 weeks before antigen sensitization did not affect IgE levels but did reduce the number of eosinophils in the bronchoalveolar lavage, but the form and purity of α-tocopherol were not indicated. Mabalirajan et al. [129] reported that oral administration of α-tocopherol in ethanol after antigen sensitization blocked OVA-induced lung inflammation and airway hyper-responsiveness. In this report, α-tocopherol treatment reduced airway hyper-responsiveness and mediators of inflammation including IL-4, IL-5, IL-13, OVA-specific IgE, eotaxin, TGFβ, 12/15-LOX, lipid peroxidation, and lung nitric oxide metabolites [130]. In summary, α-tocopherol supplementation without γ-tocopherol supplementation reduced allergic inflammation.

As in clinical studies, preclinical studies indicate that α-tocopherol supplementation and γ-tocopherol supplementation have opposing effects on allergic inflammation. In a mouse model of allergic lung responses to house dust mite, mouse diet supplemented with 250 mg α-tocopherol/kg of diet during house dust mite challenges reduces eosinophilia in the lung [104]. In contrast, mouse diet supplemented with 250 mg γ-tocopherol/kg of diet elevated house dust mite-induced eosinophilia in the lung [104]. Another mouse model of allergic lung inflammation is induced by intraperitoneal sensitization with chicken egg ovalbumin (OVA) in adjuvant and followed by challenge of the lung with inhaled OVA. Using this model, to determine whether tocopherols regulate the response to the OVA challenge to the lung, tocopherols were administered after OVA sensitization [51]; this is particularly relevant because patients are already sensitized. In this study, tocopherols were administered by daily subcutaneous injections after sensitization but before allergen challenge [51]. Importantly, tocopherols administered subcutaneously or in the diet have the same route of trafficking through the body. By either of these methods of administration, the tocopherols enter the lymph, then the thoracic duct, and then the liver where the tocopherols are loaded on lipoproteins that then enter circulation. The subcutaneous administration of tocopherols reaches plateaus in tissue levels of tocopherols in only a few days, whereas dietary supplementation of tocopherols takes a couple of weeks to achieve a plateau in tissue tocopherol levels [86, 131]. The subcutaneous administration of α-tocopherol or γ-tocopherol raises lung and plasma concentrations of the tocopherol isoform four- to fivefold without affecting body or lung weight [51], and this fold change is clinically relevant because it is achievable in humans. In this study, subcutaneous administration of γ-tocopherol elevates lung eosinophil recruitment by 175%, and α-tocopherol reduces lung eosinophil recruitment by 65% [49]. Furthermore, in these mice, α-tocopherol blocks and γ-tocopherol increases airway hyper-responsiveness [51]. The levels of tocopherols in these studies did not alter numbers of blood eosinophils, indicating that sufficient numbers of eosinophils were available for recruitment [51]. Tocopherol supplementation does not alter the expression of adhesion molecules, cytokines, and chemokines required for the leukocyte recruitment [51]. This modulation of leukocyte infiltration in allergic inflammation, without alteration of adhesion molecules, cytokines, or chemokines, is similar to several other reports of in vivo inhibition of lung inflammation by inhibition of intracellular signals in endothelial cells during leukocyte recruitment [132–134].

These pro-inflammatory allergic effects of γ-tocopherol in mice are partially reversible by switching supplements from γ-tocopherol to α-tocopherol at these doses for 4 weeks [75]. There is full reversibility of the elevated inflammatory effects of γ-tocopherol but only with highly elevated levels (10 × supplemental levels) of α-tocopherol [75] that may be potentially risky in humans because very high levels of α-tocopherol may increase the incidence of hemorrhagic stroke and elevate blood pressure [63, 65, 135]. A possibly safer alternative to reverse pro-inflammatory effects of γ-tocopherol on allergic inflammation may be longer supplementation with modest levels of α-tocopherol supplementation.

When α-tocopherol and γ-tocopherol are administered at the same time, γ-tocopherol opposes the anti-inflammatory benefit of α-tocopherol [51, 75]. Supplementation of α-tocopherol plus γ-tocopherol during challenge with OVA does not alter the level of allergic inflammation such that the numbers of lung eosinophils and airway responses are similar to that of the allergic mice with the vehicle control [49]. This suggests that these two tocopherols have competing opposing functions. Moreover, γ-tocopherol is very potent because this opposing function of γ-tocopherol occurs even though γ-tocopherol was about 5–10 times lower in concentration in vivo than α-tocopherol.

In summary, in adult mice, raising tissue concentrations of α-tocopherol fivefold is anti-inflammatory and blocks airway hyper-reactivity, and raising tissue concentrations of γ-tocopherol fivefold is pro-inflammatory and increases airway hyper-reactivity during eosinophilic allergic lung inflammation [20, 50–52, 75]. These studies are consistent with the clinical studies, demonstrating that a fivefold increase in human plasma γ-tocopherol associates with a reduction in lung function in adult humans [66]. To relate this to the prevalence of disease, a fivefold difference in plasma γ-tocopherol concentrations is consistent with fivefold higher γ-tocopherol in Americans versus most Western Europeans and Asians (Table 25.1) and higher prevalence of asthma in Americans [45, 77]. Mechanistically, during allergic inflammation in the lung, tocopherol isoforms regulate eosinophil migration on VCAM-1 [5, 6], VCAM-1 signals through PKCα [136], and tocopherols regulate PKCα directly by binding to the C1A regulatory domain of PKCα [50]. Upon binding to PKCα, α-tocopherol is an antagonist of PKCα, and γ-tocopherol is an agonist of PKCα. Thus, a mechanism for the opposing regulatory functions for α-tocopherol and γ-tocopherol on allergic inflammation in adult mice is, at least in part, a result of tocopherol regulation of signals for leukocyte transendothelial migration from the blood into the tissue.

Maternal Tocopherol and Offspring Development of Allergy

Clinical Studies of Maternal Tocopherols and Allergy/Asthma

There has been an increase in the d-γ-tocopherol isoform of vitamin E in the diet and in infant formulas that contain soybean oil [19, 20, 51, 137, 138]. Thus, tocopherol isoforms that regulate allergy and asthma in mothers may affect the risk of development of allergy and asthma in offspring. Some studies suggest that development of allergen responsiveness may occur prenatally [139–141] and it is suggested that in utero and early exposures to environmental factors are critical for increased risk of allergic disease [21]. Higher intake of vitamin E is associated with lower odds of wheeze in childhood, but in this analysis, the isoforms of vitamin E are unclear [142]. Increasing maternal α-tocopherol during pregnancy in humans negatively associates with production of inflammatory mediators in vitro in endotoxin-stimulated nasal airway epithelial cells isolated from neonates shortly after birth [143]. Also, maternal plasma α-tocopherol at 11-week gestation associates with reduced asthma treatments in children in the United Kingdom [144]. In studies of human maternal and paternal asthma and development of allergies in offspring, most associations are with maternal allergy/asthma [24, 30, 145–151], suggesting that sensitization can occur prenatally or early postnatally. There is an association of

higher risk of eczema, wheezing, and lower respiratory tract infections in early life with increases in human maternal and cord blood C-reactive protein, which is an acute-phase protein produced during inflammation [152]. It is reported that, by age 21, human plasma α-tocopherol associates with better lung spirometry and human plasma γ-tocopherol associates with worse lung spirometry [66], suggesting that in human development, tocopherols may have early life regulatory functions on responsiveness to allergen and perhaps to other environmental challenges to the lung. It has been demonstrated that maternal α-tocopherol dietary intake is inversely associated with cord blood mononuclear cell proliferative responses to allergen challenge in vitro [140, 153]. Also, from ultrasound studies of the fetus, maternal α-tocopherol levels are reported to associate with fetal growth [154]. In rats, maternal α-tocopherol supplementation during pregnancy results in larger lungs with normal structure in offspring [155]. In several clinical studies, α-tocopherol did not associate with asthma [156–158], but these studies did not measure tissue or plasma tocopherol isoforms or include analysis of potential opposing functions of γ-tocopherol. Moreover clinical reports demonstrate that the risk for allergy in children has been associated with mothers with existing allergic disease before conception [24, 30, 145–151], but whether maternal tocopherol isoforms regulate development of asthma and allergic diseases in offspring needs further study. Specifically, additional clinical studies are needed to address the balance of α-tocopherol and γ-tocopherol in healthy and asthmatic/allergic pregnant women and in infants that may influence the development of risk of allergies and asthma in children.

Preclinical Animal Studies Demonstrating a Maternal Contribution to Offspring Allergy and Asthma

Before discussing tocopherol isoform regulation of maternal influence over development of offspring allergies, it is imperative to first discuss mechanisms of the maternal effect on offspring of allergic mothers. In pregnant mice, tocopherol supplementation of the mother alters the risk of development of allergies in the offspring. The mouse model of maternal transfer of risk of allergy to offspring reflects many of the parameters of development of allergic disease [24, 30, 34, 35, 145–151, 159, 160], including the fact that the allergic responses of the offspring are not specific to the allergen of the mother [34]. In this mouse model, allergy is induced in adult female mice by sensitizing with intraperitoneal injection of OVA with the adjuvant alum on weeks 1 and 2 and then challenging with aerosolized OVA on weeks 4, 8, and 12 [30, 34–40, 160]. On the day of the last OVA challenge on week 12, these allergic female mice are mated [30, 33–39]. Another OVA challenge during mouse pregnancy does not increase risk of allergy in the offspring [34], likely because it takes about 2 weeks for resolution of allergic lung inflammation during the 3-week gestation. The OVA challenge just prior to mating is required for the offspring responsiveness (unpublished observations). The allergic response during pregnancy in mice is consistent with what occurs in humans, as it is anticipated that allergic mothers would have an allergen challenge during their 9-month pregnancy. In the neonatal mouse model, after birth, all of the offspring from allergic mothers and non-allergic mothers are treated with a suboptimal OVA protocol. This suboptimal protocol is comprised of neonates receiving only one instead of two OVA/alum treatments at postnatal days 3–5, and then starting 7 days later, the neonates are challenged with aerosolized OVA for 3 consecutive days [30, 33–39]. The offspring from allergic mothers develop allergic lung inflammation and airway responsiveness, whereas pups from non-allergic mothers do not develop allergic inflammation (Fig. 25.3). This allergen responsiveness of the offspring of allergic mothers is sustained for up to 8 weeks of age, which is adulthood in the mouse, but then declines after 8 weeks old [35]. The response of the offspring can be abrogated by blocking the allergic response of the mothers at preconception with anti-IL-4 antibody administration [34] or by antibody depletion of T cells in allergic mothers [36]. Further evidence indicates that maternal T cells are sufficient for the maternal effect on offspring because adoptive transfer of allergen-specific T cells from OVA TCR transgenic mice DO11.10 mice to females prior to mating

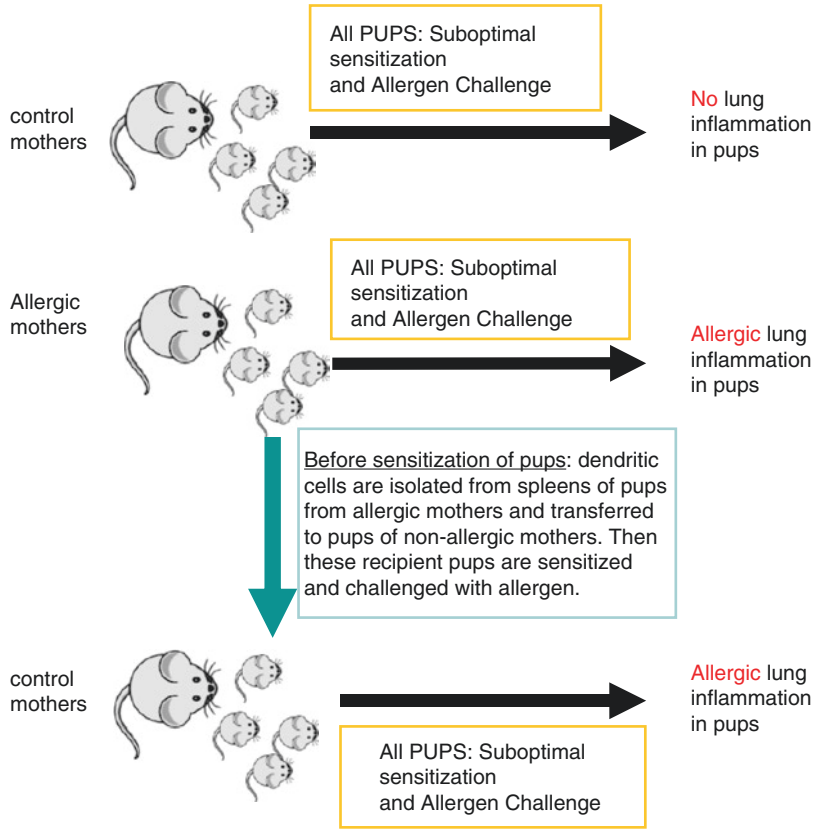

Fig. 25.3 Dendritic cells in maternal transmission of risk for allergy in offspring

results in offspring with responsiveness to suboptimal challenge of antigen [37]. However, the maternal factors are not entirely clear. IL-4 and IgE which are elevated in the mother do not pass to the fetus [141, 159, 161]. Th2 cytokines (IL-4, IL-5, and IL-13) are elevated in the placenta, but transplacental crossing of these cytokines has not been demonstrated [162–164]. Only 2% of maternal GM-CSF crosses the human placenta in ex vivo perfusate studies [165], but whether maternal GM-CSF increases the risk of offspring for allergic responses is not known. Allergens have been reported to perhaps cross the placenta, but it has been demonstrated that offspring are able to respond to BLG, whereas mothers were stimulated with OVA, suggesting that the process is antigen-independent [141]. In another report demonstrating antigen independence of the maternal effect, offspring are responsive to casein, whereas the mothers were sensitized and challenged at preconception with OVA [34]. This antigen-independent effect of maternal allergy on allergen responsiveness in pups is also demonstrated in canines [166]. Similarly to animals, human offspring respond to different allergens than the allergic mother [30]. Thus, the offspring responses are not specific to the allergen that the mother responds to, but instead, the offspring have an increased ability to respond to allergen sensitization. Therefore, female mice that are allergic before conception and develop a Th2 response during pregnancy produce offspring that have augmented responsiveness to suboptimal allergen.

The antigen-independent effect of maternal allergy on allergen responsiveness in pups is a result of changes in dendritic cells of the offspring (Fig. 25.3). The increased responsiveness of the offspring is not through changes in pup macrophages [160]. In adoptive transfer studies, transfer of splenic dendritic cells from non-challenged neonates of allergic mothers into neonates from non-allergic mothers confers increased allergic susceptibility in recipient neonates (Fig. 25.3) [160]. In contrast, the transfer of macrophages from non-challenged neonates of allergic mothers into neonates from

non-allergic mothers does not confer increased allergic susceptibility in recipient neonates [160]. The dendritic cell changes include changes in numbers of discrete subsets of dendritic cells [32, 33] and in responses by dendritic cells [160, 167, 168].

There are an increased number of discrete subsets of dendritic cells in offspring of allergic mothers. In the fetal livers from allergic mothers and in the OVA-challenged pup lungs from offspring of allergic mothers, there are increased numbers of CD11b + subsets of CD11c + dendritic cells [169], a dendritic cell subset that is critical for generation of allergic responses [168]. In contrast, in these tissues, there are no changes in CD11b- regulatory dendritic cell subsets, including plasmacytoid dendritic cells and CD103+ dendritic cells [169]. Furthermore, before antigen challenge of the pups, the dendritic cells of pups from allergic mothers had little transcriptional changes but extensive DNA methylation changes [170]. Then, after allergen challenge, there were many transcriptional changes in the dendritic cells of pups of allergic mothers as compared to pups of non-allergic mothers [170]. These studies suggest that maternal mediators, which do not direct allergen specificity, may be transferred from the mother to the offspring and these mediators regulate offspring dendritic cells and heighten the responsiveness of offspring to challenge with suboptimal doses of allergens.

A maternal effect on offspring allergic responses has also been demonstrated for maternal exposure to environmental irritants. Maternal inhalation of titanium oxide or diesel exhaust particles during pregnancy increases responses of offspring to allergen challenge [22]. Also, skin sensitization to toluene diisocyanate (TDI) induces a Th2 response in the mother, and when the mother is mated after a second dose of TDI, the offspring have increased allergic responses to suboptimal OVA [171]. In contrast, a Th1 response in the mother may protect the offspring from developing allergic responses. When females are sensitized to dinitrochlorobenzene (DNCB) which induces a Th1 response and then mated, the offspring do not develop an allergic response to suboptimal OVA [171]. Also, offspring are protected from development of asthma by prenatal challenge of the mother with LPS, which induces a Th1 inflammation, an increase in the Th1 cytokine IFNγ, and a decrease in the Th2 cytokines IL-5 and IL-13 [172–175]. Injection of non-allergic mothers with IFNγ on gestational day 6.5 protects against the development of allergic responses in offspring [176]. Fedulov et al. [177] demonstrated that treatment of the offspring from allergic mothers on postnatal day 4 with CpG oligonucleotides, a TLR9 agonist and Th1-type stimulant [178], protected the offspring from development of allergic responses to suboptimal OVA challenge. Therefore, exposure of allergic mothers or offspring from allergic mothers to a Th1 stimuli inhibited offspring responses to allergen challenge.

After birth, early in life, breast milk of allergic mother mice may influence development of allergic responses in offspring. However, milk of allergic mothers is not necessary for the offspring allergic responses, because the in utero maternal effects are sufficient for allergic responses by offspring of allergic mothers. Briefly, pups from allergic mothers that are nursed by non-allergic mothers still have an allergic response to suboptimal challenge with OVA [159]. Therefore, maternal effects in utero mediate development of allergen responsiveness in offspring of allergic mothers [159]. However, breast milk is sufficient, but not necessary, for maternal transmission of asthma risk in the offspring because when pups from non-allergic mothers are nursed by allergic mothers, the pups exhibit a response to suboptimal allergen challenge [159]. In that study, the breast milk from allergic and non-allergic mothers contained no detectable IFNγ, IL-2, IL-4, IL-5, IL-13, or TNFα, suggesting that other mediators increase the risk of offspring allergy through breast milk [159]. In clinical studies of other nutrients in breast milk, the mediators, omega-3 and omega-6 polyunsaturated fatty acids, in human milk associate with asthma and atopy, but the mechanism is not known [179, 180]. It is also reported that omega-3 fatty acids during pregnancy associate with lower infantile wheeze [181].

In contrast to studies of allergic mothers, the function of breast milk has been studied for mothers that were not allergic at preconception. In these models, the non-allergic mother mice were exposed during pregnancy or lactation to allergen or to an antigen tolerance protocol. In mouse models where the mother was not allergic at preconception but then exposed during pregnancy or lactation to allergen or an antigen tolerance protocol, there was protection of offspring responses to allergen sensitization/challenge [182]. In these studies, exposure of normal female mice during lactation to OVA results

in the transfer of antigen and TGFβ in milk, and this inhibited the development of inflammation of offspring treated later in life as adults (6–8 weeks old) by two sensitizations with OVA/alum and five OVA challenges [182]. These adult offspring had elevated regulatory CD4+ T cells, and the increase in regulatory T cells was dependent on milk TGFβ but not milk immunoglobulins [182], consistent with inhibition of allergic responses as adults. In another approach, it was demonstrated that sensitization of females before mating and then extensive antigen challenges (ten OVA challenges) during lactation resulted in the transfer of IgG immune complexes in the milk and induction of regulatory T cells and tolerance in the offspring when offspring were challenged with OVA at 6–8 weeks old; in this model, immune complexes but not TGFβ in the milk were required for tolerance [183]. In summary, depending on timing, doses, and number of antigen challenges, factors in breast milk can contribute mediators that either increase or decrease offspring responses to allergen. However, consistently, if the mother is allergic before mating, the offspring have elevated responses to allergen.

An endogenous transplacental maternal mediator in allergic mice has been suggested to contribute to the increased responsiveness in the offspring to suboptimal OVA challenge [184]. In adult mice and rats, OVA sensitization and challenge increase stress [185–190] and increase endogenous serum corticosterone [191, 192]. Symptoms of stress/anxiety are commonly associated with allergy/asthma in adult mice and in humans [193–197], but whether this contributes to allergic responses in children is not known. During pregnancy, maternal corticosterone is elevated, can cross the placenta, can affect fetal cortisol levels [198, 199], and is a strong inducer of Th2 responses [200, 201]. Cortisol is also present in human breast milk [202] and has the potential to affect allergic responses in neonates. Consistent with the mechanistic studies in mouse models, in pregnant asthmatic women without treatment for asthma, a deficiency in the placenta of a cortisol-metabolizing enzyme 11beta-hydroxysteroid dehydrogenase 2 leads to increased fetal cortisol and low birth weight which is predictive of lower lung function later in life [203, 204]. In adult 4-week-old mice, stress exacerbates OVA-induced allergic responses, and this is blocked by pretreatment with a glucocorticoid receptor antagonist [192]. Subjecting pregnant female mice to stress increases endogenous corticosterone, increases offspring allergic responses to suboptimal allergen, and increases offspring airway responsiveness after suboptimal allergen challenge [184, 198]. Glucocorticoid during pregnancy may be sufficient for allergic responses in offspring because administration of a low dose of glucocorticoid to non-allergic mothers on day 15 of gestation increases offspring allergic responsiveness to suboptimal allergen challenge [184]. When the mother mice are subjected to stress and treated during pregnancy with an inhibitor of endogenous corticosterone synthesis, there is a reduction in the allergic response by the offspring [184]. Therefore, elevated corticosterone in allergic pregnant mice might contribute as a mediator that is transferred from the mother to the fetus or in the breast milk to the neonate, resulting in enhanced responses of offspring to suboptimal allergen challenge. However, the studies with corticosterone were done with BALB/c mice, and in our hands, administration of corticosterone to C57BL/6 does not result in offspring with allergic responses (unpublished observations), even though offspring of allergic C57BL/6 mice consistently exhibit responsiveness to allergen [32, 33]. This suggests that there are mouse strain-specific effects of corticosterone. Thus, perhaps maternal corticosterone contributes, but there are likely additional mechanisms as well. Understanding mechanisms of maternal transfer of risk for allergy to offspring and mechanisms for tocopherol isoform regulation of this risk will have an impact on limiting the development of allergic disease early in life.

In Preclinical Studies, α-Tocopherol Supplementation of the Mother Reduces Allergic Responses in Offspring

The allergic responsiveness of offspring of allergic mothers is inhibited when allergic female mice are bred and then receive α-tocopherol supplementation during the pregnancy/lactation (Fig. 25.4) [169]. In this study, mothers received 250 mg α-tocopherol/kg diet or a basal α-tocopherol diet

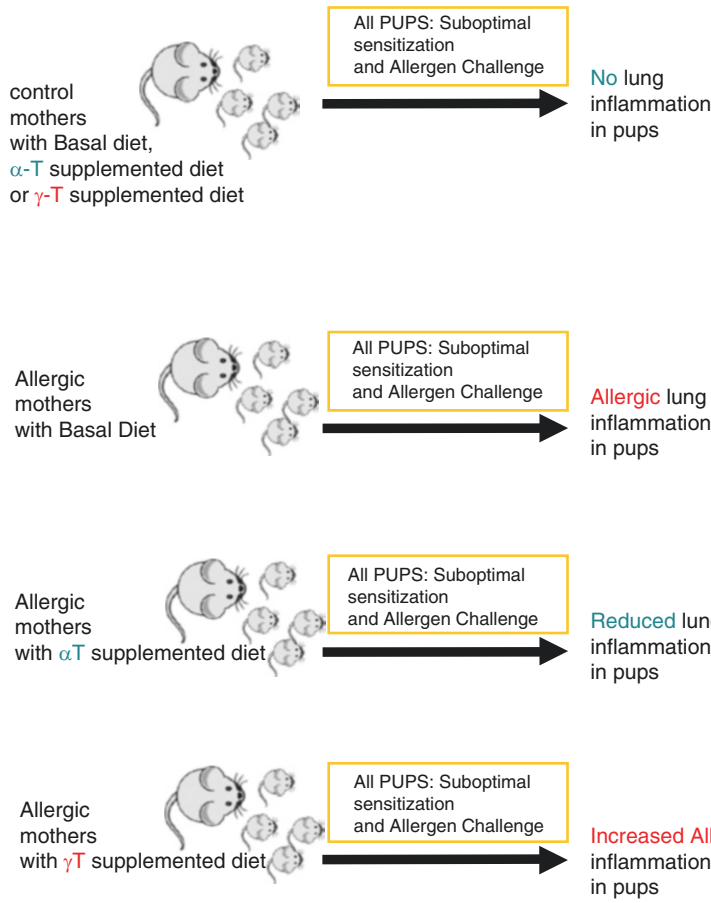

Fig. 25.4 Maternal tocopherol supplementation regulates development of allergic lung inflammation in offspring of allergic mothers

(45 mg α-tocopherol/kg diet) [169]. A basal α-tocopherol diet is used as the control rather than a tocopherol-deficient diet because adequate α-tocopherol levels are required for placental development and thus the fetus [46, 47]. There are no effects on pup weight, pup numbers, or pup gender distribution by tocopherol supplementation or OVA treatments [169]. The α-tocopherol-supplemented diet increases liver α-tocopherol in the saline-treated mothers threefold as compared to basal diet controls [169], which is consistent with previous reports for this diet in adult female mice [51, 75]. The α-tocopherol tissue concentrations are lower in allergic mothers than non-allergic mothers after α-tocopherol supplementation, which is consistent with reduced α-tocopherol levels in human asthmatics [112–114, 205]. In the future, studies of levels for sufficient α-tocopherol supplementation with disease are needed in nonpregnant and pregnant humans, especially since prenatal vitamins often contain multiple tocopherol isoforms. The maternal α-tocopherol supplementation increases pup liver α-tocopherol 2.5-fold [169]. Regarding allergic inflammation in the pups, the maternal α-tocopherol supplementation during pregnancy/lactation results in a dose-dependent inhibition of lung eosinophils [169] in the OVA-challenged pups from allergic mothers as compared to OVA-challenged pups from non-allergic mothers [169]. Maternal α-tocopherol supplementation of allergic female mothers reduces OVA-induced pup lung mRNA expression of cytokines that regulate allergic inflammation (IL-33 and IL-4) and chemokines for eosinophil recruitment (CCL11 and CCL24) [169]. Therefore, α-tocopherol supplementation of allergic mothers inhibits allergic inflammation in OVA-challenged pups from the allergic mothers.

There is a regulatory effect of α-tocopherol in utero and in the milk as determined by cross-fostering pups at birth. Cross-fostering pups from allergic mothers with 250 mg α-tocopherol/kg diet to allergic mothers with basal diet (45 mg α-tocopherol/kg diet) indicated that α-tocopherol supplementation of

the allergic mother during pregnancy was sufficient to inhibit the OVA-induced increase in neonate lung eosinophils [169]. In addition, α-tocopherol supplementation during lactation reduces the allergic responses in the neonates [169], suggesting a contribution of α-tocopherol after birth [169]. In summary, α-tocopherol supplementation of allergic mothers during pregnancy is sufficient to reduce development of allergic responses in the offspring.

α-Tocopherol supplementation starting at conception of a second pregnancy of allergic female mice also inhibits development of allergic lung inflammation in offspring. The offspring from allergic mothers that were supplemented with α-tocopherol at the time of a second mating had a > 90% inhibition of lung lavage eosinophils in the OVA-challenged pups [169]. Moreover, in OVA-challenged pups from allergic mothers, α-tocopherol reduces pup lung mRNA expression of several mediators of allergic inflammation: the cytokines IL-4, IL-33, and TSLP and the chemokines CCL11 and CCL24 [169]. There are no effects of maternal α-tocopherol supplementation on pup low levels of the Th1 cytokine IFNγ or the regulatory cytokine IL-10 [169], indicating that α-tocopherol does not switch the response to OVA to a Th1 response.

In Preclinical Studies, γ-Tocopherol Supplementation of Allergic Mothers Highly Elevates Offspring Allergic Responses

Maternal diets supplemented with 250 mg γ-tocopherol/kg diet during pregnancy/lactation increase the maternal liver γ-tocopherol level twofold and the pup liver γ-tocopherol fivefold, consistent with the fold tocopherol changes in human and mouse tissues after supplementation [20, 50–52, 73, 75]. This γ-tocopherol supplementation of allergic female mice increased offspring lung eosinophils in response to suboptimal allergen (Fig. 25.4) [33]. It is important to note that γ-tocopherol does not induce allergic inflammation in the OVA-challenged pups from non-allergic mothers [33], indicating that endogenous maternal factors of allergic mothers are required for offspring inflammation. In pups from allergic mothers, maternal d-γ-tocopherol supplementation increases inflammatory mediators including the Th2 mediator amphiregulin, IL-5, CCL11, CCL24, activin A, and GM-CSF. Of concern and potential relevance in humans, maternal γ-tocopherol supplementation also decreases the percentage of female mice that had pups. However, for those females that had pups, there is no affect numbers of pups per litter or pup body weight. It is not known whether γ-tocopherol influences placentation, placental development of fetal development. The data suggest that reduced numbers of mothers with pups and increased pup allergic responses with γ-tocopherol supplementation have potential important implications for children of allergic mothers that consumed γ-tocopherol in the diet or prenatal vitamins as well as for infant formulas supplemented with γ-tocopherol. To better define implications of early life tocopherol regulation in humans, further clinical studies are needed.

In Preclinical Studies, Offspring Dendritic Cells Are Regulated by Maternal γ-Tocopherol and α-Tocopherol Supplementation

The increase in the numbers of dendritic cell subsets in offspring of allergic mothers is regulated by tocopherol isoforms. Maternal supplementation with α-tocopherol reduces the OVA-stimulated pup lung and fetal liver numbers of CD11b + subsets of CD11c + dendritic cells, including resident dendritic cells, myeloid dendritic cells, and CD11b + alveolar dendritic cells, without altering CD11b- subsets of CD11c + dendritic cells, including plasmacytoid dendritic cells, CD103+ dendritic cells, CD11b- alveolar dendritic cells, and alveolar macrophages [169].

Most remarkably, α-tocopherol supplementation does not completely deplete CD11b + dendritic cells, but instead, α-tocopherol supplementation of allergic mothers reduces the numbers of pup CD11b + dendritic cells to the numbers of these dendritic cells in pups from non-allergic mothers [169]. This suggests that α-tocopherol does not block the baseline dendritic cell hematopoiesis but instead may block the signals from allergic mothers that specifically induce the increase in differentiation of CD11c + CD11b + dendritic cell subsets in offspring. Consistent with this, the offspring CD11b- subsets of CD11c + dendritic cells are not altered by maternal supplementation of α-tocopherol [169]. It is also reported that in the pup lungs and fetus of allergic mothers, the changes in CD11c + CD11b + dendritic cells occur without altering the expression of the antigen-presenting molecule MHCII and the costimulatory molecules CD80 and CD86 [169].

In contrast to α-tocopherol supplementation, maternal supplementation with γ-tocopherol increases fetal liver and pup development of CD11c + CD11b + dendritic cells but not numbers of offspring regulatory CD11b- dendritic cell subsets [33]. In addition, with γ-tocopherol supplementation, there is an increase in pup cytokines, chemokines, and lung IRF4 + CD11c + CD11b + dendritic cell subsets which are critical to development of allergic responses. γ-Tocopherol supplementation of the allergic mothers also increases generation of IRF4 + CD11c + CD11b + dendritic cells in the fetal liver [33]. In the fetal livers of γ-tocopherol-supplemented mothers, there are fewer regulatory CD11b- CD11c + pDCs [33], suggesting that with γ-tocopherol supplementation, there may be a reduced control of magnitude of responses to allergen challenge early in life. However, in the OVA-challenged pup lung, the number of pDCs was not altered with γ-tocopherol supplementation [33]. For the fetal liver and pup lung, there was no effect of d-γ-tocopherol on the level of expression per dendritic cell of MHCII, CD80, or IRF4 [33].

Maternal d-γ-tocopherol supplementation also partially increases numbers of resident DCs in the fetus and OVA-challenged pup lung of offspring of non-allergic mothers but not as much as the increase in resident dendritic cells of the OVA-challenged pups from d-γ-tocopherol-supplemented allergic mothers [33]. This is consistent with the increased GM-CSF with γ-tocopherol supplementation in pup lungs from allergic mothers [33]. γ-Tocopherol also increased activin A in pups from non-allergic and allergic mothers [33]. Activin A is a member of the TGFβ superfamily of cytokines and regulates allergic inflammation [206]. Activin A can induce differentiation of monocytes to mDCs, and recruitment of DCs and activin A is produced by several cell types including epithelium, endothelium, mast cells, fibroblasts, and dendritic cells [207]. Therefore, with maternal γ-tocopherol, activin A may function in concert with other mediators to increase numbers of DCs and allergic inflammation. Consistent with γ-tocopherol increasing inflammation, it increases the chemokine CCL11 in pups from allergic and non-allergic mothers, and it increases the chemokine CCL24 and the cytokine IL-5 in the pups from non-allergic mothers [33]. OVA challenge in pups from allergic mothers with d-γ-tocopherol does not result in further increases in CCL24 or IL-5 [33], which may indicate that a maximum response was achieved with OVA challenge. Nevertheless, the pups from the allergic mothers with d-γ-tocopherol have elevated CCL11 and amphiregulin suggesting that in combination, these signals as well as the presence of GM-CSF, CCL24, IL-5, and activin A may function to amplify recruitment of eosinophils in pups from γ-tocopherol-supplemented allergic mothers.

The tocopherol isoforms can directly regulate bone marrow dendritic cell differentiation. α-Tocopherol supplementation during 8-day cultures of GM-CSF-stimulated bone marrow cells reduces the generation of CD45+ CD11b + CD11c + dendritic cells and the number of cells with resident dendritic cell phenotype (CD45 + CD11b + CD11c + Ly6c-MHCII- dendritic cells) without affecting the percentage of viable cells in the culture [169]. γ-Tocopherol also directly regulates hematopoietic development of dendritic cells because d-γ-tocopherol increased the generation of IRF4 + CD11c + CD11b + bone marrow-derived dendritic cells in vitro [33].

In summary, maternal supplementation with γ-tocopherol increases and maternal supplementation with α-tocopherol decreases generation of CD11c + CD11b + DCs and signals for allergic inflammation during development. Studies of tocopherol isoform-specific regulation of inflammation provide a

basis toward designing drugs, supplements, and diets that more effectively modulate these pathways in allergic disease. The function of tocopherol isoforms on allergic inflammation and asthma has implications for tocopherol isoforms in prenatal vitamins, in infant formula, and in the diet which may impact risk for allergic disease in future generations. More studies are needed in humans to examine short-term versus long-term outcomes of a range of plasma concentrations of tocopherol isoforms.

Conclusion

The differences in outcomes of clinical studies with tocopherol isoforms are consistent with the mechanistic studies of opposing regulatory functions of these tocopherol isoforms in animal asthma models and in cell cultures with physiological doses of the tocopherol isoforms. The anti-inflammatory function of α-tocopherol and potent pro-inflammatory function γ-tocopherol, at least, reflects the opposing function of these in the regulation of signaling pathways essential to the inflammatory process. Understanding of the differential regulation of inflammation by isoforms of vitamin E provides a basis toward designing drugs and diets that more effectively modulate inflammation and improve lung function in disease in adults and during development.

The rapid increase in rates of asthma implies that the environment influences the generation of asthma and allergic inflammation. Therefore, changes in diet and/or lifestyle could modify disease. Forthcoming studies in preclinical models and clinical studies need to include measurements of tocopherol isoforms in the supplements, vehicles for the supplements, and, most importantly, the plasma and/or tissues before and after intervention. The measurement of plasma or tissue levels is necessary for adequate interpretation of study outcomes. Further studies are necessary to define and provide a basis for recommendations for doses for tocopherol isoforms in healthy individuals and particularly in inflammatory disease states in adult human females and males as well as ethnic groups that differ in prevalence of asthma [208, 209]. The potential of dietary manipulation and supplementation in allergic pregnant mothers and children with asthma requires further work. Interventions in diet early in life, in relation to childhood asthma, raise the possibility of limiting development of allergic disease.

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Chapter 26
Vitamin E, Immune Function, and Protection Against Infection

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Keywords Aging · Immune function · Infection · Nutrition · Vitamin E

Key Points

- Studies in animal models demonstrate that vitamin E deficiency impairs immune functions that can be corrected by vitamin E repletion.
- Intakes of vitamin E above recommended dietary allowances enhance T cell function, particularly in older adults.
- The immunoenhancing mechanisms of vitamin E involve the promotion of T cell activation and effector function and suppression of prostaglandin E_2 production.
- Vitamin E-induced enhancement of immune functions has significant clinical implications, as vitamin E supplementation is associated with an increased resistance to respiratory infections in older adults.
- The totality of evidence suggests the vitamin E requirement to maintain optimal immune response in older adults is higher than currently recommended dietary allowances.

Introduction

The primary functions of immune system are to protect the body against infection from invading pathological microorganisms, to clear damaged tissues, and to provide constant surveillance of malignant cells that grow within the body. The immune system also develops appropriate tolerance to avoid unwanted response to self or harmless foreign substances. The vigor of immune function varies from person to person, which is collectively determined by multiple factors such as genetics, environment, and lifestyle choices including nutrition. Nutrition as a modifiable factor in impacting immune function has been extensively studied, and several decades of research in this field have grown into a multidisciplinary field called nutritional immunology. Being no exception to the other bodily systems, immune system depends on adequate nutrients to function properly. It has long been known that nutritional status is closely associated with immunity and host resistance to infection. Studies have

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_26

demonstrated that deficiency in both macronutrients and micronutrients causes compromised immune function, which can be reversed by nutrient repletion. Malnutrition, which is prevalent in less developed parts of the world as well as in segments of population in developed world, is an important factor in determining the incidence of morbidity and mortality from infectious diseases. While in developed world general nutritional deficiencies are uncommon, depending on age, socioeconomic status, and environmental factors, specific nutrient deficiencies, less ideal diet composition, and excess calorie consumption and exposure to environmental toxins may impair immune system and increase susceptibility to infections and other immune-related diseases.

Numerous animal and human studies have demonstrated that nutritional intervention can modulate immune function and resistance to infection. Nutritional intervention can be undertaken by altering amount and type of macronutrients and micronutrients, adding non-nutrient phytochemicals, either as single or combination of multiple dietary components. Among the dietary components studied, vitamin E has been shown to be an effective nutrient to favorably modulate immune and inflammatory responses leading to improved protection against infection and the resulting complications. In this chapter, we will provide a critical review of the research on vitamin E for its immunomodulating effect, the underlying working mechanism, and clinical relevance. To help better understand the effect of vitamin E on different components and functions of the immune system, we begin with a brief overview of immune system, function of the major components, and the methods commonly used for assessing efficacy of nutritional intervention in affecting immune system.

Immune System and Assessment of Its Function in Nutritional Studies

Overview of Immune System

The immune system is a complex network composed of a variety of cell types and functional molecules produced by these cells. Immunity is commonly classified into two arms based on the features of immune response to the challenges: the innate (or natural) and adaptive (or acquired, specific) immunity. Innate immunity represents the first line of defense against invading infectious agents, and it reacts early upon challenge but lacks specificity and memory. By contrast, the adaptive immunity takes longer time to develop, but it is more effective, efficient, and has greater precision and memory. The innate immune system is mainly composed of physical and chemical barriers, cellular components including phagocytic cells [neutrophils, macrophages (MΦ), and dendritic cells (DC)] and natural killer (NK) cells, circulating effector proteins such as complement and C-reactive protein, and various cytokines and chemokines that regulate the movement and activities of immune cells. The adaptive immune response includes humoral and cell-mediated immunity. Humoral immunity mainly depends on the protein molecules called antibodies (Ab), either circulating in blood or generating locally from mucosal secretions. Cell-mediated immunity is based on direct interaction between cells that provides defense against intracellular microbes, which are inaccessible by circulating Ab. The major cellular components of the adaptive immune system are B cells and T cells. B cells secrete Ab that block and eliminate extracellular microbes and, thus, induce humoral immunity. The helper function and cytotoxic activity in cell-mediated immunity are accomplished by specialized subpopulations of T cells, i.e., $CD4^+$ or helper T (Th) cells and $CD8^+$ or cytotoxic T cells, respectively. In addition, antigen (Ag)-presenting cells (APC), including DC, MΦ, and B cells, can link innate and adaptive immunity by taking up and processing Ag to make them recognized by T cells. In an effective immune response, different immune cells with

specialized functions work together in a highly coordinated manner to protect our body from the harmful entities and maintain a homeostasis state. Malfunction of the immune system is not only the common cause for susceptibility to various infections and autoimmune diseases; it is also implicated in the increased risk of developing cancer and several degenerative diseases.

Outcome Measurements Commonly Used in Nutritional Immunology Research

Efficacy of nutritional intervention in impacting immune function is often evaluated using selected outcome measurements. A variety of markers reflecting different aspects of immune system status and functionality are available to suit specific study objectives. In the technical term, interventional studies are often categorized as in vivo, ex vivo, and in vitro studies based on the way tested agents are administered and the outcomes are acquired. In the in vivo setting, both nutritional intervention and immune tests are conducted on a living subject (human or animal), and it thus best reflects the physiological condition. In the ex vivo setting, the test agents are administered to a living subject, but the immune cells are isolated from the body and often processed (such as incubation and stimulation) before analysis is conducted. The in vitro setting refers to the cell-based ("test tube") study, in which both agent supplementation and immune test evaluation take place outside of the body. Here we only provide a brief description of the immune parameters that have appeared in the reviewed literature involving vitamin E intervention. For more information regarding marker selection and result interpretation in nutritional immunology research, the readers are referred to previous publications [1–5].

In Vivo Indices of Immune Status and Functionality

Immune Cell Phenotype This type of assay determines composition of cells in circulating blood, lymphoid organs, and various tissues concerned. Of the most common cell types identified are total white blood cells (WBC) and WBC differential, different types of lymphocytes including NK cells, DC, B and T cells, and subtypes of T cells (CD4+ and CD8+ T cells as well as their naïve and memory subpopulations). The immune cell phenotype is usually determined using flow cytometry method.

Circulating Soluble Molecules Produced by Immune Cells Total immunoglobulin (Ig) and Ig subclasses, antigen-specific antibody (Ab), cytokines and soluble cytokine receptors in blood, and IgA in secretion liquid can be quantified or semiquantified using biochemical, ELISA, and bioassay methods.

Delayed-Type Hypersensitivity (DTH) Response DTH is a favorable test for assessing immune competence because its clinical relevance is supported by the observation that DTH response is negatively associated with mortality [6–9]. DTH response represents antigen-specific, T cell-dependent, recall response manifested as an inflammatory reaction that peaks 24–48 h after antigenic challenge, i.e., a small amount of antigen intradermally injected to the forearm skin of a person or footpad of a mouse. The DTH response (induration) can be measured with a caliper.

Antibody Response to Vaccine Vaccination response is another very useful in vivo test, which measures antigen-specific, coordinated immune response. Immunization with appropriate antigens (viral or bacterial) can elicit Ab production by activated B cells, which is facilitated by helper T cells through recognizing Ag presented by APC and producing cytokines. Ab production is often used as the best surrogate indicator for host response to infection.

Ex Vivo Indices of Immune Function

Phagocytosis Phagocytosis is a process by which specialized phagocytic cells (or phagocytes, including neutrophils, monocytes/macrophages, and immature dendritic cells) engulf and internalize solid matter such as bacteria, aged red blood cells, and foreign particles. Phagocytosis can be assessed by incubating phagocytic cells in the presence of one of these substrates (bacteria, yeast, or inert particles) labeled with fluorescence and then analyzing the cellular uptake of these substrates under microscope or using a flow cytometer. This assay can be coupled to oxidative burst and bacterial killing assays.

Oxidative (Respiratory) Burst Triggered by phagocytosis or exposure to certain inflammatory mediators, neutrophils and monocytes/macrophages quickly increase oxidative metabolism resulting in generation and release of reactive oxygen species, which can kill pathogens in the cells. Oxidative burst is commonly measured based on the reduction of cytochrome C with a photometry method or change in the fluorescence properties of dihydrorhodamine-123, which can be measured by flow cytometry.

Cytotoxicity Assay Activity of both cytotoxic T lymphocytes (CTL, CD8+ T cells) and NK cells can be assessed by a cytotoxicity assay. While CTL target cells on the basis of cell-surface antigen recognition, NK cells directly lyse tumor cells or virally infected cells. In the CTL activity assay, target cells can be lymphoblasts, cultured tissue cells, or tumor cells. In NK activity assay, tumor cells serve as target cells. The cytotoxic activity of both cell types can be determined by ^{51}Cr release assay or non-isotope method such as flow cytometry.

Lymphocyte Proliferation This is the most commonly used technique to assess cell-mediated immune response. Cell proliferation can be quantified by measuring incorporation of [3H]-thymidine into DNA, or nonradioactive methods such as bromodeoxyuridine incorporation, and fluorescence dye dilution assays. Common agents used to stimulate lymphocyte proliferation are T cell mitogens Con A and PHA and TCR Ab for T cell proliferation, LPS for B cell proliferation, and pokeweed mitogen for proliferation of both T and B cells.

Cytokine Production Cytokines are key signaling molecules in regulation of virtually every aspect of immune cell function, and thus, information about cytokine profile will help estimate general immune status and specific effector functions of particular immune cells. While serum cytokines are measured as an in vivo parameter, only a few cytokines are confidently measurable. In contrast, cytokines are more commonly measured in the supernatant of cultured cells after stimulation with the agents as mentioned above for lymphocyte proliferation assay. Cytokine concentrations can be conveniently quantified using ELISA method. In addition, intracellular cytokine levels in particular types of cells can be detected, without need of previous cell purification, by using flow cytometry method.

Natural Infection and Experimental Disease Models: Clinical Implication of Altered Immune Function

Since a key function of immune system is to defend the host against infection, the promising candidates with immune-enhancing properties are sometimes further tested for their effect on infection caused by various pathogens to validate the clinical relevance. Almost all human studies use natural infection episode as endpoints; however, in a very limited number of studies, experimental infections are conducted using mild or easily controlled pathogens such rhinoviruses [10, 11] or respiratory

syncytial virus [12] to induce respiratory infections and *Shigella* [13] or *Escherichia coli* [14] to induce gastrointestinal tract infections. By contrast, experimental infection studies are commonly conducted using various animal models.

Vitamin E and Immune Function

Vitamin E is a generic term for all tocopherols and tocotrienols that exhibit the biological activity of α-tocopherol. α- and γ-Tocopherols are the main forms of vitamin E present in the common diet with comparable levels; however, α-tocopherol is about tenfold higher than γ-tocopherol in blood because the body has preference toward α-tocopherol in vitamin E transport and metabolism. All the other forms of vitamin E are very low or undetectable in the tissues due to both the low dietary intake and less preferable metabolism. α-Tocopherols, both synthetic and natural forms, are used in a great majority of published studies. The current Dietary Reference Intake (DRI) for vitamin E (DRI 2000) is 15 mg/day for teens and adults [15], which is solely based on the in vitro assay of hydrogen peroxide-induced hemolysis [16]. Even though a substantial portion of population is estimated not to meet the recommended daily intake, vitamin E deficiency is rare. The symptoms of vitamin E deficiency in humans include peripheral neuropathy, skeletal myopathy, reduced red blood cell half-life, and immunological impairments [17, 18]. While the current DRI for vitamin E is effective to prevent those symptoms in general population, it may not be adequate to meet the need in other bodily systems or individuals at different life stages. In other words, biological significance of vitamin E should be viewed beyond the point of meeting minimum intake needed for prevention of previously defined deficiency symptoms. Instead, it has been increasingly recognized that increased vitamin E intake may bring additional health benefits in several bodily systems, in particular the immune system, which is the topic of this review.

Vitamin E Deficiency and Immune Function

Vitamin E is a chain-breaking, lipid-soluble antioxidant present in the membrane of all cells, and immune cells contain particularly high levels of vitamin E, which protects them from oxidative damage related to high metabolic activity, as well as high PUFA content in these cells [19, 20]. Impaired immune function has been found in different species of animals under vitamin E deficiency condition. Vitamin E deficiency in mice caused a lower antibody production (humoral immunity) [21], and vitamin E-deficient rats had impaired activity in macrophage antigen presentation [22], polymorphonuclear cell phagocytosis [23], and lymphocyte proliferation [24]. Depressed T cell function was also found in vitamin E-deficient non-rodent animals including dogs [25], lambs [26], pigs [27], and chickens [28]. More recently, a study reported that grass carp fish fed with vitamin E-deficient diet showed retarded growth, reduced resistance to bacterial infection (A. hydrophila), and impaired immune function, which were reversed by vitamin E repletion [29]. A limited number of observational studies conducted in humans seem to point toward the same direction. Impaired neutrophil phagocytic and bactericidal activities have been observed in the preterm infants with vitamin E deficiency [30]. Healthy children (3 y old) with low-serum vitamin E levels (<10% percentile) had lower levels of lymphocyte proliferation and serum IgM compared to those with higher vitamin E levels (>90% percentile). In a case report, we described that a 59-year-old woman developed severe vitamin E deficiency due to intestinal malabsorption; this patient showed impaired T cell-mediated functions as indicated by both the in vivo (DTH) and in vitro (T cell proliferation, IL-2 production) tests, which was improved after vitamin E supplementation [17].

Vitamin E Supplementation and Immune Function

As mentioned above, vitamin E deficiency impairs immune function, which can be reversed by vitamin E repletion. However, given that vitamin E deficiency is not common, research interest has been directed toward determining whether vitamin E supplementation above the current DRI can bring immune benefit to the non-deficient individuals. While many studies in the past few decades have provided evidence to support this notion, some studies suggest otherwise.

The immunomodulating effect of supplemental vitamin E was first established in the studies using animals of various species. Dietary vitamin E supplementation above recommended level was shown to improve antibody production after vaccination in rabbits [31]; to enhance T cell-mediated functions such as T cell differentiation in rat thymus [32], lymphocyte proliferation in mice [33–35], rats [36, 37], and pigs [38], and helper T cell activity and IL-2 production [34, 35] in mice; and also to promote innate immune function including natural killer cell activity and phagocytic ability of alveolar macrophages in rats [37]. In tumor-bearing mice, vitamin E reduced immunosuppression caused by myeloid-derived suppressor cells but enhanced antigen-specific CD8+ T cell activity, which together enhanced antitumor activity [39]. Even in the Iberian green lizard, a species rarely used as laboratory animals, oral administration of vitamin E enhanced T cell mitogen PHA-induced DTH skin response, an in vivo indicator of immune response [40]. Similar results observed across a variety of other animal models suggest that vitamin E's effect on immune function may be universal rather than species-specific.

The impact of vitamin E supplementation on immune response has also been studied in human. Baehner et al. reported that volunteers (characteristics not provided) receiving 1600 IU/d vitamin E (α-tocopherol) for 1 week had increased phagocytic rate but decreased bactericidal activity of polymorphonuclear leukocytes [41], which might be related to a reduction of H_2O_2 as the consequence of the free radical-scavenging property of vitamin E. Similarly, Prasad found a decreased leukocyte bactericidal activity in young male subjects consuming 300 mg/d of vitamin E (dl-α-tocopheryl acetate) for 3 weeks. In the same study, they also observed reduced lymphocyte proliferation and unchanged DTH after stimulation with T cell mitogen PHA [42]. In a later randomized, double-blind, placebo-controlled trial (RCT) by Meydani et al. [43], healthy older adults (≥60 y) who received 800 mg/d vitamin E (dl-α-tocopheryl acetate) for 1 month showed improvement in DTH response, and ex vivo T cell proliferation and IL-2 production, as well as reduced production of prostaglandin (PG)E_2, a T cell suppressive eicosanoid. To define dose-response pattern for vitamin E's immunomodulating effect, Meydani's group subsequently conducted another RCT in which free-living elderly (≥65 y) received 60, 200, or 800 mg/d of vitamin E for 4.5 months [44]. While all three vitamin E groups had a significant increase in DTH response from their respective baseline levels, a significantly greater increase relative to the placebo group was observed only in the 200 mg/d group. Furthermore, it was also this group who showed a significant increase in antibody titers in response to hepatitis B and tetanus toxoid vaccines (T cell-dependent antigens) [44]. To a large extent, consistent with these findings, Pallast et al. [45] showed that healthy older subjects (65–80 y) receiving 50 or 100 mg/d of vitamin E (dl-α-tocopheryl acetate) for 6 months had a significant increase in DTH compared to their own baseline values, and this increase tended to be greater in the 100 mg/d group than in the placebo group ($p = 0.06$). A further subgroup analysis showed that among participants who received 100 mg/d vitamin E, those with lower DTH at baseline showed significant increase in DTH. These effects of vitamin E were concurred by De la Fuente et al., who showed that administration of 200 mg/d vitamin E for 3 months to elderly participants enhanced both T cell-mediated (mitogen-stimulated lymphocyte proliferation and IL-2 production) and innate immune responses (natural killer cell activity, neutrophil chemotaxis and phagocytosis), but inhibited neutrophil adherence and superoxide anion production [46]. While this latter study did not include a placebo group when the subjects were tested again 6 months after completion of the trial, majority of the outcomes that were altered following vitamin E

supplementation returned to their baseline levels. By reviewing the results from several vitamin E trials in which DTH was used as an in vivo indicator of immune function and plasma vitamin E levels were measured, Meydani et al. have concluded that supplementation-induced increase in plasma vitamin E levels is linearly associated with the increase in DTH response before a plateau is reached around a net increase in plasma E level by 25 μmol/L, which may be achieved by consuming 200 mg/d of vitamin E [47]. Thus, the authors suggest that vitamin E intake at 200 mg/d should be recommended as an optimal dose in terms of improving T cell-mediated function in the elderly.

The results from both animal models and clinical trials summarized above strongly suggest a positive effect of vitamin E on cell-mediated immune response. This contention is further reinforced by the mechanistic research as discussed later in this chapter.

Compared to adaptive immunity, information regarding vitamin E's effect on innate immunity is limited and less characterized, thus calling for further research. Toward this end, in addition to the results reviewed above, some recent studies have reported that vitamin E can modulate neutrophil function in both in vivo and in vitro settings using a mouse infection model and cells collected from human donors, respectively [48, 49].

Working Mechanisms for Vitamin E's Effect on Immune Function

Although research has made steady progress in advancing our knowledge about how vitamin E affects immune function, we still only partly understand the mode of its action. While the underlying mechanisms have been investigated in both human and animal studies, it is primarily the results from animal studies combined with cell-based tests that have contributed to what we know today.

Unlike vitamins A and D, which function by acting on their nuclear receptors to regulate the transcription of target genes, no cognate receptors have been found for vitamin E. As a lipid-soluble antioxidant, vitamin E may improve immune cell function by preventing cellular damage caused by uncontrolled oxygen species generated as a result of intensified metabolism during immune response. Vitamin E is localized in lipid compartments of the cell membranes where it protects both membrane lipids and proteins from oxidative damage. Cell membrane plays a critical role in immune cell activity. Immune cells depend on proper membrane activity in carrying out a variety of important functions from early activation events to ultimate effector functions. Lipid peroxidation can damage cell membranes and membrane-associated functions. For example, lipid peroxidation can reduce membrane fluidity, which is implicated in a decreased ability of lymphocytes to respond to challenges [50]. It is thus conceivable that vitamin E may help maintain the integrity and functionality of immune cell membranes by preventing membrane lipid peroxidation. Further, by residing at proximity of the membrane lipids, vitamin E is proposed to directly modulate certain properties of membranes, such as lipid raft mobility, which in turn may influence the lateral movement and activation condition of the signaling molecules [51]. More importantly, evidence has been reviewed to conclude that many functions of vitamin E are independent of its antioxidant property [52–54], and this is very likely to be true for its effect in immune system as well.

As previously reviewed [47, 55], current evidence in the aged indicates that vitamin E enhances T cell-mediated function not only by directly promoting membrane integrity and influencing the signaling events in T cells, and it can also protect T cell function indirectly by reducing production of T cell-suppressing factors such as PGE_2 from MΦ. The direct effect of vitamin E on T cell response is mainly established in animal models using both in vitro and in vivo supplementation methods. In vitro supplementation with 46 μmol/L vitamin E (d-α-tocopherol) was shown to reverse the age-associated reduction in activation-induced T cell division and IL-2 production in naïve but not memory T cells [56], probably due to the relatively higher susceptibility of naïve T cells to oxidative damage [57]. More in-depth studies indicate that vitamin E improves the early

events in T cell activation including formation of effective immune synapses, which is impaired during the aging process. For example, both in vivo (500 mg d-α-tocopherol/kg diet) and in vitro (46 μmol/L d-α-tocopherol in culture medium) vitamin E supplementations are shown to improve effective immune synapse formation and restore the defective redistribution of signaling molecules Zap70, LAT, Vav, and PLCγ in the immune synapse formed between antigen-presenting cells and naïve CD4+ T cells from old mice [51]. Furthermore, the improved LAT distribution in immune synapse by vitamin E may be related to increased phosphorylation of LAT [58], which is required for recruitment of adaptor and effector proteins including Grb2, Gads, SLP76, Vav1, PLCγ1, and phosphoinositide 3-kinase [59, 60].

Vitamin E's indirect effect that contributes to its T cell-enhancing function is built upon the research investigating production of PGE_2, an eicosanoid possessing potent pro-inflammatory and T cell-suppressing activity. Early studies reported that PGE_2 suppressed T cell response by activating adenylyl cyclase, thus increasing cAMP levels [61, 62]. PGE_2 has a broad effect on different components in both the innate and adaptive immune system [63–66], such as inhibiting T cell proliferation, IL-2 production, and IL-2 receptor expression [64]. The suppressive effect of PGE_2 on T cells concerns inhibition of several early signaling events that occur after T cell activation [66], and for some of them, the PGE_2-induced inhibition can be prevented by vitamin E. The role of PGE_2 in immunosenescence has been investigated. Significantly more PGE_2 is produced in macrophages and spleen cells from the aged mice and peripheral blood mononuclear cells from older individuals compared to their young counterparts [34, 43, 67, 68], and this age-associated change is partially responsible for impaired T cell function in the aged [69, 70]. Wu et al. reported that macrophages from old mice fed with vitamin E (500 mg/kg diet) had significantly lower LPS-stimulated PGE_2 production compared to those fed with the control diet (30 mg/kg diet). This effect was mediated by posttranslational inhibition of the enzymatic activity of cyclooxygenases (COX)-2, a rate-limiting enzyme for prostaglandin synthesis [71]. These findings were for the most part corroborated by other investigators [71–73]. Further, we showed that vitamin E may regulate COX-2 activity through reducing peroxynitrite production, a COX-2 enzyme activity promoter [74].

Vitamin E and Infection

Given that the primary function of the immune system is to defend the body against infection, determining whether vitamin E improves host's resistance to pathogens is a highly relevant issue to address. Early evidence suggesting protective effect of vitamin E against infection mainly came from animal studies that used various infection models. These animal models remain a primary tool today due to ethical issues that limit the use of experimental infections in humans. In contrast, human studies for this purpose thus far have been conducted by determining the impact of vitamin E on naturally occurring infections.

Animal Study

Interventional studies using animal models for infection have indicated that vitamin E may improve host resistance to infections including *Escherichia coli* in chicks [75] and pigs [76], *Diplococcus pneumoniae* type I [77] and *Streptococcus pneumoniae* [48] in mice, *Listeria monocytogenes* in turkeys [78], influenza infection in mice [79, 80], and also secondary *Staphylococcus aureus* infection after influenza infection in mice [81]. High incidence and mortality for respiratory infection in older adults are a serious concern, which is associated with compromised immune response, and thus have

been the main focus of studies on vitamin E and infection in the aged. The authors' laboratory has conducted several vitamin E intervention studies using animal models of respiratory infection as reviewed below in more detail.

Hayek et al. [80] infected young and old mice with influenza A/Port Chalmers/1/73 (H3N2) after these animals were fed vitamin E (500 mg/kg diet) or control diet (30 mg/kg) for 6 weeks. They reported that vitamin E treatment reduced viral titers in both young and old mice but more prominently in the aged [80]. In another study with the similar study design, Han et al. [79] further indicated that the protective effect of vitamin E on influenza infection was associated with improved cell-mediated immune function. They found that mice with more severe symptoms and delayed viral clearance had lower IL-2 and higher PGE_2 production before infection and, more importantly, a lower IFN-γ response after viral infection compared to those with less severe infection. In a later study using a bacterial respiratory infection model, Bou Ghanem et al. found higher pulmonary bacterial burden, lethal septicemia, and lung inflammation (neutrophil infiltration) in old versus young mice after infection with *Streptococcus pneumoniae*; this age-related difference in response to infection was prevented by 4 weeks of dietary vitamin E supplementation (d-α-tocopheryl acetate, 500 mg/kg diet) prior to infection challenge [48]. These authors further indicated that the vitamin E's protective effect might be mediated via its inhibitory action on neutrophil transepithelial migration, which in turn may result from altered expression of several epithelial and neutrophil adhesion molecules involved in neutrophil migration [48].

Human Studies

There is little direct evidence from the epidemiological studies supporting the beneficial effect of vitamin E intake in infection. Although a retrospective study in healthy persons (≥ 60 y) reported a negative relationship between plasma vitamin E levels and the number of infections over the past 3 years, this finding appeared not to be related to effect of vitamin E on immune system as no correlations were found between vitamin E status and any of the measurements of immune response including T cell phenotype, PHA-induced lymphocyte proliferation, and DTH response to seven ubiquitous antigens [82]. Few intervention trials have been conducted to establish the causal effect of vitamin E in improving the host's resistance to infection. This is largely due to the challenge that this type of study depends on recording the natural infections, the incidence of which is relatively low and thus a large sample size would be needed to detect the effect of intervention. Nevertheless, the results generated from limited number of such studies are promising; however, there is a need for more clinical trials to reach consensus for the efficacy of vitamin E in reducing the risk or severity of infection.

In an RCT to determine the effect of vitamin E on immune function, Meydani et al. [44], in a dose-response study designed to determine the impact of vitamin E on immune response of older adults, noticed a trend ($p = 0.1$) toward lower incidence (by 30%) of self-reported infections in all the vitamin E groups combined (60, 200, or 800 mg/d as dl-α-tocopheryl acetate) than that in the placebo group. To specifically address whether vitamin E has a protective effect against respiratory infection (RI), this group later conducted an RCT in nursing home residents (>65 y), a cohort that has a high incidence of RI infection [83]. In this study, 617 participants received 200 mg/d vitamin E, a dose determined to be optimal in their previous study [44], or placebo for 1 year, during which time RI was objectively recorded. The authors reported significantly fewer incidences of upper RI, particularly common cold in the vitamin E group than those in the placebo group. These studies support the notion that the immunostimulatory effect of vitamin E has clinical benefit in protecting against respiratory infections.

However, not all other studies conducted to date have reported similar findings. For example, Hemila et al. [84–86] using data generated from Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) study showed a positive, no effect, or even a negative effect of vitamin E on pneumonia and the common cold depending on the age, smoking history, residence, exercise, and other subject characteristics. In a

secondary analysis of ATBC study, Hemila [87] found that in 7469 participants who were male smokers aged 50–69 y at the baseline, vitamin E supplementation (50 mg/d) for 5–8 years significantly reduced the incidence of pneumonia. The author further indicated that the efficacy of vitamin E in this regard varied depending on the amount of cigarette smoking and exercise, which is based on the observation that participants who smoked 5–19 cigarettes/d and exercised had larger reduction in incidence of pneumonia compared to those who smoked ≥20 cigarettes/d and did not exercise (69% versus 14%) [87]. Although the cohort in this study was quite different (all were male, smokers, younger adults included, lower vitamin E dose but longer supplementation period) compared to those in study by Meydani et al., a protective effect on RI was observed in both studies. In another RCT conducted in a Dutch elder (≥ 60 y) cohort living in the community, Graat et al. [88], however, found no effect of 200 mg/d of vitamin E on the incidence of all RI. Since this study was conducted in independently living participants versus those living in nursing homes in the study by Meydani et al., it is speculated that the efficacy of vitamin E might be more pronounced in nursing home residents who are more frail but live under controlled condition, compared to those who are relatively younger, healthier, living independently, and more influenced by other lifestyle factors. As another example of complexity, in a cohort study which followed 1509 Swedish men and women (20–60 y) for 4 months, the authors found no effect of vitamin E from food on the risk of upper respiratory tract infection (URTI) in either men or women; however, use of supplemental vitamin E was found to reduce the URTI incidence in men [0.56 (95% CI 0.33–0.95)], but not in women [89]. Given the fact that the participants in this study were younger adults rather than the elderly, and vitamin E intake from food is much less (2–20 mg/d) compared to that provided by supplements, these negative results do not negate the notion that optimal intake (200 mg/d, from 60, 200, 800 mg/day tested) of vitamin E may be protective in reducing the risk of RI in older adults.

Concluding Remarks

Vitamin E's beneficial effect on the immune system has been supported by strong experimental evidence, in particular from the research on T cell-mediated function. The clinical significance of this beneficial effect of vitamin E is confirmed by some animal model studies and clinical trials using naturally occurring infection as an outcome; however, the reports from human studies are inconsistent. The discrepancy may be partly due to the difference in study design and experimental protocols (supplementation dose and duration, selection of and methods used for outcome variables) and cohort characteristics (age, health status, lifestyle, genetic background).

Related to this Belisle et al. reported that the effect of vitamin E on cytokine production and pneumonia incidence in elderly may vary based on polymorphism in pro-inflammatory cytokine genes [90–92].

Although the recommended dietary intake of vitamin E may not be met in a significant number of people, vitamin E deficiency, based on acceptable serum vitamin E levels and the known deficiency symptoms, is not common. In fact, research on vitamin E's impact on immune function has been focused on the benefit of increased vitamin E intake above the recommended level (15 mg or 22 IU/day) in improving the immune function in targeted populations such as the older adults. The totality of evidence to date suggests that vitamin E requirement to maintain optimal immune response in older adults may be higher than that currently recommended. This is supported by animal studies and some human studies reporting protection from RI when older adults are supplemented with higher than currently recommended level of vitamin E. Additional clinical trials to confirm causal role of vitamin E in protecting against infection in humans are needed. Such information will be helpful in developing dietary guidelines for achieving optimal intake of vitamin E in different populations.

Acknowledgments The authors' work is supported by the USDA/ARS under contract #58-1950-4-003 and various grants from NIA.

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Chapter 27
Vitamin E and Air Pollution

Rebecca F. McLoughlin, Bronwyn S. Berthon, Evan J. Williams, and Lisa G. Wood

Keywords Air pollution · Particulate matter · Vitamin E · Alpha tocopherol · Dietary antioxidants · Disease

Key Points

- 80% of the global population is exposed to unacceptably high levels of air pollution.
- Particulate matter (PM) air pollution exposure alone is the 5th leading risk factor for mortality worldwide.
- PM exposure is associated with asthma, chronic obstructive pulmonary disease, respiratory infections and cardiovascular, metabolic, gastrointestinal, skin and central nervous system (CNS) diseases.
- Such diseases have a common underlying pathology of inflammation and oxidative stress.
- Dietary supplementation with vitamin E is hypothesised to be beneficial due to its antioxidant and anti-inflammatory properties.
- Emerging evidence from clinical trials support a role of vitamin E and C in ameliorating the effects of air pollution in asthma and reducing the risk of respiratory infections.
- Further work is needed to assess the efficacy of vitamin E supplementation in protecting against the numerous other health consequences of air pollution exposure.

Introduction

For centuries it has been known that substances inhaled from the air, otherwise known as air pollution, can be deleterious to health [1]. Over time, the widespread exposure and damaging health effects of air pollution have driven it to become one of the leading causes of death worldwide [2]. Historically, concerns related to the inhalation of harmful compounds were concentrated on the effects of quartz, asbestos and coal dust, which were linked to the serious and sometimes fatal conditions of silicosis, asbestosis, lung cancer, mesothelioma and pneumoconiosis arising from occupational exposure. Later

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_27

the industrial burning of coal in urban areas was linked to thousands of deaths during dangerous winter coal smogs [1]. In more recent decades, while exposure to these traditional pollutants has decreased via reduction and control methods, air pollution has continued to be driven by vehicle engine combustion, fossil fuel combustion, power plants and other industrial sources [3]. Air pollution remains a serious concern, with epidemiological evidence clearly showing death rates are related to the concentration of particulate air pollution [4]. Strategies for reducing the damaging health effects of air pollution are urgently needed.

Various nutritional interventions have been assessed for their protective effect against air pollution exposure. In particular, it has been hypothesised that dietary supplementation with vitamin E may be beneficial, due to its antioxidant and anti-inflammatory properties. In intervention studies, vitamin E is often delivered in combination with vitamin C, as these are the key lipid-soluble and water-soluble dietary antioxidants in humans, and vitamin C has a regenerative role as vitamin E becomes oxidised. A study in healthy individuals exposed to air pollution highlighted the potential benefit of these nutrients; a combination of vitamins E and C was effective in decreasing markers of lipid and protein damage and improving both enzymatic and non-enzymatic antioxidant defences [5]. Here we review the literature describing health effects of air pollution and the role of vitamin E in modifying the detrimental consequences of air pollution.

The Nature of Air Pollution

Air pollution can include contamination of both outdoor (ambient) and household (indoor) environments. Air pollution can be emitted directly from the source as is the case for carbon monoxide, lead, nitrogen dioxide and sulphur dioxide. Air pollution can also be formed in the atmosphere through reactions of gases, emissions and organic compounds. For example, ozone is created when sunlight reacts with air pollutants and volatile organic compounds which leads to photochemical smog [6].

Particulate matter (PM) air pollution affects the greatest number of people worldwide, with mortality and morbidity associated with both acute and chronic exposure [7]. PM includes emissions from both natural and manmade sources and is a heterogeneous mix of solid and liquid particles that vary in terms of size, mass, source and chemical composition [3]. PM can contain a variety of pollutants such as sulphate, nitrates, ammonia, sulphur, chloride, black carbon and mineral dust [8]. PM is primarily categorised by its mass concentration, ranging from particles less than 10 μm in aerodynamic diameter (PM_{10}) to particles less than 2.5 μm in diameter ($PM_{2.5}$) and particles which have a diameter of 0.1 μm or less ($PM_{0.1}$) [9]. The size of PM is a major factor in determining adverse health effects as it controls how far the particle can penetrate the respiratory system. $PM_{10,}$ or coarse, 'inhalable' particles, can be deposited into the nasopharynx and sometimes into the thorax, while $PM_{2.5}$, which is known as fine or respirable particles, can pass into the ciliated airways and into the alveolar gas exchange areas of the lung [3, 10]. Ultrafine particles, or $PM_{0.1}$, present the greatest threat to health due to their deep penetration into the lungs and ability to cross the air-blood barrier [3].

The health and environmental risks posed by air pollution have led to the World Health Organization (WHO) introducing air quality guidelines in 2005, with recommended thresholds and limits for the key pollutants including particulate matter (PM), ozone (O_3), nitrogen dioxide (NO_2) and sulphur dioxide (SO_2) [8]. The US Environmental Protection Agency (USEPA) under the Clean Air Act has developed National Ambient Air Quality Standards (NAAQS) for these pollutants, as well as carbon monoxide (CO) and lead (Pb) [11]. While these standards are in place, many states and countries do not comply, and most concerning is the advice from the WHO stating that there is no safe threshold for which exposure to PM has no adverse effects on health [8].

Latest estimates reveal that worldwide, the problem of air pollution has reached epidemic proportions. Only one in ten people resides in a city that complies with WHO air quality standards, and from

the global air quality data, only 16% of the populations assessed were exposed to levels of particulate matter within the thresholds set by the standards [12]. In terms of changes over time, PM pollution is decreasing in high-income areas of America, Europe and the Western Pacific, while concentrations are increasing in other areas including the Eastern Mediterranean, Southeast Asia and low- to middle-income countries in the Western Pacific [12]. Indoor air pollution from the burning of biomass fuels for heating and cooking is a major source of exposure in low- to middle-income countries, where the concentrations of ambient air pollution are also increasing [7]. In terms of ozone exposure, there was a 7% increase globally, from 1990 to 2015, with the highest concentrations seen in the United States, Western and Central sub-Saharan Africa, the Mediterranean, the Middle East, South Asia and China [13].

Populations at Risk and Mortality Attributed to Air Pollution

Evidence suggests that children [14–17], the elderly [16–18] as well as individuals with poor health and pre-existing health conditions (e.g. asthma, COPD, hypertension, ischaemic heart disease, obesity and diabetes) [19], suboptimal lifestyles or genetic predisposition may be more susceptible to the toxicity of air pollution [16, 20]. Children are among those most vulnerable to the adverse effects of air pollution. Children have higher minute ventilation than adults and often spend more time outdoors, resulting in higher internal doses of air pollutants per unit of body size [21]. In fact, it is estimated that the minute ventilation of a 6-year-old child is double that of an adult [22]. Studies examining individual exposure to air pollution have demonstrated that despite being exposed to similar outdoor concentrations of PM_{10} (children, 41.5 μg/m^3 [23]; adults, 38.5 μg/m^3 [24]), personal exposure of children was much higher than adults (66.8 versus 26.9 μg/m^3 above ambient levels) [23, 24]. Children also have underdeveloped respiratory tract and immune systems making them more susceptible to the toxic effects of air pollutants [25, 26].

At the other end of the age spectrum, elderly individuals are more susceptible to the adverse effects of air pollution exposure than the general population [16]. Immunosenescence, which refers to the age-related deterioration of the immune system, reduces our ability to compensate for the damaging effects of air pollutants. Particle clearance may also become less efficient with age [17]. Furthermore, elderly individuals are more likely to suffer from chronic diseases. Pre-existing diseases may determine susceptibility, with evidence suggesting that air pollutants may exacerbate chronic diseases such as cardiovascular disease, hypertension, lung diseases (e.g. asthma and COPD) and diabetes [18].

Genetic predisposition may also increase susceptibility to the adverse effects of air pollution [16, 27]. Endogenous antioxidant defences present in the body, such as glutathione S-transferase (GST), catalase and superoxide dismutase, can neutralise reactive oxygen species (ROS) produced in response to air pollutants and subsequently prevent oxidative stress [28]. There is evidence that polymorphisms in genes encoding for antioxidant enzymes, such as those from the GST family, can result in altered gene expression and antioxidant function and subsequently increase susceptibility to oxidative stress and inflammation in response to air pollutants [16, 29, 30].

According to the most recent analysis of the global burden of diseases (2015) [31], exposure to $PM_{2.5}$ is the 5th leading risk factor for mortality worldwide, accounting for approximately 4.2 million deaths (7.6% of global deaths) from various diseases (Fig. 27.1). Globally, it was estimated that $PM_{2.5}$ exposure was responsible for 27.1% of deaths from COPD, 24.7% from lower respiratory infections, 16.5% from lung cancer, 17.1% from ischaemic heart disease, and 14.2% from stroke [31]. In additional to this, in 2015 ozone-attributed mortality from lung disease was estimated at 245,000 deaths [31]. A number of studies have also suggested associations between short- and long-term air pollution exposure and mortality from other diseases such as central nervous system disorders, diabetes and adverse pregnancy outcomes, as described in the following sections. However, to date there is insufficient data to estimate deaths attributable to air pollution from these specific diseases.

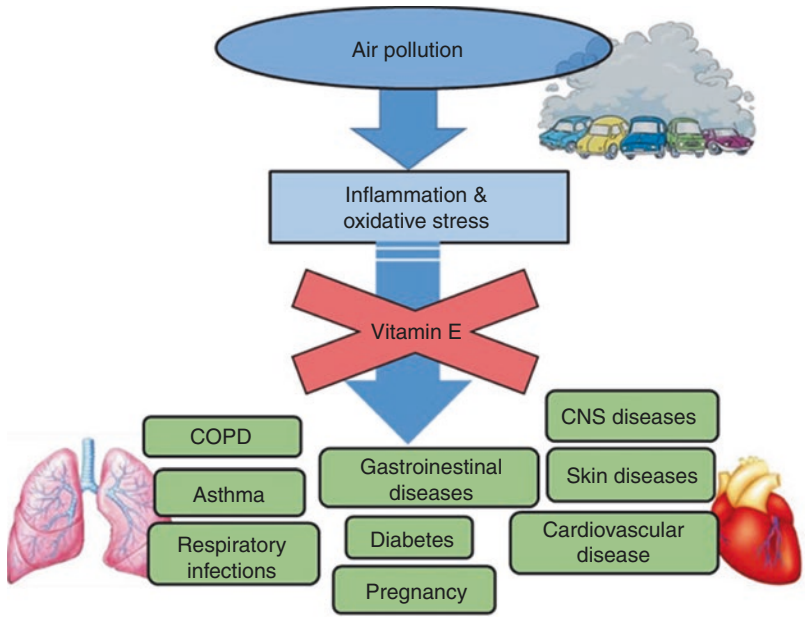

Fig. 27.1 Health consequences of air pollution exposure

Respiratory System Diseases, Air Pollution and Vitamin E

Asthma

Asthma is a major non-communicable disease, estimated to affect more than 235 million people worldwide [32]. Recently, a number of narrative and systematic reviews of epidemiological studies have demonstrated an association between air pollution exposure during early childhood and the development of asthma [33–35]. It is suggested that air pollution may contribute to the development of asthma by altering lung structure and size, and subsequently lung function and volume [36, 37], as well as through altering the developing immune system [38, 39]. Furthermore, it is widely accepted that short-term exposure to air pollutants (including $PM_{2.5}$ and PM_{10}) can worsen asthma symptoms in those with existing asthma and increase the incidence of asthma-related hospitalisation [38, 40], with long-term exposure associated with poorly controlled asthma and decreased lung function in both adults and children [36, 40, 41].

Two key features of asthma that are exacerbated by air pollution are airway hyperresponsiveness [40, 42] and airway inflammation [39, 40]. This likely relates to increased reactive oxygen species production and the stimulation of the innate and adaptive immune responses [43]. Once in contact with the respiratory epithelium, inhaled air pollutants such as PM stimulate the generation of reactive oxygen species (ROS) by inflammatory cells [16, 44], leading to oxidative stress in the airways [45], activation of nuclear factor kappa B (Nf-κB) and synthesis of inflammatory mediators (e.g. cytokines and chemokines) [16]. This results in airway inflammation and subsequent tissue damage, airway remodelling and enhanced sensitisation to inhaled allergens [40]. In addition to inducing the production of ROS, there is evidence that air pollutants such as PM directly stimulate the release of inflammatory cytokines (e.g. IL-6, IL-8 and TNF-α) from phagocytic immune cells (e.g. neutrophils and macrophages) as well as epithelial cells [46]. Furthermore, it has been demonstrated in animal studies that PM exposure is associated with T cell subset imbalance in the airways [47], which is associated with lung injury.

Individuals with asthma have been reported to have low levels of antioxidants including vitamin E and increased levels of oxidised glutathione in their lung lining fluid [48, 49], suggesting that these individuals are more susceptible to oxidative stress. Furthermore, vitamin E status appears to be important in both individuals with and without asthma, with evidence that serum antioxidant levels are positively associated with lung function measures such as FEV_1 [50]. Lower dietary vitamin E intake has also been associated with reduced lung function and increased asthma severity [51]. This highlights the potential for vitamin E supplementation to prevent and/or treat asthma, in particular air pollution-induced exacerbations of asthma, through mechanisms such as decreasing airway inflammation, oxidative stress and Th2 immune responses [52].

A number of studies have examined the effectiveness of vitamin E supplementation against air pollution-induced reductions in lung function and expression of asthma, using both observational and experimental controlled exposure studies [53]. For example, in one randomised controlled trial (RCT), adult male street workers exposed to high levels of ozone were given either an antioxidant supplement (75 mg vitamin E, 650 mg vitamin C and 15 mg beta carotene) or placebo daily for 6 months while average ambient air pollutant concentrations were recorded [54]. Antioxidant supplementation attenuated air pollution-associated decline in lung function measures (FEV_1, FEV and FEF). Similarly, in a study conducted in children with asthma, antioxidant supplementation (50 mg vitamin E and 250 mg vitamin C per day for 12 weeks) attenuated air pollution-induced reductions in lung function [55].

Experimental studies using controlled exposure to ozone have also demonstrated the protective effects of vitamin E supplementation in those with [56] and without asthma [57]. In a RCT examining the effect of dietary antioxidants on ozone-induced bronchial hyperresponsiveness, asthmatic adults supplemented with 800 IU vitamin E and 1000 mg vitamin C per day for 2 weeks responded less severely to a post-ozone exposure sulphur dioxide challenge compared to those given the placebo [56]. Similarly, in a RCT in healthy adults supplemented with 100 IU vitamin E, 250 mg vitamin C and 26 mg carotenoids per day for 2 weeks, ozone-induced bronchial hyperresponsiveness was reduced [56]. Overall, there is increasing evidence that supplementing with a vitamin E and vitamin C combination is protective against air pollution-induced lung function decline and airway hyperresponsiveness, in people with and without asthma.

COPD

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of mortality worldwide, and by the year 2030, it is predicted to become the third leading cause [58]. In 2015 it was estimated that 3.17 million deaths were attributed to COPD, accounting for approximately 5% of all deaths globally [58]. While COPD is most commonly associated with cigarette smoking, there is increasing epidemiological evidence from both developed and developing countries that air pollution exposure is associated with COPD incidence, acute COPD exacerbations resulting in hospitalisation, morbidity from COPD (such as decreased lung function and increased respiratory symptoms) and mortality from COPD [59–68]. Furthermore it has been demonstrated that the risk of COPD exacerbation, hospitalisation and mortality significantly increases with every 10 $\mu g/m^3$ increase in PM_{10} concentration [60, 69].

Oxidative stress is recognised as a major predisposing factor in the development of COPD, with antioxidant capacity in COPD shown to be decreased [70]. Inhaled air pollutants such as PM that are not effectively cleared from the airways are suggested to induce oxidative stress by promoting the synthesis of ROS in alveolar macrophages, granulocytes and respiratory epithelial cells [60]. These ROS can induce tissue and cellular damage, cell necrosis and apoptosis, increased tissue permeability and airway inflammation [70]. Airway inflammation is also suggested to be induced by PM directly [67, 71], as following phagocytosis of inhaled PM, inflammatory cells (e.g. alveolar macrophages) and

respiratory epithelial cells produce pro-inflammatory cytokines and chemokines [71]. Additional pro-inflammatory mediators are released from respiratory epithelial cells in response to macrophage-derived cytokines, which contribute to the overall inflammatory response [67]. Chronic inflammation in COPD causes progressive and irreversible airflow obstruction due to narrowing of the small airways, structural modifications and destruction of the lung parenchyma. As such, it is suggested that inflammation is central to the association between long-term exposure to air pollution and COPD [67].

Studies have shown that individuals with COPD have lower plasma [72] and peripheral skeletal muscle [73] α-tocopherol (vitamin E) concentrations compared to their non-COPD counterparts, with higher serum α-tocopherol concentrations associated with better lung function (FEV_1) [74] and lower risk of mortality from respiratory diseases [75]. Additionally, air pollutants, in particular cigarette smoke, have also been shown to further deplete endogenous vitamin E levels in COPD [76].

Vitamin E supplementation has been examined in COPD, with conflicting results reported. While some studies of relatively short duration (8 weeks–3 years) have showed no beneficial clinical effect [72, 77, 78], vitamin E supplementation has been shown to reduce biomarkers of oxidative stress [72] and improve lung function (FEV_1 and FVC) [79], with higher dietary vitamin E intake associated with reduced risk of COPD [80]. Furthermore, a large 10-year randomised controlled trial reported that vitamin E supplementation (600 IU every second day) led to a 10% reduction in the risk of COPD in females >45 years [81]. While further research is needed to specifically examine the effect of vitamin E on PM-induced damage in COPD, these studies suggest that vitamin E may be protective against the development and/or exacerbation of COPD due to air pollution exposure.

Respiratory Infections

Respiratory tract infections, in particular lower respiratory infections (such as bronchitis and pneumonia), are the most deadly communicable diseases worldwide and the third leading cause of overall mortality accounting for 3.2 million deaths in 2015 alone [32]. While infections of the upper respiratory tract (e.g. the common cold, tonsillitis, rhinitis, laryngitis, sinusitis, pharyngitis and certain types of influenza) are not considered severe health outcomes, they are the most common respiratory infections worldwide, causing significant morbidity. There is increasing epidemiological evidence that exposure to air pollutants, in particular PM, is associated with increased exacerbation of both upper and lower respiratory tract infections as well as associated hospitalisations and emergency department visits [82–92]. For example, 3 days following a 10 $\mu g/m^2$ increase in PM_{10} levels, one study reported a significant increase in medical visits for upper and lower respiratory diseases of 1.5% (CI 0.3, 2.7) [93]. Furthermore, animal and in vitro studies have demonstrated that air pollution exposure can increase susceptibility to and enhance the severity of viral respiratory infection [94–96].

The mechanisms driving the development of respiratory symptoms following exposure to air pollution are not completely understood. However, as previously mentioned in this chapter, air pollutants such as PM induce the production of ROS and subsequently oxidative stress. This oxidative stress can have damaging effects on the respiratory system, subsequently increasing the susceptibility of the lung tissue to viral and bacterial infection [92]. Air pollution-induced oxidative stress is also proposed to exacerbate inflammation during lower respiratory tract viral infections, enhancing the severity [97]. Modulation of antiviral defences is likely involved [92]. Studies have shown that exposure to air pollutants can impair the ability of alveolar macrophages to inactivate or phagocytose viruses, thus increasing susceptibility to viral infections [92, 98]. Furthermore, PM has been shown to stimulate ROS synthesis in alveolar macrophages [99], as well as inhibit alveolar macrophage production of the

antiviral agent interferon (IFN) which increases susceptibility to bacterial and viral respiratory infections [94]. Air pollutants have also been suggested to disrupt epithelial tight junctions, thus increasing respiratory epithelium permeability to viruses [92].

Several studies have examined the effectiveness of vitamin E against air pollution-induced respiratory infections. In a randomised controlled trial conducted in workers in Jakarta exposed to poor-quality air, daily multivitamin supplementation (containing antioxidants including vitamin E and C) over 3 months was reported to significantly reduce the frequency of acute respiratory tract infection symptoms compared to placebo [100]. In another study, 4-year vitamin E supplementation (50 mg/day, ~ 75 IU) was found to reduce the incidence of the common cold among smoking individuals >65 years (5–14 cigarettes per day) living in the city [101]. Furthermore, vitamin E supplementation (50 mg/day for 5–8 years) was associated with reduced incidence of pneumonia in male smokers 50–69 years of age [102]. Overall, there is increasing evidence regarding the protective effects of vitamin E supplementation against air pollution-induced respiratory infections.

Cardiovascular Diseases, Air Pollution and Vitamin E

Cardiovascular disease (CVD) is the number one cause of death globally. It was estimated that 17.7 million people died from CVD-related illnesses in 2015, which accounts for approximately 31% of all global deaths according to WHO statistics [103]. A number of epidemiological studies have found an association between exposure to air pollution and an increased risk of cardiovascular morbidity and mortality, including cardiovascular disease (CVD), ischaemic heart disease (IHD), myocardial infarction (MI) and atherosclerosis [104–109]. Studies also show that CVD risk increases in proportion to the amount of PM exposure, with a significant increase to CVD risk with every 10 μg/m^3 increase in PM [106, 107]. Increases in atherosclerotic plaques have also been associated with PM; a 10-year prospective cohort study in Germany found that exposure to high ambient air pollution ($PM_{2.5}$) was associated with an increase in coronary artery calcium (CAC) [110]. Similarly, another study also found that 2.5 years exposure to $PM_{2.5}$ resulted in an increase in mitral annular calcium (MAC) [104].

There are multiple mechanisms proposed to mediate the effects of PM on CVD risk. Firstly, inhaled fine particles can enter the bloodstream due to their ability to cross the lung-blood barrier, leading directly to endothelial dysfunction and platelet aggregation. Secondly, PM can cause inflammation and oxidative damage. This may be due to the action of fine particles in the bloodstream, which interact with circulating immune cells, causing systemic oxidative stress and inflammation [111–113]. Alternatively, exposure of the lungs to PM can cause airway oxidative stress and inflammation, and subsequent 'spillover' of inflammation into the systemic circulation [114, 115], leading to vascular dysfunction, hypercoagulability, etc. Thirdly, inhaled particles can cause an imbalance in the autonomic nervous system, which regulates cardiac function. This can lead to decreased heart rate variability (HRV) and enhanced susceptibility to cardiac arrhythmia, which is associated with cardiac morbidity and mortality [116]. Exposure to high concentrations of $PM_{2.5}$ has been shown to cause cardiac autonomic dysfunction in the elderly, which was worst in individuals with pre-existing hypertension [117].

Outside the context of air pollution, the ability of vitamin E supplementation to reduce CVD risk is controversial, with some studies linking vitamin E with increased mortality risk [118–120]. To our knowledge, there are no human studies that have used vitamin E supplementation to improve the cardiovascular effects of air pollution. In mice, vitamin E supplementation was shown to mediate the effects of cigarette smoke exposure (CSE), by decreasing hepatic lipid peroxidation, LDL and triglycerides as well as increasing HDL [121]. As vitamin E supplementation was able to reduce these CVD risk factors in exposed mice, further investigation in PM-exposed humans is warranted.

Metabolic Diseases, Air Pollution and Vitamin E

In recent years, metabolic diseases have gained increasing attention, due to their increased prevalence in western society. Obesity and its related comorbidities such as type 2 diabetes (T2D) have increased substantially, with worldwide obesity rates tripling since 1975 [122]. Recent epidemiological studies have shown that in children there is an association between air pollution exposure and both BMI [123, 124] and subcutaneous abdominal adipose tissue mass [124]. Associations between air pollution exposure and metabolic syndrome [125] and T2D [126, 127] have also been reported. A 10-year mean PM_{10} increase of 10 μg/m^3 lead to a significant increase in risk of developing metabolic syndrome [125], and higher exposure to PM was associated with elevated markers of T2D risk, including higher levels of HbA1c, fasting glucose lower insulin sensitivity and decreased β-cell function [124, 126, 127].

The mechanisms linking air pollution and metabolic diseases are complex, multifactorial and continuing to emerge. Chronic systemic inflammation, which occurs as a result of PM exposure, plays a key pathogenic role in the development of both insulin resistance and T2D [128]. Many studies of T2D in both animal and humans describe increased levels of inflammatory cytokines and innate immune cell activation, which are crucial to the pathogenesis of the disease [129]. In addition, air pollutant-mediated autonomic imbalance, which leads to HRV (described above), also exacerbates systemic insulin resistance, due to overactivity of the nervous system [130].

No previous studies, to our knowledge, have looked at the effects of vitamin E supplementation on T2D risk or insulin resistance in the context of PM or air pollution. However, some studies have shown that vitamin E alone, and in combination with other nutrients, can reduce both oxidative stress markers and systemic inflammation caused by PM [5, 131, 132]. This suggests a potential protective role of vitamin E against the development of metabolic diseases, which warrants further exploration.

Gastrointestinal Tract Diseases, Air Pollution and Vitamin E

The gastrointestinal tract is exposed to high concentrations of air pollutants that are inhaled and absorbed systemically, transported from the lungs via mucociliary clearance or ingested via contaminated food and water [133]. Emerging evidence suggests that acute and chronic exposure to air pollutants, in particular PM, is associated with gastrointestinal diseases such as irritable bowel syndrome (IBS), inflammatory bowel diseases (i.e. Crohn's disease (CD) and ulcerative colitis (UC)), appendicitis [134] and enteric infections in infants [133].

There are several possible mechanisms by which air pollution exerts detrimental effects on the intestines [135]. Air pollutants, such as PM, can be metabolised by gut microbes producing toxic metabolites [136] which can alter the gut microbiota composition, resulting in dysbiosis (microbial imbalance) [137, 138] and subsequent decreased production of beneficial metabolites such as short-chain fatty acids (SCFAs) [138]. Furthermore PM can alter gut epithelial tight junctions through ROS production, subsequently increasing gut permeability [138, 139]. This allows PM and microbial products to move into the lamina propria, inducing a pro-inflammatory response from dendritic cells and macrophages which can drive systemic inflammation, increase ROS production, enhance gut permeability and alter the luminal environment of the gut contributing to further dysbiosis, which is associated with increased disease risk [135].

There is evidence from both animal and human studies demonstrating that vitamin E supplementation is effective in reducing oxidative stress and inflammation in gastrointestinal diseases. For example, in rats with acetic acid (AA)-induced ulcerative colitis, vitamin E given intraperitoneally reduced inflammatory cytokine production and suppressed acetic acid-induced mucosal injury [140]. Similarly,

in humans with mild and moderately active ulcerative colitis, vitamin E enemas (800 IU/day for 12 weeks) significantly decreased disease activity, with 64% of participants achieving remission by the end of the study [141]. Furthermore, vitamin E supplementation (800 IU for 4 weeks) has been shown to significantly reduce measures of oxidative stress in individuals with CD [142]. However, to our knowledge, there have been no human studies examining the effectiveness of antioxidant supplementation against air pollution-induced gastrointestinal damage, which also represents an important area for future research.

Skin Diseases, Air Pollution and Vitamin E

Collectively, skin conditions are the 4th leading cause of disability and the 18th leading cause of health burden worldwide [143]. Epidemiological research indicates that air pollutants, in particular PM, can have diverse adverse effects on the skin such as contributing to extrinsic skin ageing (characterised by pigment spots, coarse wrinkles, solar elastosis and telangiectasia) [144] and the development and progression of skin diseases such as atopic dermatitis, acne, psoriasis, allergic reactions and skin cancer [145]. Interestingly, it is estimated that the amount of environmental pollutants absorbed by the skin is equivalent to that of respiratory uptake [146].

PM is proposed to exert detrimental effects on the skin primarily by depleting enzymatic (e.g. glutathione peroxidase and superoxide dismutase) and non-enzymatic (i.e. glutathione and vitamin E and C) antioxidant defences in the epidermis [144], inducing oxidative stress and subsequently inducing inflammation [145]. ROS generated by PM impair the ability of the skin to prevent pathogen entry and repair DNA damage [147]. Furthermore, ROS activate mitogen-activated protein kinase (MAPK) signalling pathways which contribute to the activation of transcription factors including activator protein 1 (AP-1) and NF-kB [147], synthesis of pro-inflammatory cytokines (i.e. TNFα, IL-1α, IL-6 and IL-8) and accumulation of phagocytic cells such as neutrophils [144]. Matrix metalloproteases (MMPs) which promote collagen degradation are also synthesised as a result of the activation of these transcription factors [147]. Together, the adverse effects of PM-induced ROS generation are implicated in the development and progression of inflammatory skin diseases and skin ageing [147].

There is evidence from both animal and human studies that air pollution exposure is associated with lower levels of vitamin E in the stratum corneum (outermost layer of the skin) [148] and sebum [149]. There is evidence that both topical application [150] and oral vitamin E supplementation [151–153] are associated with improvements in inflammatory skin conditions. For example, several studies have demonstrated that oral vitamin E supplementation (400–600 IU/day for 2–8 months) is associated with improvements in skin disorders such as atopic dermatitis [151–154], with atopic dermatitis risk in children inversely associated with vitamin E intake (OR = 0.33, 95% CI = 0.16–0.67) [155]. However, to our knowledge no studies to date have examined the effect of vitamin E supplementation on PM-induced skin damage, which is an important area for further investigation.

Central Nervous System Diseases, Air Pollution and Vitamin E

There is increasing evidence that both acute and chronic exposures to air pollution are associated with adverse effects on the central nervous system (CNS) including neuroinflammation [156]; ischaemic stroke [157–159]; cognitive dysfunction [160]; CNS diseases such as multiple sclerosis [161], Alzheimer's disease (AD) and Parkinson's disease (PD) [160, 162]; and psychiatric disorders which induce anxiety, aggression and anti-social behaviour [160]**.** Furthermore, prenatal exposure to air pollution is suggested to induce neuroinflammation and oxidative stress and may be a potential risk factor

for developmental delay [162–164] as well as neurodevelopmental (i.e. autism) [162, 164] and neuropsychiatric (i.e. schizophrenia) [162, 165] disorders.

Air pollution is proposed to have both direct and indirect effects on the CNS. Air pollutants, in particular PM ($PM_{2.5}$ and $PM_{0.1}$), are able to enter the CNS where the physical characteristics of the particle itself and/or the toxic compounds present on the surface of the PM (e.g. polyaromatic hydrocarbons) may stimulate the innate immune system in the brain [166]. Air pollutant-induced systemic inflammation has also been implicated in CNS damage, with inflammatory mediators suggested to penetrate the blood-brain barrier (BBB) [166]. While the mechanisms by which air pollutants induce damaging effects in the CNS are not completely understood, emerging evidence suggests that alterations in the BBB, activation of micro- and astroglia, mitochondrial dysfunction, cerebral vascular damage, increased ROS production, neuroinflammation and subsequent loss of neural tissue are involved [166].

Due to its ability to reduce oxidative damage, vitamin E is suggested to have protective effects against CNS diseases. In rats exposed to ozone, vitamin E supplementation has been shown to inhibit oxidative stress-induced memory deterioration and lipid peroxidation [167]. In humans, epidemiological studies have found vitamin E consumption to be protective against PD [168, 169]. Similarly, the use of vitamin E and C supplements in combination [170] and high dietary intakes of vitamin E [171] have been shown to be associated with lower risk of AD. However, to our knowledge there are no human studies examining the effectiveness of vitamin E supplementation against air pollution-induced CNS damage specifically, and evidence regarding the protective effects of vitamin E against CNS disease in humans is limited and conflicting [172–176]. More research is needed in this area.

Pregnancy, Air Pollution and Vitamin E

Foetuses are considered to be particularly susceptible to toxicants such as air pollution due to their physiological immaturity [177]. Furthermore, timing of air pollution exposure in addition to the dose absorption rate has been proposed to be more detrimental than overall dose, with the developing organ systems more vulnerable to toxicants during certain periods [177, 178]. There is a growing body of epidemiological research showing an association between prenatal air pollution exposure and adverse pregnancy outcomes such as low birth weight (LBW) [179–186], intrauterine growth restriction (IUGR) [177, 185, 187, 188], preterm birth [180, 187, 189, 190], congenital malformations (such as cardiovascular defects, neural tube defects and genital organ anomalies) [180, 187, 191–195] and stillbirth/neonatal mortality [177, 184, 187]. Exposure to air pollutants prenatally is also proposed to be associated with long-term effects such as increased risk of hypertension later in life [20]. In addition, it has recently been suggested that maternal comorbidities have a modifying effect on the association between air pollution and adverse pregnancy outcomes; women with pre-existing comorbidities such as diabetes, preeclampsia and asthma are at greater risk of preterm birth [196]. Air pollution has also been implicated in infertility, with PM exposure associated with DNA fragmentation, reduced sperm performance and abnormal sperm morphology in men, as well as reduced general fertility in females [187].

The mechanisms by which air pollutants are involved in adverse pregnancy outcomes are yet to be completely understood. However, Kannan et al. propose five possible biologic mechanisms whereby air pollutants, in particular PM, may influence pregnancy outcomes [197]. As previously mentioned in this chapter, PM increases systemic oxidative stress. Oxidative stress-induced DNA damage may disrupt DNA transcription [197] resulting in increased placental DNA adducts [198] and subsequently low birth weight [199] and IUGR [200]. It is also proposed that inhaled PM can induce acute placental inflammation which may impair transplacental nutrient and oxygen exchange, resulting in foetal growth restriction and impaired development [197]. This is supported by evidence that PM exposure during pregnancy is associated with increased foetal C-reactive protein (CRP) levels [201], with elevated CRP levels shown to be associated with adverse outcomes such as preterm delivery, preeclamp-

sia and foetal growth restriction [202, 203]. Increasing blood coagulability and viscosity, as well as altering epithelial functions (i.e. increasing vasoconstriction and endothelin levels and triggering endothelial dysfunction), are also mechanisms by which PM is suggested to be associated with adverse pregnancy outcomes [197]. Furthermore, it has been proposed that PM exposure is associated with increased blood pressure (BP). Elevations in BP during pregnancy resulting in hypertension could increase the risk of adverse outcomes such as LBW, preterm birth and IUGR [197].

It has been demonstrated in animals that vitamin E supplementation can have protective effects against environmental pollution toxicity during pregnancy [204, 205]. One study conducted in pregnant mice exposed to cadmium (a major environmental pollutant) showed that vitamin E supplementation prevented foetal resorption and foetal malformations and improved growth and bone formation [204]. Similarly, in pregnant rats, vitamin E has also been shown to reduce the risk of oxidative stress-induced intrauterine growth retardation and foetal mortality [205]. To our knowledge, there are no studies to date that have examined the use of vitamin E against air pollution-induced adverse pregnancy outcomes in humans. Furthermore, a meta-analysis of 21 trials involving 22,129 pregnant women reported that vitamin E in combination with other supplements does not reduce the risk of pregnancy outcomes such as preterm birth, low birthweight, stillbirth, intrauterine growth restriction, foetal mortality or pre-eclampsia [206]. In addition, the study reported an association between vitamin E supplementation and increased abdominal pain and preterm labour rupture of membranes [206]. This highlights that there is a need for further research examining not only the benefits of vitamin E supplementation as a modulator of air pollution-induced adverse pregnancy outcome but during pregnancy in general.

Summary and Conclusion

Over 80% of the global population is exposed to unacceptably high levels of air pollution. This presents a major environmental risk to human health, with children, the elderly and those with pre-existing co-morbid disease being among the most vulnerable. Exposure to pollutants increases the risk of many common diseases, including asthma, COPD, cardiovascular, metabolic, gastrointestinal, skin and CNS diseases. While the specific mechanisms leading to the development and progression of each of these diseases vary, all have a common underlying pathology of inflammation and oxidative stress. This highlights the potential protective role of antioxidative and anti-inflammatory interventions. Nutritional strategies for protecting against air pollution hold great promise. Being simple, inexpensive and having minimal side effects, they are likely to be widely accepted and adopted. Most human trials to date have used a combination of vitamin E and vitamin C, which has been shown to improve antioxidant status and reduce air pollution-induced oxidative stress and inflammation. Clinical trials also provide evidence of a role for combined vitamin E and C in ameliorating the effects of air pollution in asthma and reducing the risk of respiratory infections. Further work is needed to assess the efficacy of vitamin E supplementation in protecting against the many other health consequences of air pollution exposure.

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Chapter 28
The Role of Vitamin E in Pregnancy

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Keywords Pregnancy · Vitamin E · α-Tocopherol · γ-Tocopherol · Preeclampsia · Pregnancy outcomes · Oxidative stress · Fertility · Prematurity · Intrauterine growth restriction · Preterm delivery

Key Points

- Vitamin E plays an important role in fertility, implantation, and neural development early in embryo development.
- Oxidative stress is associated with untoward maternal and neonatal outcomes of pregnancy.
- The pharmacokinetics and placental transport of dietary and supplemental vitamin E during pregnancy and their relationship to fetal status and function are not well characterized.
- Randomized clinical trials of vitamin E supplementation on pregnancy and neonatal outcomes provide equivocal results and suggest the need for more carefully designed protocols.

Introduction

Vitamin E was first reported to have an impact on fetal development when Evans and Bishop noted that rats fed a diet deficient in an unnamed dietary substance (factor X) had reduced reproduction success, specifically, reabsorption of the embryo was noted by day 9 [16]. In the 1930s, the factor was subsequently named vitamin E, and Olcott and Mattill begin to describe a potent antioxidant function for this micronutrient [46]. Since that time, the important antioxidant functions of vitamin E have been shown to have significant impacts and/or associations in pregnancy outcomes. In 1982, Freeman

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_28

and Crapo [19] described the nonenzymatic antioxidant properties of vitamin E in what was termed a quenching reaction, reducing lipophilic free radical species, thereby decreasing toxicity and resulting in a beneficial decrease in tissue injury. Vitamin C and β-carotene were consistently noted to have synergistic mechanisms of action helping to recycle α-tocopherol back to its more potent antioxidant form [19]. Many complications of pregnancy and the neonatal period (including but not limited to preeclampsia, infection, chronic lung disease, necrotizing enterocolitis, and intraventricular hemorrhage) are augmented by inflammation making the task of defining optimal vitamin E status and antioxidant capacity during pregnancy a worthwhile endeavor.

Shortly after its discovery, vitamin E was found to include a group of up to eight individual compounds, α-, β-, γ-, and δ- tocopherols and tocotrienols. The origin of the nomenclature tocopherol came from the Greek roots *toco-* or offspring and *phero,* bear, as suggested to Evans by George M Calhoun, a Professor of Greek at the University of California [15, 17]. This name recognized the early link of vitamin E to fertility and reproduction, though almost 100 years later, considerable scientific work remains necessary to fully understand the role of vitamin E in fertility and pregnancy and its ability to optimize maternal and neonatal outcomes.

Vitamin E in Pregnancy

Oxidative stress has been associated with low birth weight, preterm delivery, and preeclampsia, all clinically important adverse maternal or neonatal outcomes of pregnancy [8, 57, 58]. Vitamin E, specifically RRR-α-tocopherol, has potent antioxidant effects, which may mitigate these risks if intake is optimal during pregnancy. A more complete understanding of vitamin E status of the pregnant woman and its impact on the developing fetus as well as associations with important pregnancy outcomes such as length of gestation, mode of delivery, and associations with preeclampsia, pregnancy-induced hypertension, and gestational diabetes among others is critical to improving pregnancy outcomes. At best, the current literature provides incomplete and conflicting data on baseline status, placental regulation of transfer of vitamin E compounds to the fetus, and the impact of diet and supplementation on both levels and outcomes. This chapter will discuss in detail the current available body of literature, its strengths and gaps in study design, and current knowledge with a recommendation for future design considerations for clinical trials in this area.

Vitamin E concentrations in plasma rise throughout gestation during an uncomplicated pregnancy with levels rising 40–60% in the second to third trimester; this increase may be attributed to an increase in maternal lipoprotein status during pregnancy. Vitamin E is a fat-soluble molecule transported in circulating lipoproteins [26, 45, 62, 64, 65]. Low-density lipoprotein (LDL) is the major carrier of vitamin E during pregnancy with a higher proportion of vitamin E than high-density lipoprotein (HDL), very low-density lipoprotein (VLDL), or chylomicrons [1].

An increase in vitamin E during pregnancy provides a balance in the ability to counter the increased burden of oxidative stress associated with pregnancy, potentially decreasing oxidative injury to or impact on the mother/fetal dyad. It has been suggested that maternal antioxidant capacity may be reduced during pregnancy in light of decreased bioavailability α-tocopherol if a large proportion is bound to lipoproteins and therefore less biologically available [10].

Vitamin E and Placental Transport

Fetal or cord blood measurements of vitamin E are typically reported as 20–30% of maternal levels [39]. This is consistent with data demonstrating progressively increasing cord levels with increasing gestational age, supporting the theory that vitamin E transfer across the placenta is highly regulated

as cord levels of vitamin E rise with advancing gestation in proportion to increasing maternal levels over the same time period. Using isolated placental cotyledons in vitro, Schenker et al. demonstrated slow transport of vitamin E at only 10% of the rate of L-glucose. Additionally, he noted that free forms of the RRR form of vitamin E had the most efficient transport [55]. A study in 15 pregnant women using deuterium-labeled RRR and all-*rac*-α-tocopherol acetate given in a range of doses 5 days prior to delivery found more efficient transport into cord blood of the d3RRR form than the d3all-*rac* form regardless of maternal dose ($p < 0.5$) [1].

Recent information may shift this paradigm, as Capper et al. have shown in a lamb model that, although fetal blood levels remain steady with increasing maternal vitamin E administration, fetal tissue vitamin E content increases with increased supplementation. Fetal growth was significantly increased in the vitamin E supplemented ewes [7]. This concept is further be supported by evidence from Didenco et al. who report higher concentrations of the α-tocopherol metabolite 2,5,7,8-tetramethyl-2- (â-carboxyethyl)-6-hydroxychroman 2 (α-CEHC) in the cord blood of infants whose mothers had higher vitamin E intake during pregnancy as measured by 24-h diet recall [13].

Pressman et al. supplemented mothers with vitamin E and measured vitamins E and C in maternal and cord blood as well as chorioamnion and amniotic fluid and found varying correlations between these parameters in a manner that suggests difficulties with relying on vitamin blood levels to infer tissue or system antioxidant capabilities [48]. Nonetheless, data such as these are valuable in highlighting that antioxidant capabilities happen at the cellular level in the tissues, and, although blood is the most available matrix for sampling, it does not reflect the complete status of the antioxidant defense system.

Vitamin E and Preeclampsia

Preeclampsia comprises a significant component of pathology of pregnancy contributing to 10–15% of maternal mortality associated with pregnancy. Approximately 2–10% of pregnancies worldwide and 3.4% of US pregnancies are impacted by this condition, defined as hypertension during pregnancy, typically developing after the 20th week of gestation. Incidence of this condition has risen by 25% in the United States, and there is evidence that this may be associated with rising rates of obesity in pregnant women leading to an increased inflammatory state and significantly more oxidative stress during the pregnancy [4, 30]. As a potent antioxidant, maternal vitamin E status and its impact on oxidative stress load may play a role in the development of this condition [26, 50]. A range of maternal α-tocopherol levels have been reported in preeclampsia; similarly, severe preeclampsia, which carries the most risk to fetus and mother, has been associated with a wide range of α-tocopherol levels, including high [36, 66, 67], moderate [25, 45, 66], and low [42, 47, 54].

In order to evaluate impact of antioxidant supplementation on prevention preeclampsia in higher-risk populations, Chappell et al. supplemented 238 women with 400 IU/day of vitamin E and 100 mg/day of vitamin C as compared with placebo from the 16th to the 22nd week of gestation. Supplementation was determined to be safe for the fetus, with no untoward impact. There was a significant reduction in preeclampsia at 8% in the vitamin group vs. 17% in the placebo group. Interestingly, there was additional biochemical evidence that antioxidant supplementation was effective in decreasing the ratio of plasminogen activator inhibitor (PAI) 1 to 2 by 21%, a biomarker that typically rises in preeclampsia [8].

Another link of vitamin E to preeclampsia involves evaluating an oxidative metabolite as a measure of oxidative stress or load. Scholl and Stein found pregnant women with a poor pregnancy outcome as defined by intrauterine growth restriction (IUGR) or prematurity have increased oxidative damage to their DNA early in the third trimester of pregnancy as measured by urinary 8-oxo-7,8 dihydro-2-deoxyguanosine [58].

In a larger cohort of 307 pregnant women, Scholl et al. evaluated by urinary isoprostanes, 8-iso-prostaglandin $F_{2\alpha}$, a biomarker of oxidative stress, at entry to prenatal care (15 weeks estimated gestational age ± 0.49 weeks), preeclampsia risk increased fivefold with increased urinary isoprostane excretion and was decreased threefold in women who had increased antioxidant capacity as measured in early pregnancy urine sampling. This study also measured antioxidant intake in diet by 24-h diet recall and found no association between antioxidant intake and antioxidant capacity as measured in sequential urine sampling throughout pregnancy; however, it is worthwhile noting that intake of vitamin E as measured in α-tocopherol equivalents (ATE) ranged from 6.6 to 7.5 ± 0.4/day across tertiles of isoprostane, consumption well below the daily recommended intake for pregnancy. These relatively low maternal daily dietary intakes complicate any assessment of the impact dietary of antioxidant intake on pregnancy outcomes in this cohort [57]. Nevertheless, oxidative stress compounded by low vitamin E or ATE intake may be associated with increased risk of pregnancy outcomes such as low birth weight, preterm delivery, and preeclampsia. A mother's antioxidant status has the potential to protect the maternal-fetal unit, thus increasing intrauterine growth and infant weight at birth and reducing the risk of development of preeclampsia.

Several mechanisms may mediate the impact of vitamin E on the development of preeclampsia, including the increased antioxidant capacity seen with higher status and subsequent decreases in lipid peroxidation and decreased inflammation as well as modulation of the α-tocopherol transport protein and its impact on placental implantation.

Vitamin E and Fetal Development

The role of lipid peroxidation in suboptimal pregnancy outcomes may be modulated by vascular endothelial cell dysfunction [27]. Vitamin E is essential in the development of the vascular connections required by the placenta developed at the site of placental implantation and subsequent embryogenesis. Miller et al. have published a series of studies describing the role of both α-tocopherol and α-tocopherol transfer protein (αTTP) in embryogenesis using a zebra fish model. Alpha-tocopherol-deficient zebra fish present with developmental delay and increased mortality days after fertilization despite having viable eggs [43]. αTTP in zebrafish just after fertilization shows an increase in mRNA transcription in the first 24 h. Early brain and axis development appears to be impacted as these transcripts are localized to these areas [44]. Although expressed in the adult liver, the presence of αTTP has been documented in the human yolk sac, as well as in the placenta and in the uterus at the site of implantation; therefore, αTTP is likely to be important in delivering α-tocopherol to the embryo at critical points in development [29, 31].

The transport of vitamin E by αTTP at the cellular level moves α-tocopherol from endocytic organelles to other cellular locations [49]. αTTP is also critical for the normal development of placental labyrinthine trophoblasts in mice [29, 31–33]. The elucidation of instrumental roles for αTTPs in the development of normal placentation highlights the potential impact of vitamin E in consideration of causes of preeclampsia, as abnormal placentation has been strongly implicated in the development of this serious condition of pregnancy.

Vitamin E and Recurrent Pregnancy Loss

Simsek et al. postulated that an imbalance in lipid peroxidation and antioxidant capacity in early pregnancy may impact early loss in 40 women with habitual abortion (mean pregnancy loss of 2.3) as compared to 40 matched controls. Plasma α-tocopherol was significantly lower in women with

habitual abortion as compared to controls ($p < 0.05$), in addition to other antioxidants including vitamin A and β-carotene [60]. In clinical situations, one antioxidant alone may be unlikely to substantially drive significant outcomes such as changes in habitual abortion as numerous oxidative and inflammatory pathways are involved, but it is important to recognize the goal of balancing antioxidant defenses and prooxidant insults to promote healthy pregnancy and birth outcomes.

Vitamin E and Male Infertility

Male infertility is noted to impact 50% of the infertility experienced by up to 15% of couples attempting pregnancy over a 12-month period [2]. In a systematic review of the effect of oral antioxidants (vitamins C and E, zinc, selenium, and carnitine) on male infertility, 17 randomized trials, including a total of 1665 men, were identified. Of these trials, 14 (82%) showed an improvement in either sperm quality or pregnancy rate after antioxidant therapy [52]. The levels of 8-hydroxy-2′-deoxyguanosine (a measure of oxidatively damaged DNA) in sperm DNA were significantly higher in infertile male than in control patients. Antioxidant treatment including vitamin E supplementation demonstrated a positive effect on the sperm concentration in ejaculate ($p < 0.05$). Supplementation with the antioxidant vitamins E (200 mg) and C (200 mg) as well as glutathione (400 mg) led to decreased levels in spermatozoa of 8-hydroxy-2′-deoxyguanosine (1.5 ± 0.2 to 1.1 ± 0.1 per 10^5 deoxyguanosine, $p < 0.05$). The ultimate clinical impact on male fertility with antioxidant supplementation remains to be determined [34].

Vitamin E and Pregnancy/Neonatal Outcomes

Growth In a prospective cohort study of 239 healthy pregnant women, heaviest birth weight and greatest birth length were associated with the largest intake of vitamins E and C. There was no significant association in this cohort between serum vitamin E and birth weight and length; however, maternal serum levels were evaluated at mid-gestation, and it is well accepted that maternal serum vitamin E levels rise and peak (increasing by approximately 60% from prepregnancy levels) shortly before healthy term delivery [35].

Many studies have documented increasing serum concentrations of α-tocopherol with advancing gestation, and higher circulating concentrations of α-tocopherol are positively associated with several indicators of fetal growth including birth weight and length. In addition to α-tocopherol associations with increased birth weight for gestation, relationships with an increased proportion of infants with birth weight ≥ 90th percentile (large for gestational age) and decreased proportion of infants with birth weight ≤ 10th percentile (small for gestational age) have been described. It is plausible that circulating concentrations of α-tocopherol could be associated with increase in fetal growth by greater blood flow and nutrient supply to the fetus due to decreased inflammation, improved placental implantation, or other yet to be described mechanisms. It is also plausible that α-tocopherol could be a marker for other maternal factors associated with general good health that are more directly involved in the causal pathway impacting fetal growth.

Preterm Delivery There are limited data on baseline status of maternal and cord blood vitamin E levels and their association with prematurity. α-Tocopherol was demonstrated to be lower in mothers delivering very preterm as compared to mothers delivering preterm >32 weeks. No difference was noted in colostrum levels, and there was no relation between maternal serum α-tocopherol levels and colostrum levels in the first 12 h after delivery [14, 40].

Older data from 1987, evaluating vitamin E in cord blood of preterm and growth restricted infants as compared to term appropriately grown infants, found significantly lower maternal and cord vitamin E levels in the preterm and small infants [59]. The limited data available provides the basis for further evaluation of this potential association.

Fetal Pulmonary Development Vitamin E has been shown to impact expression of cyclin D, cyclin E1, P27, P53, and Bcl2-L, genes involved in both regulation of the cell cycle and cell signaling. Close evaluation of these data indicates that vitamin E status in early gestation may impact airway development [5]. Maternal serum α-tocopherol in the first trimester has been associated with crown rump length in the first trimester ($p = 0.002$), the earliest time point in gestation that α-tocopherol has been associated with growth. Turner et al. followed this cohort and described 5-year respiratory follow-up associations with first trimester crown rump length status with the respiratory outcomes of improved lung function as measured by forced expiratory volume and forced vital capacity and asthma diagnosis [63].

When evaluating vitamin E and fetal pulmonary outcomes, Devereux [12] proposes that vitamin E may impact pulmonary development via modulation of T cell development, with naive T cells being more responsive to vitamin E than mature T cells. Some clinical studies have associated improved pulmonary outcomes in childhood with more optimal maternal vitamin E status [12, 37]. These studies suggest that if maternal intake were increased to 15 mg/day in mothers, then asthma diagnosis would be halved at age 5 based on data from this Scottish cohort. Devereux suggests this change might best be accomplished by dietary modification as opposed to supplementation as a modest increase of 5 mg/day would likely bring significant numbers of pregnant women into an ideal vitamin E status [12].

Allergy/Immunology Maternal vitamin E intake or status may impact additional cell lines and clinical outcomes in addition to inflammation and growth. Mothers with a family history of atopy were evaluated for vitamin E intake during pregnancy, and those with the highest vitamin E intake were found to have offspring with a decreased allergic response as measured by cord blood mononuclear cell proliferative responses to allergens. This study provides some evidence that maternal intake of vitamin E during pregnancy may modulate the development of the fetal immune system. These results warrant further investigation to elucidate the exact mechanisms of action leading to impacts on a variety of biological systems [11].

Neurodevelopmental Outcomes In a Chinese cohort of 150 mother-infant pairs, maternal and cord levels of vitamin E were associated with developmental outcomes at 2 years as measured by Gesell Developmental Schedules. Specifically, cord vitamin E levels in a multiple linear regression model positively correlated with motor and average developmental quotients ($p < 0.05$). Placental transfer rates of vitamin E were associated with a statistically significant protection against motor, language, personal, and social behavior delays at 2 years [9]. These preliminary findings provide a foundation for more extensive studies following associations with antioxidant status, in particular vitamin E in pregnancy and neurodevelopmental outcomes.

Birth Defects One study using food frequency questionnaire (FFQ) recalls data collected 25 months after conception associated all types of congenital heart disease (CHD) with maternal diets that were higher in total vitamin E intake from diet and supplements. Although the FFQ has shown to be valid over this time frame, the risk margin of error due to recall bias increases with extended time frames, and this relationship is confounded by life-changing events such as pregnancy and parenting an ill child in the interim [24]. Additionally, there was no analysis of impact on CHD between α- and γ-tocopherol intake [61]. Subsequently, Gilboa et al. refuted this finding with a study evaluating the association of vitamin E with major birth defects in 4525 infants with a birth defect and 8665 control infants showing no apparent impact of periconception maternal vitamin E intake on fetal birth defects including CHD [22].

Confounding Risk Factors Maternal smoking plays a significant impact on fetal oxidative stress exposure. Fayol et al. evaluated maternal smoke exposure (none, environmental, and smoker) and its impact on maternal vitamin E status as well as cord blood levels. Increased smoke exposure was associated with decreased maternal serum vitamin E ($p = 0.03$), but no impact on cord blood levels [18]. These results are consistent with Capper et al. using a sheep/lamb dyad, which demonstrated increasing tissue incorporation of α-tocopherol with improved maternal serum α-tocopherol status despite minimal impact on fetal serum levels [7].

An additional example of a confounding condition is maternal obesity. In the evaluation by Scholl et al. of tocopherol levels and fetal growth outcomes, obese women (BMI > 29) had lower α-tocopherol ($p < 0.01$) and higher γ-tocopherol ($p < 0.0001$) status. γ-Tocopherol was not associated with growth in this cohort [56].

Cochrane Review of Vitamin E Supplementation in Pregnancy

The Cochrane review by Rumbold et al. [53] reviewed the available literature for safety and impact on pregnancy outcomes (maternal and fetal) of vitamin E supplementation with or without vitamin C and/or other vitamin supplements given at any time during pregnancy. The Cochrane Pregnancy and Childbirth Groups Trials Register of March 2015 included 21 trials involving 22,129 women identified as well as data from 17 trials with varying degrees of bias from low risk (10 trials), unclear risk (6 trials), and high risk (5 trials). The review included studies from sites around the world where dietary intake could be presumed to be either inadequate or adequate in vitamin E and other micronutrients. A study was included if it compared vitamin E supplementation (with or without another vitamin or mineral) to placebo, no placebo, or intake of other supplements.

Rumbold et al. [53] evaluated vitamin E supplementation during pregnancy regarding primary outcomes of stillbirth, preterm delivery (prior to 37 weeks gestation), clinical preeclampsia, intrauterine growth restriction (IUGR), prelabor rupture of membranes, and placental abruption. Additional analyses were performed examining differences in 31 other clinical domains by any vitamin E supplementation, with an added sensitivity analysis by trial quality, as a subgroup analysis based on gestation at trial entry (greater than/equal to or less than 20 weeks), as a subgroup by dietary intake with low intake defined as "intake less than the recommended dietary intake in that setting as measured by dietary questionnaire," and as a subgroup analysis based in risk of adverse pregnancy outcome at trial entry.

This Cochrane review was initiated in part due to results from earlier clinical trials and observational studies in adults of vitamin E supplementation that suggested null or adverse outcomes in different studies [6, 51]. Rumbold et al. [53] suggest that the tolerable upper limit of vitamin E at 1000 mg/day [28] might be inappropriately high for a pregnant woman and her fetus. This review found "no convincing evidence that vitamin E supplementation in combination with other supplements results in other important benefits or harms" but suggested further research is warranted to evaluate the potential impact of vitamin E supplementation in preventing placental abruption. This Cochrane review had several strengths in evaluating the evidence for supplementation with vitamin E during pregnancy, including the identification of all available studies and a complete assessment of their qualities and potential impact. Eligible studies included subjects with a global distribution such that the results might be helpful in considering recommendations for broad range of women. The included studies involved a large number of participants, 22,129 subjects before exclusion criteria for analysis began. The statistical analysis is described in detail and designed to best deal with the weaknesses of the individual studies which were selected for inclusion.

The weaknesses of this review are worth noting before considering its conclusions. The review's broad definition of vitamin E supplementation included a wide range of doses, from 100 to 800 mg/day with three studies employing an unknown dose. The form of vitamin E, i.e., RRR- vs. all-*rac*-α-tocopherol, was not considered, and no assessment was conducted of relevant biomarkers of compliance, antioxidant status, or antioxidant capacity. The inclusion of studies testing the effect of vitamin E in combination with vitamin C and/or other micronutrients also confounds conclusions about the role and actions of vitamin E alone. The comparison of vitamin E supplementation (alone or in combination with vitamin C or beta-carotene to placebo, no placebo, and/or other supplements) also confounds the ability to draw specific conclusions regarding vitamin E supplementation.

While including studies from a wide variety of countries and their cultures and dietary patterns provides some strength to the review, it presents a challenge to capturing these differences and distributing them evenly into intervention groups given their disparate study designs. This approach introduces the potential for inclusion of an unknown number of subjects with socioeconomic factors known to impact biology and pregnancy outcomes, including those that could impact antioxidant capacity and inflammation. Given the lack of these data, the review is unable to control for these factors by its meta-analysis design.

In the evaluation of IUGR, the 3rd percentile was selected rather than the 10th percentile as a cutoff for this diagnosis. The 10th percentile is the typical cut point for this diagnosis and would have been more effective in evaluating growth failure impacted by oxidative stress and inflammation. Infants who fall below the third percentile often have other associated birth defects or diagnoses, such as trisomy 13 or 18, not an isolated diagnosis of IUGR.

In considering the strengths and weakness of this Cochrane review, it is necessary to appreciate the inherent difficulty in designing and conducting any dietary supplement intervention in pregnant women due to their protected status and the desire to avoid any potential harm to the fetus. In contrast to animal model studies, it is impractical and unethical to design a diet with the elimination of one or more nutrients in the control group. Thus, any proposed study design must consider the impact of the baseline status of nutrients selected for the intervention as well and those other nutrients that may impact its function. Consideration of the potential for no benefit or even potential harm of supplementing a nutrient replete individual is also imperative in the study design. This also can dilute out the beneficial impact from supplementing an individual who is insufficient in a particular nutrient.

Forms of Dietary and Supplemental Vitamin E

γ-Tocopherol, found primarily in corn oil, soybean oil, and margarine, is plentiful and inexpensive. It occurs in high levels in many processed foods in a typical western diet, and intakes have increased as dietary patterns have shifted to an increase in processed foods over the last half-century. Due to this shift, γ-tocopherol is now the most common form of vitamin E in the western diet as compared to historical diets where the α-tocopherol form found in seeds, nuts, and leafy green vegetables was more plentiful [41]. Examining the 1994–1996 Continuing Survey of Food Intakes by Individuals, Maras et al. [38] found that among American women, only α-tocopherol intake in adult women 2.4% had adequate vitamin E intake from diet alone. Gao et al. [20] also evaluated US diets using the National Health and Nutrition Examination Survey data set and found significant modifications in diet would be needed to increase α-tocopherol intake to the recommended 15 mg/day.

Maternal diet has been associated with tocopherol levels during gestation; in particular, serum α-tocopherol levels at mid-gestation appear higher with maternal vitamin E intake from diet as well as use of prenatal vitamins, while γ-tocopherol levels positively correlate with increased maternal fat in the diet and negatively correlate with use of prenatal vitamins. This is not surprising considering

the high levels of γ-tocopherol in corn and soy oils frequently consumed in the modern western diet. Clinical outcomes of this same study of 1231 pregnant women showed association of α-tocopherol maternal serum levels at 16 and 28 weeks gestation with increased fetal growth as measured by birth weight [56]. Hanson et al. reported associations with γ-tocopherol in maternal and cord blood [23]. Unlike α-TTP, which is known to be active in the placenta and likely plays a significant role in moving α-tocopherol into the developing fetus, the mechanism for transplacental movement of γ-tocopherol is not yet described. This association is accompanied by correlations with cord blood γ-tocopherol and birth weight and weight percentile ($p = 0.04$ and $p = 0.05$, respectively) and maternal γ-tocopherol and birth weight ($p = 0.04$) in models adjusted for maternal smoking and gestational age [23]. Further research on the dietary and supplemental intake of both α- and γ-tocopherol and its impact on maternal and cord blood levels are warranted.

Knowing the source and molecular configuration of vitamin E supplementation is critical to appropriate interpretation of results of clinical trials of supplementation with vitamin E. α-Tocopherol is the desired tocopherol as it has in vivo antioxidant activity. A tocopherol in the RRR configuration is the naturally occurring configuration, but there are eight existing α-tocopherol molecules with differences in the position of carbon at the 2′, 4′, and 8′ positions, (RRR-, RSR-, RRS-, RSS-, SRR-, SSR-, SRS-, and SSS-α-tocopherols); only the forms with an R configuration in the second position, RRR-, RRS-, SRR-, and SRS-, should be considered for supplementation during pregnancy [41].

The Institute of Medicine (IOM) sets the Recommended Daily Allowance (RDA) for pregnancy at 15 mg/day of vitamin E, specifically regarding the 2R-stereoisomeric forms of α-tocopherol; the RDA for adult men and women is the same as that for pregnant women. The RDA for lactating women is the highest recommended by the IOM at 19 mg/day of vitamin E, with the aim of providing the infant 4–5 mg/day of vitamin E through the first year of life [28].

Supplementation should be in the RRR-α-tocopherol form, as opposed to the synthetic all-*rac*-α-tocopherol form that includes all of the isoforms of α-tocopherol, only half of which have antioxidant action, but all of which are absorbed and found in human serum, breast milk, and animal tissues after supplementation. A study evaluating pregnancy sow supplementation with natural source α-tocopherol or three times the dose of synthetic α-tocopherol noted that a lower dose of natural vitamin E supplementation maintained oxidative status in vivo and achieved similar piglet and sow serum concentrations of α-tocopherol. In muscle tissue samples from piglets on day 39, the RRR-α-tocopherol stereoisomers were higher ($P < 0.001$), while RRS-, RSS-, and RSR-α-tocopherol were lower ($P < 0.001$) in piglets exposed to the lower-dose natural form as compared to the higher dose of the synthetic form of vitamin E [3]. These results may be important in evaluating recommendations for dietary modifications in pregnancy as compared to supplement trials or recommendations even though the long-term human implications of this alteration are currently unknown.

Supplementation of lactating women with natural RRR or all-*rac*-α-tocopherol produced significantly different stereoisomers ratios of α-tocopherol in maternal milk [21]. This may be of concern given the greater antioxidant impact of RRR-α-tocopherol. The 2R-α-tocopherol available in synthetic supplements has the potential to decrease antioxidant availability in human milk and may alter the positive impact of human milk on neonatal outcomes.

Knowledge Gaps and Consideration of Future Research

Current evidence shows an association of maternal intake of vitamin E during pregnancy with fetal growth, and there is emerging evidence in animal models for incorporation of α-tocopherol into tissues of the fetus, while serum levels remain lower than maternal throughout gestation. The impact of supplementation with vitamin E during pregnancy on fetal outcomes such a growth and pregnancy outcomes is less clear.

The shifting pattern globally of dietary vitamin E intake due to greater consumption of processed foods, including those formulated with γ-tocopherol-rich soy oils, during fetal development is an area where further research is necessary. Similarly, evaluation of fetal tissue levels of γ- and α-tocopherol and their metabolites with varying diet concentrations of each is an important area identified for further research. This may best be completed in the lamb, piglet, or rat models in order to build upon our existing data of vitamin E concentrations in fetal tissue. A major gap in the current body of knowledge in maternal/fetal vitamin E includes placental transport. It is necessary to better understand the mechanisms by which each form of vitamin E transport across the placenta is regulated. Evaluation of α- to γ-tocopherol circulating ratios in maternal and cord blood, as well as their metabolites and how they impact placental transport of α-tocopherol, is also an area worthy of more attention.

New clinical research studies should include the measurement of baseline vitamin E status before supplementation is initiated, as well as carefully assessing dietary intake throughout the study. Consideration should be given to study designs involving a control group to a group that modified diet to increase α-tocopherol to recommended intakes and a third group who would receive a targeted supplement dose of RRR-α-tocopherol based on their estimated α-tocopherol dietary intake. The supplement selected should also be evaluated to ensure that the action of the included compound (natural or synthetic) is identical to the action of natural sources of vitamin E obtained through the diet.

Supplementation of mothers who already have adequate vitamin E status could have unintended consequences and potentially cause harm to the mother and fetus if levels are driven too high. Another important consideration when evaluating the design of a clinical maternal vitamin E supplementation study is determination of the maternal status and dietary intake of vitamin C and β-carotene. These levels need to be taken into account given their demonstrated impact on either increasing the availability of active α-tocopherol, potentially increasing its antioxidant impact on the developing fetus, or concomitant impact on total antioxidant capabilities.

Conclusion

Vitamin E, particularly α-tocopherol, plays an important role in fertility, implantation, and neural development early in embryo development. α-TTP has been implicated in α-tocopherol delivery to the developing fetus across the placenta and is a protein that needs further evaluation in its placental distribution and activity. Maternal serum levels of vitamin E, in particular α-tocopherol, have been associated with improved fetal growth, but serum levels in pregnancy are just one value and may be difficult to interpret based on the complex physiological changes that occur progressively throughout gestation. More complete information including fetal levels and tissue levels of both α- and γ-tocopherol in addition to evaluation of metabolites of these molecules will better inform our current understanding of their impact on pregnancy and fetal/developmental outcomes. It is also important to acknowledge that, in its antioxidant role, vitamin E acts in conjunction with vitamin C and that the impact of other antioxidants such a vitamin A, beta-carotene, and other carotenoids may confound research outcomes unless there are adequate controls in the study design.

Clinical trials of vitamin E supplementation during pregnancy are challenging to design and conduct. The results from vitamin E supplementation studies in pregnant women to date have been inconsistent, and no authoritative recommendations have been promulgated to ensure vitamin E adequacy is available. Improvement in the design of new clinical trials regarding dietary vitamin E requirements and/or the use of vitamin E supplements has the potential to lead to increased understanding of tocopherols and their impact on fertility, inflammation, preeclampsia, fetal growth, prematurity, and other important pregnancy outcomes. It is important to recognize that many of the pregnancy outcomes targeted by clinical supplementation studies have a physiologic basis in lipid peroxidation.

Even with targeted vitamin E supplementation using baseline maternal levels and accounting for desired increases of maternal serum levels of 40–60% as gestation progresses, a level of sophistication has not yet been achieved by past clinical trials. However, it is worth noting that marked limitations are associated with expectations that focusing on any single antioxidant nutrient will significantly impact clinical outcomes if the multitude of other factors affecting pregnancy are ignored. Concerted efforts to impact preconception health with reductions in obesity, smoking, and alcohol consumption while increasing dietary intake of a range of antioxidants, including but not limited to α-tocopherol, retinols, and carotenoids, are much more likely to impact public health outcomes.

Acknowledgments We would like to gratefully acknowledge the help of Ammar Kamil and Matthew VanOrmer for their help in the preparation of this chapter.

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Part V
Public Health Implications of Vitamin E

Chapter 29
Vitamin E and Healthcare Costs: Models to Assess the Impact

Mark Nuijten

Keywords Health economics · Vitamins · Nutrients · Cost-effectiveness · Market access Reimbursement · Model · Costs · Stratified medicine

Key Points

- Cost-effectiveness analyses can be applied to nutrients, if clinical evidence exists.
- Clinical evidence for vitamin E to reduce cardiovascular risk in diabetics carrying the Hp 2-2 genotype is convincing, and it is based on RCTs and meta-analyses.
- The use of vitamin E in type 2 diabetes mellitus is a cost-effective treatment because of a reduction of CVD events and subsequent lower costs and higher quality of life (QALY).
- Vitamin E offers value for money in terms of cost per QALY: the incremental cost-effectiveness ratio is close to zero, whereas the lower threshold is £ 20,000 per QALY in the UK.
- This assessment illustrates that concepts of health economics can be applied to vitamin E, and the results show that vitamin E is extremely cost-effective.

Rational

Vitamins are a specific nutrition category either covering specific dietary needs and/or nutrient deficiency of individuals and more recently have attracted more interest for the treatment of patients who are, for instance, at risk for cardiovascular diseases. Although these products have existed for a long time, the health economic evidence of vitamins tends to be limited. An important reason is that vitamins do not always fall under the coverage requirements for reimbursement, like for pharmaceuticals, which often require health economic data. Economic evaluations for pharmaceuticals and other health technologies, including devices, are common practice since 2000. Since that time, reimbursement agencies in different countries have developed evaluation guidelines, resulting in a large number of published research papers, comments, letters and editorials on economic evaluations of health technologies and policies [1]. Cost-effectiveness analysis has become a requirement in reimbursement submissions for pharmaceuticals in the market access process. The objective of this chapter is to

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_29

describe the applications of health economic theories and models to obtain a better understanding of the possible impact of this essential micronutrient on healthcare costs.

Background Vitamin E

Clinical Background

Cardiovascular disease (CVD) is the most frequent, severe and costly complication of type 2 diabetes mellitus (T2DM) [2]. It is the leading cause of death among patients with T2DM regardless of diabetes mellitus (DM) duration [3]. Myocardial infarction (MI) and stroke are the most severe manifestations of CVD. In the UK, the prevalence of diabetes is 6% in people aged 20–79 years [4]. The number of cases of diagnosed diabetes in the UK increased to 3.2 million in 2013, with an estimated 850,000 people having the disease without knowing it and as many as 7 million more at high risk of developing it [5]. By 2025, if current trends continue, an estimated 5 million people will have diabetes and T2DM accounts for about 90% of cases of diabetes [5]. It is increasingly prevalent, with the T2DM population in the UK expected to rise to 3 million by 2017 [5]. Due to increasing prevalence rates in the past decade, DM has become a major public health issue. Both macrovascular and microvascular complications are common long-term sequelae of the disease. Cardiovascular disease is the single most major cause of death among DM patients, accounting for approximately 65% of mortality in DM [6]. Overall, DM accounts for 35% of hospitalizations due to CVD. The total economic cost of T2DM amounted to £ 21.8 billion in 2010–2011 [4], including both direct and indirect costs. Despite advances in the healthcare of patients with diabetes as well as in prevention efforts for the development and treatment of diabetes complications, the life expectancy for such patients remains suboptimal compared to the general population [7, 8]. Population-based studies have shown that the relative risk of CVD in DM is severalfold higher compared with those without DM [9]. Over the past decade, evidence has been accumulating that haptoglobin (Hp) gene is an independent risk factor for the development of vascular disease risk among individuals with diabetes, both type 1 and type 2 diabetes (see Chap. 21, Howard Hodis) [7].

Vitamin E Reduces Risk for Cardiovascular Complications

The proof of concept that vitamin E supplementation provides concrete cardiovascular benefit to Hp 2-2 DM individuals has been provided in two independent studies and a combined analysis:

- The Israel Cardiovascular Events Reduction with Vitamin E (ICARE) study, a prospective study in which Hp 2-2 DM individuals were randomized to vitamin E or placebo, demonstrated a significant reduction in CVD death, MI and stroke in Hp 2-2 DM individuals who received vitamin E.
- Retrospective analysis of the Heart Outcomes Prevention Evaluation (HOPE) study demonstrated a significant reduction in CVD and death in individuals with the Hp 2-2 genotype who received vitamin E.
- Combined analysis of ICARE and HOPE demonstrates a significant interaction between the Hp genotype and vitamin E on CVD.
- Extrapolation of the results of the ICARE and HOPE studies to a real-life population over 50 years demonstrates that a pharmacogenomic algorithm of administering vitamin E to Hp 2-2 DM individuals would prolong life by approximately 3 years in these individuals.

Hp typing represents a once in a lifetime test that identifies those DM individuals at exceptionally high risk of CVD. The Hp type may be used to more effectively focus the attention of the clinician and

the utilization of healthcare resources on those DM individuals for whom more aggressive risk factor modification is most needed. The Hp genotype also appears to identify a very large subgroup of DM (a third of the DM population) individuals who may receive marked clinical benefit from an extremely inexpensive therapy. This innovative pharmacogenomic approach towards the reduction of cardiovascular complications in a major subset of diabetic patients may become an essential part in the arsenal of diabetes care. This pharmacogenomic modality of treatment is of a unique nature in view of its low cost and wide availability. Cardiovascular complications of DM cost over $174 billion annually in the USA alone and account for 80% of all deaths in individuals with DM [10–12]. The prevalence of DM is increasing at an alarming rate, particularly among individuals of lower socioeconomic status [11, 13, 14]. The requirements for Hp typing, although not readily available for commercial use, are expected to be simple and inexpensive.

In the broader context of public, policy-makers and professionals' attention moving towards personalized medicine, and with growing budget constraints, this pharmacogenomic paradigm seems even more sound.

Applying Nutrients Within the Current Healthcare System: Still an Emerging Concept

Cost-effectiveness analysis has become a common practice for informing reimbursement decisions for pharmaceuticals and other health technologies including devices. Nutritional interventions tend to be excluded from these processes, although healthcare decision-makers have begun to realize that food plays an important role not only in those already with disease but also in the onset and evolution of lifestyle-related disorders. Indeed, improving health through better population nutrition may contribute to the cost-effectiveness and sustainability of healthcare systems. Formal guidelines exist for pharmaceutical products, but no systematic approach exists for the cost-effectiveness assessment of nutritional interventions.

Conventional foods and dietary supplements are traditionally used to meet daily nutritional requirements and preventing deficiency in the general population. However, some nutrients, like vitamin E, have a benefit in managing disease or medical conditions in specific risk groups beyond their traditional nutritional function.

In food there may be multiple acting agents, whereas single nutrients like vitamin E have well-defined entities, like pharmaceuticals, and their mechanism of action is usually known. On the other hand, nutrients like vitamin E can be naturally found in food (corn oil, soybean oil, margarine and dressings) contrary to pharmaceuticals. Contrary to pharmaceuticals, vitamin E has several structural entities and is sold in different forms. Therefore the first question is if vitamin E in doses above the normal intake to manage a disease or medical condition can be considered a pharmaceutical product or a nutritional intervention or may have a position in between.

In the next section, we explore the introduction of vitamin E in the consumer market and the medical market.

The Medical Market (Patients)

The market access for medicinal products (drugs) differs from nutritional products in the medical market. The main difference is that market access for (outpatient) medicinal products is a central procedure with specific requirements. The main target audiences are the central health authorities and health technology assessment (HTA) bodies, e.g. the National Institute of Health and Clinical Excellence (NICE) in the UK, making decisions on reimbursement decisions. The market access for

Table 29.1 Diagram

	Pharmaceutical	Nutritional product
OTC	Sometimes	Mostly
Reimbursed	Mostly	Sometimes

nutritional products is a more decentralized process, where the local payers (health insurance companies) are the main target audiences with different data requirements than the central health authorities. The diagram in Table 29.1 shows the actual possibilities, including the rare options. Vitamins with benefits beyond their nutritional function for management of disease or a medical condition may fall in between the extreme positions for pharmaceuticals and nutritional products.

Another important difference is that most prescription drugs are usually only available in the medical market. Some over-the-counter (OTC) drugs may be available in the consumer market, but they may only be reimbursed for a patient fulfilling specific clinical criteria, e.g. severe disease. In the contrary many nutritionals and vitamins, including vitamin E, are available as OTC.

General Introduction to Health Economics

Escalating costs have become a major concern for healthcare professionals, decision-makers and the public, prompting the implementation of new cost containment measures over the last decade and focusing especially on new medical therapies and to a less extent medical devices. As a consequence, healthcare decision-makers have been forced to develop new and more stringent criteria in the decision-making process for uptake of innovative therapies in the health insurance package. Instead of only considering the clinical benefits, available at registration (traditional data: efficacy, safety and quality parameters), and the price of the new treatment, the decision-makers have taken a broader perspective, including also other related costs in the healthcare system. Cost-effectiveness analyses can provide additional evidence-based information to help decision-makers set priorities. Over approximately the last 10 years, we can distinguish various additional data requirements, which especially relate to the use of the medicinal product in real daily practice. The most important new data requirements are effectiveness, cost-effectiveness and budget impact. Other considerations may also be taken into account depending on the specific indication, e.g. equity and social values in case of lifestyle medicinal products or orphan medicinal products. As a consequence, new treatment options, including preventive treatments, are evaluated from a clinical as well as a broader economic perspective, thereby taking into consideration the overall costs and the impact on quality of life (QoL). The decision of health authorities on coverage of a medicinal product in the health insurance package is based on the value for money of that medicinal product. Health authorities will make a trade-off between the incremental, clinical benefit and the extra cost of the new medicinal product versus standard therapy. Currently formal methods of health technology assessment, such as budget impact analysis (BIA) and cost-effectiveness analysis (CEA), are applied in order to make a value for money decision, which determines the final reimbursement decision.

National health technology assessment institutes evaluate cost-effectiveness of new innovative medical products and provide recommendations to the national health authorities. A cost-effectiveness analysis provides a cost per quality-adjusted life year (QALY), which is also defined as the incremental cost-effectiveness ratio (ICER). In the UK, the National Institute of Health and Clinical Excellence (NICE) has adopted a cost-effectiveness threshold range of £20,000–£30,000 per QALY gained, which means that the English society is willing to pay up to at least £20,000 per QALY gained for a new, innovative, medicinal product [15] (Scenario 1, see insert, provides explanation of QALY concept). A budgetary impact analysis shows the impact of a new medicinal product on the annual national medicinal product budget and total health expenditures. If the budget impact is considered too high,

the prescription of the product may be restricted to a subpopulation. A positive reimbursement for restricted use may lead to a substantial reduction in potential sales, which may only be 10–30% of the total expected sales based on the registered indication. There is also a probability that the drug will not be reimbursed because of a negative assessment of the reimbursement dossier. The health authorities may not be convinced of the clinical benefit and/or the cost-effectiveness neither for the total population nor for a subpopulation. In this case there will be no formal reimbursement under the health insurance system, and manufacturers are confronted with selling the product to a highly selected population who can afford to pay for the product privately or under a private insurance.

This type of value messages, in terms of CEA and BIA, is increasingly important for new treatment modalities in high-prevalent diseases, like cardiovascular disease, because of its high budgetary implications.

Cost Assessment

In a cost-effectiveness analysis, measuring the extent of costs is an important step. Costs can be broadly divided into two discrete resource categories: direct costs and indirect costs (often labelled productivity costs). Direct costs reflect the monetary burden of the medical care and non-medical care expenditures made in response to a disease. The cost of medicinal products is one type of direct medical cost. Other types of direct medical cost include cost of hospitalizations, cost of physician visits, cost of tests and procedures and cost of durable medical equipment. Direct non-medical costs include the costs to patients, such as travel to obtain treatment. The main indirect cost is lost productivity from the patient being absent from work as a result of morbidity or premature mortality induced by the disease or its treatment. Productivity costs are only relevant when studies are conducted from a true societal perspective.

In CVD resulting from T2DM, the medical costs may consist of drugs for MI and stroke, consultations for cardiologist and neurologist, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA) and carotid endarterectomy for stroke. The total medical costs for MI are £ 6530 per patient in the first year and £ 702 in the subsequent years. For stroke these costs are, respectively, £ 3579 and £ 5892.

The direct non-medical costs may be transportation costs by patient and family to the hospital after MI or stroke. In stroke, there may also be direct non-medical costs due to required home modifications resulting from remaining disability, e.g. house lift.

The lost productivity resulting from CVD complications may be due to absence from work during the acute phase of MI and stroke. A proportion of patients may not fully recover from MI and especially stroke, and therefore lost productivity consists of the total working days lost until retirement based on the human capital approach. As we consider CVD complications in patients with T2DM in this chapter, many patients are already retired or unemployed due to previous morbidity at the time of the CVD event. Therefore the indirect costs only occur in the employed population. But these indirect costs are not included in a cost-effectiveness analysis in the UK, which only requires inclusion of costs relevant for the healthcare payer, whereas these costs are included in the Netherlands requiring a broader society perspective.

Validity

Effectiveness is the actual health benefit achieved by a medicinal product in daily practice. This concept contrasts with efficacy, which is the benefit measured under ideal conditions in a homogeneous group of patients. In cost-effectiveness analyses, effectiveness is more relevant than efficacy, because costs and QALYs in real life are a function of effectiveness instead of efficacy. Clinical trials can have

a low external validity because they have strict inclusion and exclusion criteria and treatments are protocol driven, leading to potential overestimation of units of healthcare utilization. Considering the prospective approach, the concept of validity should be addressed. Internal validity is the extent to which the analytic inference derived from the study sample is correct for the target population. External validity or generalizability is the extent to which the results found in the study sample also apply to the population from which the sample was taken. Randomization is usually applied to balance confounding variables, and, with double blinding, helps to support internal validity. However, the external validity of randomized clinical trials (RCTs) is more questionable. Inclusion criteria for patients and selection of study sites may mean that the sample is not representative of the potential patient population. In addition treatment patterns may be determined by the protocol. Therefore, both clinical and economic outcomes may not be typical and do not correspond to usual practice. Hence it should always be considered that due to its restriction on external validity, the estimates of efficacy from RCTs may not be representative of the effectiveness of the intervention in the target patient population.

In clinical trials, there may be T2DM patients with many comorbidities, like heart failure, renal failure, neuropathy, osteoarthritis, arthrosis, peripheral artery disease and polyneuropathy. This heterogeneity in the patient population may lead to power constraints in a clinical trial with a limited number of patients. Therefore inclusion and exclusion criteria may be applied in a T2DM clinical trial in order to reduce this heterogeneity and increase the probability of obtaining statistical significant clinical outcomes. On the other hand, the clinical outcomes may not be fully representative results anymore due to a reduction in external validity.

Scenario 1: QALY Concept

The "cost/QALY" is seen as the preferred cost-effectiveness outcome. The QALY gain is calculated by combining the utility gain (quality of life gain) with the number of life years gained (LYG). QALY gained may therefore be the result of longer life expectancy, utility gain or both. For example, a patient who experiences a survival gain of 4 years at a utility level of 0.4 will achieve the same 1.6 QALY gain as a patient with a survival gain of 2 years at a utility level of 0.8. Therefore, QALYs may even be gained without increasing life expectancy.

There are generic QoL scales, e.g. the EQ-5D from the EuroQoL Group, which are important in the decision-making process, but disease-specific QoL outcomes can be relevant as well. If there is a correlation between the QALY gains and improvement in disease-specific measures, it gives decision-makers more confidence that the effects of the treatment are producing the QALY gains.

For example, in T2DM the baseline QALY is 0.785. After a MI the QALY reduces further by 0.055–0.730 and after stroke by 0.164–0.621 (Fig. 29.1).

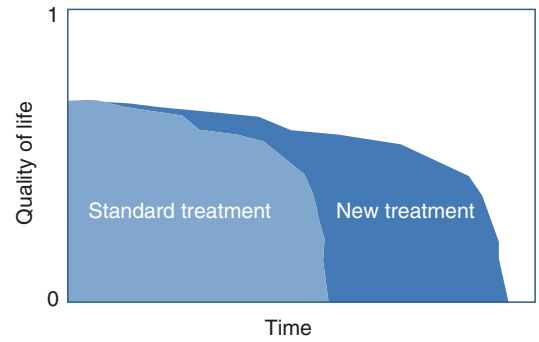

Fig. 29.1 Example of the effect of survival gain on the QALY

Application to Vitamin E

Based on the current vitamin E data, we may expect the following health economic (cost-effectiveness and budget impact) evidence, which is relevant for the target audiences (HTA institutes, payers, employers):

- Effectiveness: reduction of long-term CVD complications (MI, stroke and mortality).
- Costs: cost savings due to reduction of CVD complications stroke and MI (medical costs and productivity loss). However the reduction of CVD-related mortality increases the total costs, because more patients stay alive accruing further costs for T2DM and its complications.
- Effectiveness: gain in QALYs due to reduction of CVD complications. The reduction of MI and stroke will lead to quality of life gain, and the reduction of mortality will lead to more life years, which will results in an overall gain in QALYs.
- ICER: favourable cost-effectiveness in short-term – dominant (total cost savings and higher effectiveness) or a low cost per QALY gained.

Scenario 2 provides more detailed overview for health economic outcomes for the various perspectives.

Scenario 2: Health Economic Outcomes for Various Perspectives

HTA institutes

The preferred analytical technique is cost-effectiveness analysis, presenting the results in cost per QALY gained for vitamin E. In many countries, the HTA institutes take a broad society perspective, e.g. in the Netherlands. The society perspective includes the direct medical costs but also direct non-medical costs (e.g. transportation to healthcare facility) and indirect costs due to lost productivity. In other countries, the HTA institute only takes a payer perspective including only the direct medical costs (e.g. NICE in the UK). The costs per QALY gained are higher when only medical costs and no direct non-medical and indirect costs are included leading to a lower cost-effectiveness.Central health authorities may consider reimbursement of vitamin E, when there is clinical need for the use of vitamin E for the requested indication. The clinical data show that there is a high clinical need in T2DM patients with Hp 2-2 genotype.The following issues should be considered:

- The ICER is the main criterion for reimbursement. Therefore a value strategy may be based on the upper limit of the price potential based on the cost per QALY gained. This upper break-even number is the value for the new drug, when the cost per QALY gained remains just below the threshold, e.g. lower than £ 20,000 per QALY gained in the UK.
- The budget impact for the possible indications for vitamin E is quite high because of the population size. Therefore, the health authorities would probably impose a restriction to a subpopulation at increased risk, for example, Hp 2-2 T2DM patients with serious comorbidity (severe obesity, history of previous CVD events). A future clinical program can include a separate trial in this subpopulation. Another option is to include a predefined proportion, e.g. 30% of patients in the clinical trial belong to this subpopulation, in order to make predefined subpopulation analyses. A similar subgroup analysis may be based on the current clinical data.

Based on these considerations, we may question if a central reimbursement route for vitamin E is the most appropriate route for vitamin E, when the ICER is higher than £ 20,000 per QALY gained.

Payers

Health authorities have decentralized decision-making and introduced market mechanisms to improve system efficiencies and increase the decision-making power of payers. These payers seldom use cost-effectiveness ratios such as cost per QALY gained. More relevant criteria are efficacy, safety, total budget impact and particularly drug budget impact.

The payers may consider reimbursement of vitamin E when:

- The payers are convinced of the clinical benefit of vitamin E contributing to the quality of care and quality of life of the patient. The payers rely for judgement of the clinical benefit on the medical community, who will also consider RCT data as the most important data. Payers are also willing to consider other strong scientific data, e.g. observational evidence. The inclusion of vitamin E in a local or national clinical guideline would also contribute to listing by payers.
- A cost-effectiveness analysis is not required for payers, although it may support the societal value of vitamin E. Payers may consider potential cost savings by vitamin E more important, and therefore a cost-minimization model might be sufficient. The cost savings per patient can be extended to a budget impact analysis by including epidemiology data. The break-even price for vitamin E from the payer perspective is based on the savings in medical costs due to reduction of CVD complications stroke and MI. However the reduction of CVD-related mortality increases the total costs, because more patients stay alive accruing further costs for T2DM and its complications.
- The budget impact for the possible indications for vitamin E is quite high because of the population size of Hp 2-2 T2DM diabetes. Payers would probably also impose a restriction to a more severe subpopulation. If there is a high probability of the implementation of a prescription restriction, a proactive selection of a subpopulation for vitamin E is recommended for several reasons as mentioned in previous section for central approach.

Based on the considerations, we may consider a payers' route of market access:

- Payers may not require a formal drug status of vitamin E.
- There is no requirement for "cost per QALY" gained outcomes, but a cost-minimization approach would be sufficient, which would show cost savings (price < break-even price vitamin E) or neutral costs (price = break-even price for vitamin E) at patient level and population level (budget impact analysis is based on the total number of eligible patients).
- Payers may require price discounts, or they may want to have price-volume arrangements in order to avoid any risk.
- The payers' route for reimbursement for vitamin E may seem more attractive than a central route because of less stringent clinical and health economic requirements.

Employers

- The clinical data of vitamin E show that efforts to reduce the prevalence of CVD complications in DM patients could result in significant savings to employers. Lost productivity includes absenteeism as well as presenteeism, which is the single largest driver of the costs of poor health among full-time employees. A cost-minimization model and a budget impact model can be used to show to the employer the potential cost savings by paying vitamin E. This is not yet a real option for reimbursement and is not addressed further in this chapter, but it can become relevant in new business models in the near future.

The Consumer Market

The main target audiences are the consumers:

- There is no need for central reimbursement procedure or application for reimbursement by payers.
- There are no clear data requirements like clinical efficacy, cost-effectiveness and budget impact.

- The claims for efficacy of vitamin E may not require high-quality RCTs as for reimbursement claims, which would save costs and also increase time to launch.
- There would be no additional delay between market authorization and market access. The reimbursement procedure may require at least 9 months.
- Value potential is based on the break-even price based on the willingness to pay by consumers.
- Retailers may also require discount based on volumes.

The consumer market approach may be most relevant for vitamin E for the non-DM population. As health economics is not relevant for the consumer market approach, we focus in the remainder of this chapter on the medical market.

Stratified Nutrition

Diabetics with Hp 2-2 will benefit most from vitamin E by a reduction of CVD complications. Therefore, the use of vitamin E in the total T2DM population may be inappropriate from an economic perspective, because the cost of vitamin E in the patients without Hp 2-2 has less clinical value. But vitamin E is not associated with adverse events, which means that there is no unnecessary risk of adverse events in prescribing vitamin E to these patients.

Therefore, the choice between the use of vitamin E based on diagnostic testing for Hp 2-2 genotype and empirical use of vitamin E in all T2DM patients requires an economic assessment to answer the following questions:

– Payer perspective: Do the cost savings by a reduction of CVD complications at least balance the additional cost of a diagnostic test for Hp 2-2 and any associated medical resource utilization (e.g. extra GP visit)?
– HTA institute: Do the cost savings and QALY gain by a reduction of CVD complications at least lead to an ICER below the threshold, e.g. £ 20,000 per QALY gained in the UK?

The conditional use of a medication based on a diagnostic test is a new concept defined as stratified medicine, which we may redefine as stratified nutrition, when medication is replaced by nutrition. Stratified medicine as opposed to empirical medicine is the practice of using biomarkers or diagnostic tests to guide the choice of therapeutic treatments. By identifying groups of patients who will benefit from treatments, stratified medicine is a step towards personalized medicine, where the treatments will be completely tailored to the individual patient. The stratified nutrition approach stratifies the patient population in patients with or without a genotype (Hp 2-2) in this evaluation of vitamin E. This stratification of the population based on diagnostic testing is intended to reduce the ineffective use of vitamin E, which should translate into improved health outcomes for patients at increased risk and more efficient use of healthcare resources. This has been illustrated in several well-known examples (Herceptin®, Glivec®, Vectibix®, Erbitux®, Xalkori®, Zelboraf®), particularly in oncology. The study by Blum showed that the concept of screening T2DM individuals for the Hp genotype and treating those with Hp 2-2 with vitamin E also appears to be highly clinically effective [16].

A previous study assessed the cost-effectiveness of adding n-3 PUFAs to the current secondary prevention treatment versus standard prevention alone after acute MI in five countries: Australia, Belgium, Canada, Germany and Poland [17]. The implications of adding n-3 PUFAs to standard treatment in patients aged 59 years with a recent history of MI were analysed from the healthcare payer perspective. The ICER varied between 2788 Euros (Canada) and 5097 Euros (Belgium) per LYG. The study shows that adding highly concentrated n-3 PUFAs to standard treatment in the secondary prevention of MI appears to be cost-effective versus standard treatment alone. Although adding n-3 PUFAs is restricted to a subpopulation, after MI, no diagnostic tests are applied, and therefore this is not a full example of stratified nutrition.

Besides the outcomes of the health economic evaluation, stratified medicine (or stratified nutrition) also challenges current reimbursement approaches in many markets. Market access of stratified medicine is highly dependent on the assessment process, in particular health technology assessment (HTA) and pricing and reimbursement decisions. As a current rule, diagnostics and pharmaceuticals (or nutritionals) are considered under separated appraisal and payment processes. Third-party payers in various healthcare systems have been rather resistant to paying for costly stratification diagnostics unless the diagnostic companies can demonstrate clinical utility and/or cost-effectiveness without endangering the various healthcare budgets. Because of silo mentality in many healthcare systems, a holistic approach is required in order to assess the full health and economic value of stratified nutrition. The next section provides an application of health economic concepts to the use of vitamin E in T2DM.

Health Economic Analysis

Introduction

A health economic evaluation compares at least two alternative medical interventions by examining both their costs and consequences. Such health economic evaluations can either be performed alongside clinical trials (piggy-back study), as observational naturalistic economic evaluations (measured in real-world settings), or on the basis of health economic modelling. Economic analyses alongside clinical trials are often inconclusive due to a limited time horizon, a possible focus on surrogate endpoints, a highly selected patient population (low external validity – results are not generalizable) and protocol-driven costs. The major limitations of observational naturalistic economic evaluations are the selection bias (selection among treatment options can be influenced by the clinical and socioeconomic status of patient), and they can be conducted only after registration, as they are implemented in real-world settings.

The third type – economic modelling – is routinely used to predict the 'comparative product value' for market access and reimbursement purposes. Economic modelling has the advantage that it can handle limitations outlined for piggy-back and observational economic evaluations. For example, economic modelling may be used to translate efficacy outcomes obtained from randomized controlled trials into effectiveness outcomes (expected under routine real-world conditions), to simulate long-term therapy effects and to translate surrogate parameters into patient-relevant outcomes. Furthermore it enables estimating results to specific patient groups or local settings, which is crucial to ensure the relevance of national reimbursement decisions. Modelling techniques usually extrapolate the available short-term evidence over time in order to estimate outcomes beyond the study period or to link intermediate endpoints to final outcomes.

Modelling studies are based on decision analysis, which allows both clinical and economic consequences of medical actions and attitudes to be analysed. From treatment algorithms a model can be constructed which considers the timings of actions and their consequences over time. In effect, a model shows the consequences and complications of different therapeutic interventions, and it should correspond, as much as possible, to the real-life situation of the disease.

Models may take the form of decision-analytic trees or they may be very complex Markov models. Markov models are most appropriate for chronic diseases with health states based on severity, for instance, heart failure, which is graded according to different New York Heart Association (NYHA) classes. In our case for vitamin E, the outcomes are not time cumulative states of severity but events only (MI, stroke or mortality), and therefore an event-driven decision tree model is more appropriate. In addition, payers and employers usually are not familiar with cost-effectiveness models, which is another reason to prefer a transparent decision tree model instead of a complex Markov model.

Table 29.2 Design features of model

Features	Central health authorities	Payer/employer
Time horizon	Lifetime	5 years
Outcomes	ICER	Medical costs
Model	Complex	Transparent
Analysis	Fixed	Interactive
Uncertainty	Sensitivity analysis	Scenario analysis

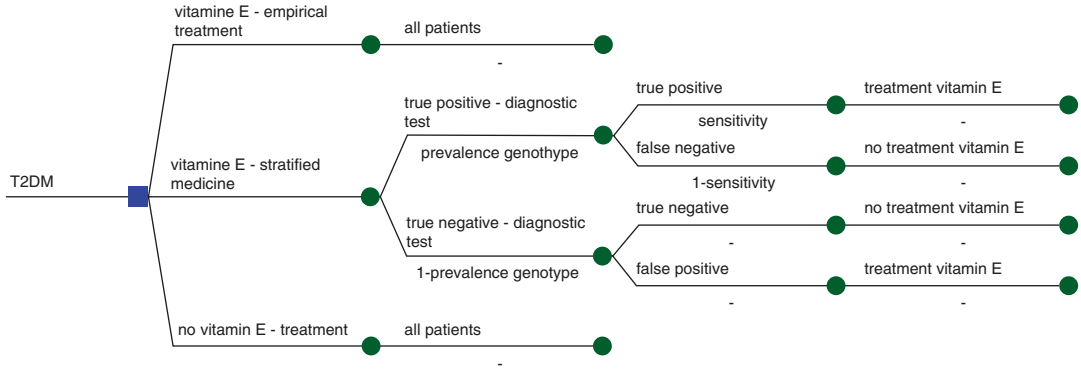

Fig. 29.2 Health economic model for vitamin E

Table 29.2 shows the relevant features of the model for payers and employers compared to central health authorities. The analysis is fixed for central authorities based on national data, but the analysis for payer or employer should be adjusted to company-specific data (epidemiology, costs).

Design

A decision tree model is used to estimate the cost-effectiveness of vitamin E. Figure 29.2 shows the structure of the model. The first node in the model reflects the treatment options:

- Vitamin E: current indiscriminate use in all T2DM patients
- Vitamin E: the use of vitamin E based on diagnostic testing for Hp 2-2 genotype
- No treatment with vitamin E

The second node reflects if patients will be treated with vitamin E and will have the benefit:

(1) Vitamin E: current indiscriminate use in all T2DM patients

- The proportion of patients being Hp 2-2 will benefit from vitamin E by a reduction of CVD complications.
- The proportion of patients being not Hp 2-2 cannot benefit from vitamin E by a reduction of CVD complications regardless of treatment with vitamin E.

(2) Vitamin E: the use of vitamin E based on diagnostic testing for Hp 2-2 genotype

- True+ for Hp 2-2: Patients will benefit from vitamin E by a reduction of CVD complications.
- False- for Hp 2-2: Patients cannot benefit from vitamin E by a reduction of CVD complications as they will not be treated with vitamin E.

- True- for no Hp 2-2: Patients have their normal risk of CVD complications.
- False+ for no Hp 2-2: Patients will not benefit from vitamin E by a reduction of CVD complications regardless of treatment with vitamin E.

(3) No treatment with vitamin E

- The proportion of patients being Hp 2-2 will not benefit from vitamin E by a reduction of CVD complications.
- The proportion of patients being not Hp 2-2 have their normal risk of CVD complications.

The time horizon of the model is lifetime in the base case analysis. Costs and QALYs are discounted at 3.5%. For central HTA organizations, the ideal time horizon of a cost-effectiveness study is lifetime. From health economic perspective, lifetime time horizon is also appropriate because T2DM is a chronic disease. For payers the time horizon of this model should be at least 3–5 years in order to show at least the cost savings from the reduction of intermediate morbidity by vitamin E.

The model includes medical resource utilization patterns for:

- Diagnosis of Hp 2-2 including the price of the diagnostic test
- Treatment with vitamin E
- CVD complications

Results

Tables 29.3 and 29.4 show the results of the base case analysis for the three management options over 5-year, 10-year and lifetime period (no treatment, indiscriminate use of vitamin E (indiscriminate vitamin E) and stratified use of vitamin E). The analysis shows that vitamin E leads to lifetime cost savings of £ 757 and a gain of 1.106 QALYs compared to 'no treatment'. Consequently, the ICER is £ 684 per QALY gained. Stratified vitamin E leads to similar QALYs gained (21.658) compared to empiric vitamin E. The cost difference is due to the avoidance of the use of vitamin E in patients with no Hp 2-2, who will not benefit from vitamin E. Therefore, stratified vitamin E leads to cost savings of £ 711. Because stratified vitamin E leads to similar QALYs gained and overall cost savings, stratified vitamin E is dominant over empiric vitamin E use, which is the most cost-effective outcome. Stratified vitamin E leads to cost savings of £ 46 compared to 'no treatment', whereas the gain in QALYs is 1.106, which results in an ICER of only £ 41 per QALY gained, which is far below the commonly used threshold of £ 20,000 in the UK and therefore also makes stratified vitamin E cost-effective. This threshold means that the UK society is willing to pay up to £ 20,000 for a gain of one QALY, and therefore vitamin E is extremely cost-effective. Finally, indirect costs due to productivity loss are not included in the base case analysis as its perspective was that of the payer. The use of a broader societal perspective that includes these indirect costs would further improve the cost-effectiveness of vitamin E.

Table 29.3 Outcomes of the base case analysis

	No treatment			Indiscriminate vitamin E			Stratified vitamin E		
	Costs (£)		QALYs gained	Costs (£)		QALYs gained	Costs (£)		QALYs gained
	Medical	Indirect		Medical	Indirect		Medical	Indirect	
5 years	27,285	209	3.630	27,392	71	3.654	27,290	71	3.654
10 years	50,326	768	6.636	50,515	262	6.712	50,309	262	6.712
Lifetime	167,688	1733	20.552	168,445	592	21.658	167,734	592	21.658

Table 29.4 Outcomes of the base case analysis – ICERs (lifetime analysis)

Comparison	Costs (£)	QALYs gained	ICER
No treatment	167,688	20.552	
Indiscriminate vitamin E	168,445	21.658	
Difference	757	1.106	684
Indiscriminate vitamin E	168,445	21.658	
Stratified vitamin E	167,734	21.658	
Difference	−711	0.000	Stratified vitamin E dominant
No treatment	167,688	20.552	
Stratified vitamin E	167,734	21.658	
Difference	46	1.106	41

Conclusion

Health economic theory is mainly used for pharmaceuticals in submissions for reimbursement. The objective of this chapter was to apply the health economic theory to vitamin E. Projections about the effectiveness and expected costs of an intervention can be modelled using realistic and explicit assumptions based on outcomes from randomized clinical studies. In this health economic application to vitamin E, it is illustrated that there is no difference between a cost-effectiveness analysis for a pharmaceutical and vitamin E, as long as the clinical evidence for vitamin E fulfils the requirements for pharmaceuticals. For vitamin E, there are clinical studies which provide high-level evidence for the cardiovascular benefit to Hp 2-2 T2DM individuals. The analysis shows that the use of vitamin E in all T2DM patients leads to a favourable cost-effectiveness ratio, which further improves, when vitamin E is only following a positive diagnostic test for Hp 2-2 genotype. Summarizing, we may conclude that vitamin E is a very cost-effective treatment to reduce CVD-associated healthcare costs in particular in T2DM patients carrying the Hp 2-2 genotype.

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Chapter 30
Do Consumers Care About Micronutrients? A Perspective on the Possible Role of Vitamin E in the Dietary Choices of Consumers

Klaus G. Grunert

Keywords Consumer behaviour · Nutrition knowledge · Food choice · Micronutrients · Behavioural change

Key Points

- Consumers have an interest in vitamins, but their general level of knowledge about micronutrients is most likely low.
- Consumers obtain nutrition knowledge mostly from the web and from sources of variable information quality.
- Nutrition considerations are only one among several factors affecting consumer food choice.
- Providing nutrition information to consumers has up till now focused on macronutrients.
- Most consumer purchase decisions are made habitually, and increasing vitamin E intake by changes in volitional buying behaviour will be difficult.

Introduction

Affected by decades of information campaigns, broad treatment in the media, and a steady flow of dietary guidelines and product-related information, most consumers have learned that there is a link between the food you eat and your health [3]. Most consumers have heard about the role of fat and sugar in a (un-)healthy diet, and many have even been acquainted with omega-3 fatty acids. They know about vitamins, and they may even have heard the term antioxidants.

However, most consumers are not nutritionists and will never achieve a level of nutritional knowledge that the average nutritionist would regard as satisfactory. They are laymen navigating in a complex subject area, where multiple information sources are available and where the messages sent out to consumers are often conflicting [39]. Consumers also differ considerably in their motivation to make sense of these messages and to use them in their daily food choices. Not everybody is highly motivated to eat healthily, because food serves other purposes than maintaining a healthy body, like being a source of pleasure, a backbone of family life, and an arena for personal growth, and consumers may perceive that the health goal conflicts with some of these other goals [8, 34]. Even when

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_30

consumers are motivated to eat healthily and make sense of and apply nutritional knowledge, they may find this difficult to do in everyday decision-making where information may be lacking and decisions are made out of habit and under time pressure.

In the broader context of nutrition information, nutrition knowledge, and consumer decision-making, vitamin E is only a small aspect. Some consumers are attempting to manage their intake of macronutrients [11], but for all we know, their attention to micronutrients is generally limited unless there has been some media hype on a particular issue, and in this case the effects are usually short-lived [44]. This does not mean that it is impossible to raise consumer attention to a particular micronutrient if special focus and effort is expended on it. But as consumers' attention is a limited resource and many messages compete for consumer attention, it will usually imply communicating some sense of urgency leading to consumer concern.

The purpose of this chapter is to address the possible role of vitamin E in consumer information seeking and decision-making. Based on a general framework described in the next section, we will discuss the concept of nutrition knowledge and present insights on how and from which sources consumers obtain their nutrition knowledge and how this nutrition knowledge affects their food choices and other decisions relevant for their dietary intake. We will then review possible options for behavioural change regarding the intake of micronutrients like vitamin E.

Health Interest, Nutrition Knowledge, Information Seeking, and Food Choices

Figure 30.1 shows a simple model on how nutrition information can affect consumer food choices. Nutrition information can affect food choices to the extent it is acquired as part of the shopping process – typically product-related information, for example, nutrition labels – or because it has been acquired earlier as part of a not purchase-related information acquisition process that has extended the body of nutrition knowledge that the consumer has. Acquiring nutrition information can increase the appetite for more information, or can have the opposite effect, when the consumer thinks that s/he now knows enough [13].

As already noted, the extent of search for nutrition information will depend on the consumer's interest in healthy eating. Measures of consumer interest in healthy eating are widely available, an

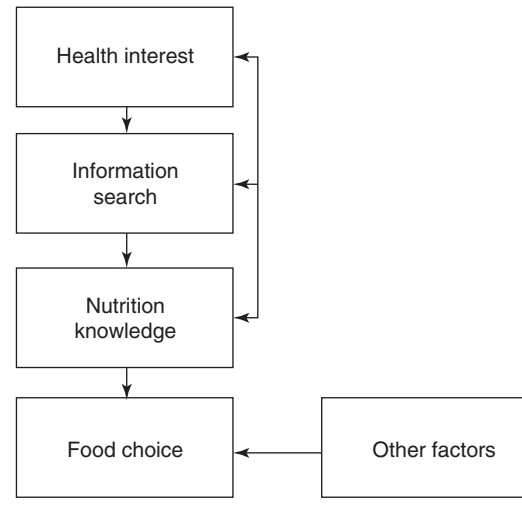

Fig. 30.1 Antecedents and consequences of nutrition knowledge acquisition

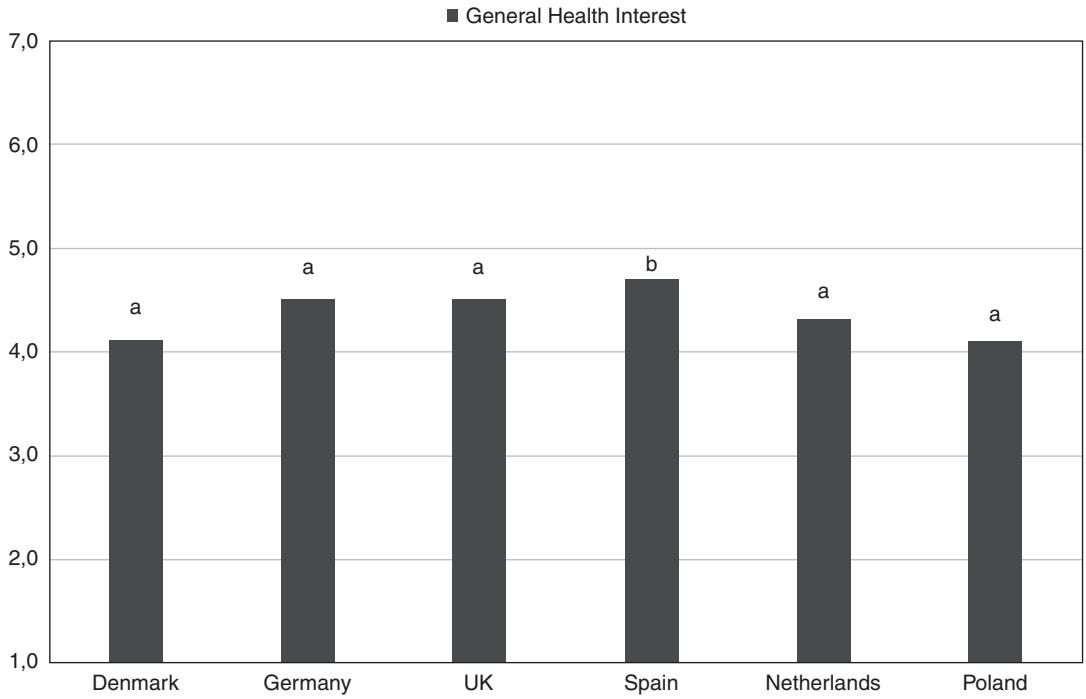

Fig. 30.2 Interest in healthy eating in six European countries. Mean of items on 7-point scale with higher values expressing more General Health Interest, total $n = 5395$. Different letters indicate significant differences, $p < 0.05$, Scheffe test. (Adapted from [13])

example is the General Health Interest scale [34]. Figure 30.2 shows the distribution of scores on the General Health Interest scale from a recent study in six European countries. It shows a wide dispersion with most consumers scoring in the middle of the scale, underlining the fact that not everybody has a high interest in healthy eating and that the health motive is not the only motive when it comes to food choice. The considerable variation in interest in healthy eating has implications for the search for nutrition information. Consumers with a lower interest in healthy eating are not necessarily completely uninterested in nutrition information, but will search less, will be less meticulous in their search, and will process the information more superficially.

While everybody knows a few things about nutrition, not all people look for nutrition information when buying food. A European study indicated that the percentage of shoppers looking for nutrition information on a food package when shopping varies between 9% and 25%, depending on the country [12]. Even when people don't look for nutrition information when shopping, they can in principle still make use of their nutrition knowledge in their decision-making, for example, by using their pre-existing knowledge about the nutrient profile of different types of food products. However, the fact that people have this type of knowledge does not mean that it is used, as many food choices are made without extensive elaboration, i.e. they are based either on habit or on the use of a few key decision cues [41]. Trying to change people's diet by expanding their nutrition knowledge and giving them product-related information on nutrient content is therefore always uphill, unless we are dealing with consumers that have a specific health concern that increases their motivation to process such information.

In the following, we will deal in more detail with the central concepts of nutrition knowledge, information search, and food choice. Based on this we will discuss options for behavioural change with a focus on change related to a micronutrient like vitamin E.

Nutrition Knowledge

'Nutrition knowledge' is not a very well-defined concept. We can make a basic distinction between declarative nutrition knowledge and procedural nutrition knowledge [9]. Declarative knowledge includes basic awareness of nutritional concepts (calories, fat, fibres), knowledge about dietary guidelines, about relationships between diet and health, and about the nutritional content of different food categories. Procedural knowledge is about applying the declarative knowledge in everyday life – in reading food labels and in making choices in what to buy, how to cook, and how much to eat. Attempts to measure nutrition knowledge mostly take the form of multiple-choice tests, where statements have to be categorized as true or false. The most comprehensive scale for measuring nutrition knowledge, developed by Parmenter and Wardle [31], distinguishes four dimensions: dietary recommendation, sources of nutrients, choosing everyday foods, and diet-disease relationships. Levels of nutrition knowledge have been shown to be related to demographic characteristics (females have more knowledge than males, and nutrition knowledge becomes higher with age) and with interest in healthy eating [14], and higher levels of nutrition knowledge facilitate the use of nutrition information when buying food [28], while results on correlations with actual food choice have been mixed [46].

Interestingly, vitamins do not play a major role in these instruments. The Parmenter and Wardle scale has only two items on vitamins, one question on which type of bread is the best source of vitamins and minerals and a question on which vitamins are antioxidants. The more recent scale developed by Dickson-Spillmann et al. [9] contains nothing on vitamins. Generally there is a much stronger focus on macronutrients than on micronutrients and a stronger focus on disqualifying as compared to qualifying nutrients [18]. This focus may actually be unaligned with consumers' own perceptions about the importance of different nutrients. Hoefkens et al. [18], in a study conducted in six European countries, asked respondents about the perceived importance of their daily diet being low in a number of disqualifying nutrients and high in a number of qualifying nutrients. Out of seven nutrients (calories, fat, saturated fat, sugar, salt, fibre, vitamins and minerals), respondents rated it as most important that their daily diet is high in vitamins and minerals, with a mean rating significantly higher than for the other six nutrients. This was true not only for the pooled data but also for all six participating countries. And while the perceived importance was not surprisingly linked to how health-conscious people were, even in the group of not health-conscious consumers, vitamins and minerals were regarded as most important.

We have therefore rather solid evidence that consumers regard vitamins as important, even though measures about the actual level of consumer knowledge on vitamins remains sparse – and information about their knowledge on particular vitamins like vitamin E is even more lacking.

Information Search

For information search about nutrition, we can distinguish between product-related information search, which is typically available on the food label and acquired (if at all) in the context of a purchase decision, and general nutritional information, which may or may not be acquired in the context of a decision process and for which a plethora of information sources is available.

Concerning information search in the context of a consumer decision-making process, numerous studies have been conducted over the years (for overviews see [2, 15]). A huge number of determinants of information search have been investigated, covering factors concerning the market environment, situational variables, product importance, knowledge and experience, individual differences, and costs of search [15]. It has been shown that information accessibility and availability increase search [35, 36], time pressure reduces search [29], pre-search brand or store preferences decrease search [5], and costs of search decrease search. Results on the effects of previous knowledge and experience have been mixed, with both positive [20] and negative [36] effects being reported. Indeed, a higher level of knowledge and experience can facilitate the processing of new information, decreasing information costs and leading to increased information search [4], but an already high level of knowledge can also decrease the felt information need, leading to lower perceived benefits of the search and hence a lower level of information search [22]. Indeed, it has been shown that a higher level of nutrition knowledge can involve less use of health claim information on food labels [19]. Schmidt and Spreng [37] have summarized much of the earlier research on determinants of information search in a model where all known determinants of information search are mediated by either motivation to search or perceived ability to search. Several reviews are available summarizing research on search for product-related nutrition information [11, 16, 24, 28]. A major result from this body of research is that the salience of a health goal at the time of exposure and decision-making plays a major role in the use of this information (e.g. [43]).

Sources of general nutrition knowledge, typically not acquired as part of a shopping process, can come from many different sources – mass media, family and friends, your physician, and of course all the information on the Internet. This plethora of sources may make it difficult for consumers to decide where to go and when to look for information and may result in confusion and uncertainty [39]. In a study carried out in five European countries, the share of respondents answering 'no' to the simple question whether they know where to find information on healthy eating was 21% in Denmark, 32% in Italy, 49% in Poland, 50% in the UK, and 63% in Belgium [30]. When asked where they look for such information, the most frequently cited source in all countries was 'websites found via search engines', which was considerably more frequently used than mass media, family/friends, and own physician, but also more frequently than websites recommended by nutrition or health experts. Other studies (reviewed in [6]) found similar results. Internet-based information is to a lesser extent subject to basic rules about correctness of information and source-checks than classical media [27] and is many times not transparent about the source of information and its motivation to post it [1], and many health-related websites have large amounts of user-generated content, which adds to the uncertainty about the quality of the information [42]. Consumers often try to handle this by consulting several sources in an attempt to triangulate the information [1, 33]. Also, consumers have idiosyncratic views on the relative reliability of different sources of information and apply a range of different criteria to evaluate the reliability of nutrition and health-related information, including quality of the argument, quality of the language, professional appearance of the website, and, if a source is cited, trustworthiness of the source. For user-generated content, user agreement plays a role ([26, 38], see also Fig. 30.3).

The processing of information may occur at different levels of depth [32], depending on the degree of motivation of the receiver and the constraints in terms of time and other tasks under which the processing occurs. As Internet-based search usually has a high element of browsing, the way information is processed will usually be a combination of deep and shallow processing. People are likely to make use of the information in a stepwise fashion: First, websites are evaluated very quickly based on heuristic criteria like visual appeal, ease of navigation, and the presence of advertising, and only those website going through that screening process and being allocated more attention will then be processed more thoroughly [38].

Information obtained from websites and other information are the input for further processing leading to inferences that go beyond the manifest content of the information and eventually leading to the development of subjective health theories, which may or may not be related to official

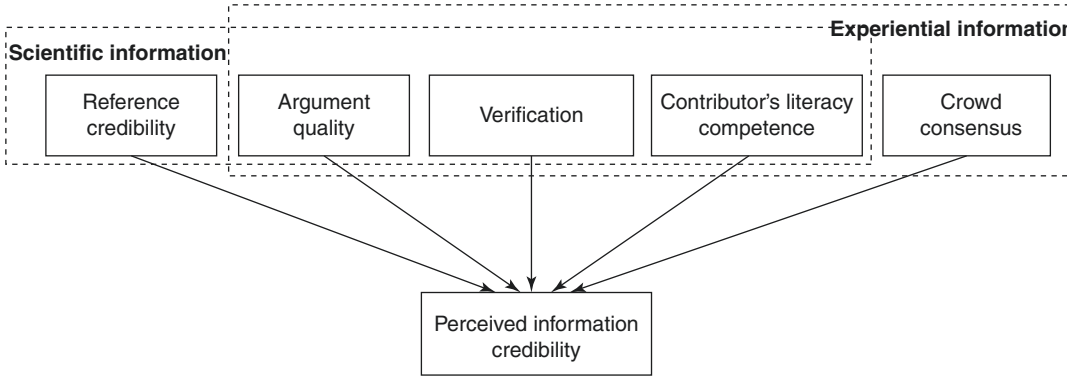

Fig. 30.3 Evaluation of the reliability of web-based information from [26]

recommendations about healthy eating. Newly learned information is always interpreted on the basis of information already known and subsequently integrated into the existing network of personal knowledge. Newly learnt information that is roughly congruent with existing views will often only reinforce those existing views without adding more detail, whereas information that is discrepant with existing views may either be discounted, for example, by source discreditation, or lead to attempts to reconcile views by searching for additional information.

As noted above, we do not presently have much insight into consumer knowledge about vitamin E. But what we know about the general characteristics of search for and processing of nutrition information suggests that such knowledge will often be diverse, loose, and idiosyncratic, which means that it will not be just a mirror of the established body of knowledge in the area.

Decision-Making

Just because people have nutrition information does not mean that they use it when making food choices. Many food choices are habitual, and of those that are not, most are made at the point of purchase. Figure 30.4 shows, for a range of products and countries, the average decision time that people use – that is, the time from arriving at the shelf to the time when leaving again with at least one product in the shopping cart (adapted from [12]). As can be seen, the average is around 25–30 s, and the distribution around the average is actually skewed, with many people only using 5–15 s and a few people using several minutes. In other words, decisions in the supermarket are made very quickly and will not involve a lot of deliberation. Research using eye-tracking methodology has also shown that when shoppers scan products on the shelf, most products only get a few seconds or less in the process (e.g. [25]).

This means that, for many shoppers and in many cases, the decision-making in the store will not be of the kind where the shopper processes several product attributes, like the ingredient list, the nutrition label, the origin, and the appearance, and tries to aggregate them into an overall evaluation before making a decision. Most food purchases will not involve a lot deliberation. In dual processing theories of decision-making, one distinguishes between conscious, deliberate decisions that involve reasoning and spontaneous, intuitive decisions with limited conscious cognitive activity [10]. Nobel Prize winner Kahneman has labelled these two types of decisions as governed by system 2 (the conscious, deliberate system) and system 1 (the spontaneous, intuitive decision) [23]. Most food purchases are most likely governed by system 1.

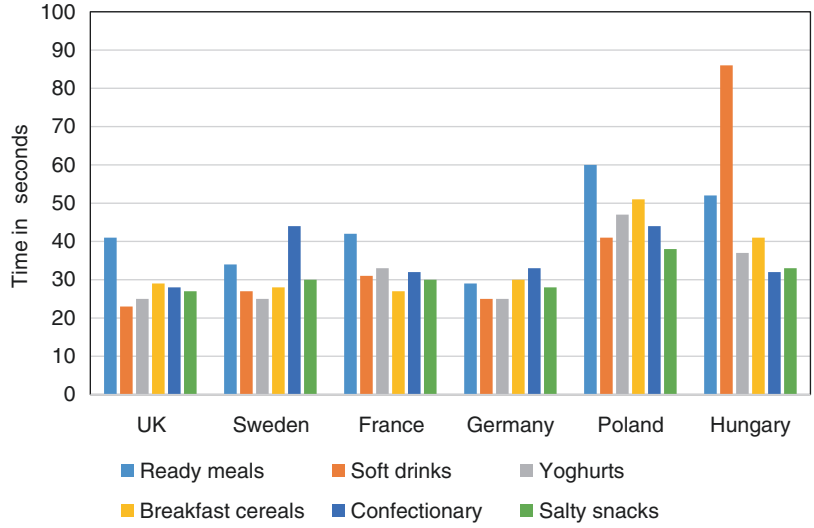

Fig. 30.4 Decision times when shopping for food. Based on data from [12]

System 1 governed decisions are triggered by a few environmental cues. Shoppers may recognize the brand as one they have used before and found satisfactory. Or they have not used the brand before, but recognize it as familiar and have some positive associations with it, possibly due to being exposed to market communication. They may like the appearance of the product or its packaging. Health-related information will not play a big role in system 1 governed decisions, unless the healthfulness of the product can be linked to those few cues that govern the decision. A product that was previously categorized as healthful may be recognized and bought again. Also, health-conscious consumers may process key cues that they think are related to healthfulness, for example, that the product is organic [41] or that the product carries a health symbol like the Choices logo or the Keyhole logo. But relatively few shoppers turn the product around to read the list of ingredients or the nutrition table [12].

This leaves relatively little room for information about micronutrients to affect food choice, unless consumers are especially motivated due to a specific health concern, in which case they would turn the package around, or unless they associate a whole product category with a specific micronutrient (like vitamin C with juice). The possible exception is when the micronutrient is mentioned in a health claim. Health claims are on the front of pack and have therefore a higher chance of being noticed than the ingredient list, which is usually on the back. One health claim on the role of vitamin E as an antioxidant is currently permitted in the EU, but we have no evidence on the extent to which it is used on products, much less to which extent consumers pay attention to it. The more general knowledge we have on how health claims affect consumers provide, however, no basis to expect any strong effects. Existing evidence suggests that health claims are largely ignored, may be misunderstood when actually perceived [40], and generally play only a limited role in people's food choices, unless people have a specific health problem that directs their attention towards a specific type of claim [17].

Options for Behavioural Change

Encouraging consumers to consume more – or less – of a particular micronutrient is a complex matter. People don't eat micronutrients. They don't even eat food – they eat meals that are being composed and prepared based on food products. The final intake of micronutrients is therefore the result of a

complex interaction of decisions about buying food, preparing meals, and quantities eaten. On top of that, people may change their micronutrient intake by food supplements. Jensen et al. [21] have illustrated this complex relationship well in the diagram shown in Fig. 30.5.

Attempted changes in micronutrient intake therefore need to change some of those behaviours and in a way where the desired effect is not counteracted by indirect changes in some other behaviours. We have already discussed the limited possibilities to change food choices based on micronutrient information. Similar reasoning applies to change the way that people prepare meals, snack between meals, and decide how much to eat. Volitional behavioural change requires learning about the consequences of the behaviour and turning this knowledge into an intention to indeed change. Both – learning and formation of intentions – presuppose motivation. Without some sense of urgency, both learning and the formation of intentions are difficult. And this is only the beginning – the intentions need to be turned into behaviour that in turn needs to become part of daily routines.

Habits depend on a stable context [45], which in turn means that habits are more easily changed when the context in which the behaviour takes place changes. Life transition phases are therefore obvious occasions for behaviour change [7], but also changes in the workplace, at home, in the kitchen, in the canteen, and in the supermarket can contribute to breaking habits – if the motivation is present.

As noted above, the motivation is not always present. People differ in the role they assign to the health theme in their food choices [8]. From the nutritionist point of view, food is a bundle of nutrients, and people should choose food with the aim of obtaining an optimal intake of nutrients. Some people indeed try to manage their food intake in this way – with different degrees of success, as also they have to balance the nutritionist perspective with other food motives like pleasure and family liking. But other people have a more holistic approach. They do not decompose food into nutrients, but evaluate them holistically mainly based on cultural and gastronomic criteria. They do not necessarily eat less healthy for that reason. But they are more difficult to address when one wants to change micronutrient intake by changes in volitional behaviour.

Obviously, industry and policy-makers have other means to induce changes in micronutrient intake than by volitional change of behaviour. Industry can enrich products in the interest of public health without promoting these changes. Menus in the catering industry, both public and private, can be adapted. Physicians can prescribe food supplements (there would still be an adherence issue, though).

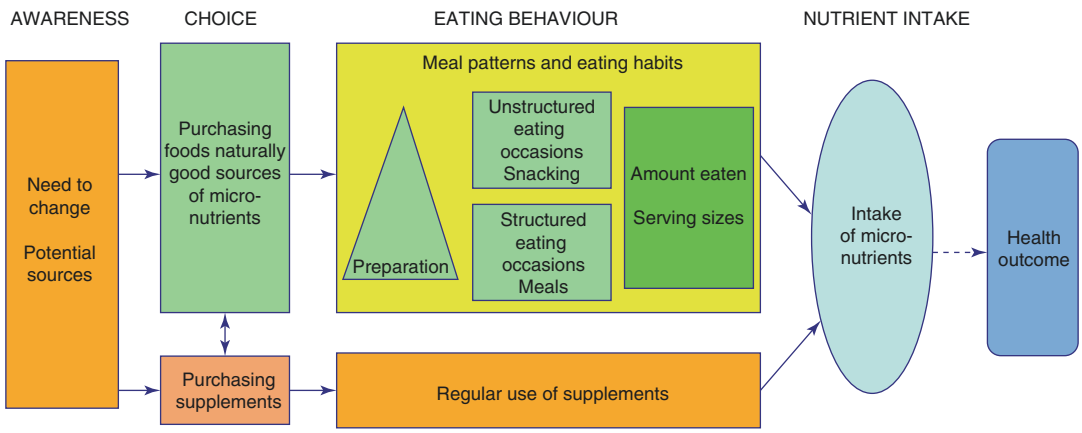

Fig. 30.5 Process of eating behaviour contributing to micronutrient intake. From [21]

Conclusions

Little is known about what consumers know about vitamin E and how this knowledge affects their behaviour. But by resorting to what we know more generally about food-related consumer decisions, about the way layman nutrition knowledge comes about, and about the role of nutrition information in consumer decision-making, we still can draw some conclusions about the likely role of vitamin E and the prospects of any consumer changes in the intake of vitamin E that might be desirable. On these grounds, the conclusion is that the role of vitamin E in consumer decision-making is probably very small and that attempts to change vitamin E intake by changes in volitional behaviour will be difficult.

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Chapter 31
Addressing Key Knowledge Gaps in Nutrition Research and the Impact of Funding Priorities in Human Nutrition

La Verne L. Brown and Paul M. Coates

Keywords Nutrient status · Dietary intake · Bioavailability · Phenotype variations · Food synergy Nutrient-nutrient interactions · Nutrition assessments · Government funding · Public-private partnerships · International collaborations

Key Points

- Addressing key knowledge gaps in research on vitamin E and other nutrients requires the development of new tools to improve dietary assessments and the identification and validation of biomarkers for determining nutrition status.
- Further enhancement of omics technologies to map the interrelationships between nutrient intake and true nutrient bioavailability as well as improved study designs to better address phenotypic variations in nutrient requirements will advance our understanding of the role of vitamin E in health promotion.
- Advances in our understanding of vitamin E and other nutrients in causal relationships between diet, nutrition, and health status will require global sharing and analysis of biomedical "big data."
- Funding opportunities to support this work are expanding beyond isolated government agency initiatives to include more interagency collaborations, public-private partnerships, and international collaborations.

Significant heterogeneity in study outcomes has resulted in a lack of confidence in reports highlighting the benefits of supplement ingredients (such as nutrients) for health maintenance. One might argue that until the role of nutrition, as a whole, in a healthy life span is fully elucidated, then there are limitations on how well a dietary supplement (with a single nutrient) can be justified as an essential component in the diet of healthy individuals. Challenges affecting the advancement of nutrition research include (1) assessments of nutritional status and dietary intake, (2) phenotypic variations in nutrition requirements (e.g., life stage, sex, genetics, and/or epigenetic differences), and (3) interrelationships between intake (via supplement or food) and true nutrient bioavailability. These challenges are confounded by ambiguous mechanisms of action, numerous active metabolites, and pleiotropic tendencies that are common features of many nutrients.

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4_31

Table 31.1 Characteristics of various clinical interventions

	Types of interventions		
Intervention characteristics	Drugs	Nutrients (modified diet)	Physical activity
Disease treatment	X		
Disease prevention	X	X	X
Health maintenance		X	X
Large effect (limited scope)	X		
Minimal effect (polyvalent scope)		X	X
Effects observed in isolation	X		
Effects observed in matrix		X	
Single endpoint	X		
Multiple endpoints (pleiotropic)		X	X
Linear response	X		
Sigmoidal response		X	X
Short-term effects	X		
Long latency		X	X

The World Health Organization defines nutrition as the intake of food, considered in relation to the body's dietary needs [1]. As such, good nutrition is considered the cornerstone of good health, and poor nutrition is believed to be the cause (at least in part) of increased susceptibility to disease or impaired physical and mental development. As of July 2017, more than 800 clinical trials with the key term "nutrient" and nearly 500 clinical trials with the key term "tocopherol" in the study title were currently registered in clinicaltrials.gov. Yet, evidence to support a causal relationship between disease and poor nutrition is subpar [2]. Randomized controlled trials are considered the gold standard for demonstrating causal relationships between an intervention and an outcome. But, do all types of interventions require the same approach when designing studies to address critical questions pertaining to nutrition sciences? An acknowledgment of key differences between various types of interventions is perhaps the first step in study design. For example, key characteristics of drug interventions vs. other types of interventions (including nutrient interventions, n-RCTs) are contrasted in Table 31.1. Drugs are intended for disease treatment/prevention. In general, drugs elicit a large effect, but with limited scope, that is often measured at a single endpoint [3]. A drug's efficacy is often observed in isolation, giving way to a linear dose response that is measurable within a short timeframe. Nutrients, on the other hand, are by definition essential for maintaining good health. They are polyvalent in scope and likely trigger a response through minuscule, sometimes immeasurable actions at multiple endpoints [3]. In addition, the food matrix or the physiological matrix of an individual may greatly impact the efficacy of a nutrient intervention. Furthermore, the sigmoidal dose responses and the long latency periods of nutrient interventions in healthy individuals are in stark contrast to common drug interventions in diseased individuals. Highlighting these differences is intended to incite a greater scrutiny of the design elements that are especially relevant to n-RCTs. One may start by ensuring that methods for characterizing various population phenotypes, nutrient interventions, and select biomarkers are appropriate for addressing the nuances that affect the science.

Phenotype-Based Nutrition Requirements

The continued advancement of nutrition sciences calls for more n-RCTs to be conducted on healthy individuals such that baseline nutrient requirements and/or nutrient status may be ascertained [4]. Yet, of the clinical studies registered with clinicaltrials.gov that listed tocopherol or vitamin E as an

intervention, only a fraction focused on outcomes in healthy individuals; and of those, there was no apparent consensus on what constituted "health." The search revealed wide discrepancies in inclusion and exclusion criteria for vitamin E intervention studies of "healthy" populations. In addition, details on how baseline health statuses were confirmed also varied greatly. Given the likelihood that nutrient interventions may impact multiple endpoints at levels that can be misconstrued as "noise," the ability to ascertain "health" (or at least reduce potential baseline confounders) from one individual to the next and draw comparisons between one study to the next becomes a crucial task [5, 6].

Nutrient requirements can vary with age and life stage, environmental influences, health status, gender, and ethnicity. For example, in the case of vitamin E, the Institute of Medicine acknowledges that persons consuming a diet high in polyunsaturated fatty acids (PUFAs) require a higher intake of vitamin E to meet the estimated average requirement (EAR) levels for adults in the USA [7]. But, on the other hand, since studies used to support the current EAR levels for vitamin E were conducted in men only [7, 8], the IOM lacked the scientific data to deliberate potential EAR distinctions between men, women, and other subpopulations. Clearly the lack of data on diverse populations (including women, various ethnic groups, seniors, and children) is a critical knowledge gap impeding the advancement of nutrition sciences. Efforts to include more diverse populations in n-RCT as well as improved characterizations (and harmonization) of clinical, behavioral, and environmental markers are expected to improve as the science gains greater appeal.

One revolutionary approach that may help to address key knowledge gaps in nutrition sciences is the *All of Us* Research Program at the National Institutes of Health (NIH). The program is expected to build upon a national research cohort of one million or more US participants. By ensuring the enrollment of participants from diverse social, racial/ethnic, ancestral, geographic, and economic backgrounds as well as from all age groups and health statuses, the program aims to take into account individual variability with respect to environment, lifestyle, and genes for health maintenance and healthcare [9].

Initiatives such as this may increase opportunities of more longitudinal crossover trials for exploring nutrient interventions. Baseline confounders may be reduced in n-RCTs where each participant is her/his own case and control. Well-characterized assessments at homeostasis, followed by a challenge, and restoration of homeostasis may offer important insights into individual variability with respect to nutrition interventions [5]. Formalized studies of this type, termed n-of-1 studies, have not been well utilized in nutrition research; and some guidelines for designing n-of-1 nutrition trials may be warranted [10]. While the design may not be suitable for providing evidence for population-wide interventions, multiple n-of-1 studies (if appropriately designed) may provide evidence for benefits of specific interventions in some subsets of the population as well as the larger population [11].

Interrelationships Between Nutrient Intake and True Bioavailability

Nutrients are an integral part of a food source. The bioavailability and efficacy of most nutrient interventions not only vary with respect to the physiological condition of the host but also the dietary patterns of the host as well as the chemical form and/or food matrix of the intervention. Vitamin E, in natural food sources, occurs as a mixture of eight different isoforms. The four tocopherols and the corresponding four tocotrienols are identified as α-, β-, δ-, and γ- isomers. While some evidence suggest that each of the vitamin E isoforms has different (and sometimes claimed superior) biological properties [12–14], α-tocopherol is most abundant in the blood (83% α, 13% γ, 2% β, 1% δ) and is the basis for the IOM recommendations for vitamin E. However, γ-tocopherol is slightly more abundant than α-tocopherol in the skin (53% γ vs. 47% α) and is present in appreciable amounts in muscle (38% γ, 62% α) and adipose tissue (31% γ, 69% α) [12, 15]. Also, in the USA, dietary intakes of vitamin E represent 60–70% γ-tocopherol and only 20–25% α-tocopherol [15]. Yet, vitamin E intervention

studies most often explore α-tocopherol in isolation; and studies of γ-tocopherol, or the naturally occurring mixture, are limited. Whether or not intervention trials of isolated or synthetic α-tocopherol represent ecologically relevant study designs is debated. What is crucial to the advancement of nutrition sciences is the appropriate interpretation and application of the study results. Since it is well established that the vitamin E isoforms are not interconvertible inside the human body and the different forms have demonstrated structure-specific effects in virtually all cellular components and biological fluids [13], it is important when designing studies and/or interpreting study results to consider the potential that each isoform (or other food components) may act as confounders when applied to the whole system. This concept was evident in "The Randomized Factorial Trial of Vitamins C, E, and Beta-Carotene in the Secondary Prevention of Cardiovascular Events in Women (WACS trial)." Participants in the trial were supplemented with 600 IU isolated α-tocopherol. No benefits were observed with respect to the primary endpoints with vitamin E (or any of the antioxidants that were tested). The authors of the WACS trial reasoned that a lack of effect was observed in this and other vitamin E trials because supplementation with α-tocopherol *in isolation* may deplete levels of the more powerful antioxidant, γ-tocopherol [16, 17].

The matrix in which vitamin E is incorporated has a significant impact on the interpretation and relevance of study results. It is well established that vitamin E is better absorbed in the presence of dietary fat, but the *type* of dietary fat and/or food matrix may be equally important factors [18]. For example, vitamin E (^{2}H-labeled α-tocopherol) absorption was shown to be significantly greater following a meal of toast and butter than it was following a meal of cereal and full-fat milk. Both meals contained 17.5 g of fat. On the other hand, little to no vitamin E absorption was observed following a meal of cereal and reduced-fat milk (2.7 g fat) or water (0 g fat) [19].

A food synergy paradigm has been suggested as an alternative to the traditional single-nutrient approach for the advancement of nutrition sciences. Supporters of the food synergy paradigm stress that food components interact in complex ways that cannot be explained via the assessment of individual chemical parts (nutrients). Instead, the mapping of nutritional profiles or dietary patterns and physical activity in relation to health status should be the focus [5, 6, 20–22]. One example of an intervention that seemed to embrace the food synergy paradigm is the DASH diet. DASH-Sodium is an NIH-funded study that compared the effects of three levels of dietary sodium (low [50 mmol/d], intermediate [100 mmol/d], high [150 mmol/d]) and two patterns of diet (a control diet and an intervention diet high in fruits, vegetables, and low-fat dairy products) on blood pressure in individuals with higher than optimal blood pressure or with stage 1 hypertension [23]. Following the 90-day intervention diet period, 63% of participants with stage 1 hypertension achieved blood pressure goals after adopting the DASH dietary pattern (without reductions in sodium intake), and 51% achieved blood pressure goals by simply reducing sodium intake (without changes in dietary patterns). The combination of the DASH diet and lower sodium intake resulted in 84% of participants achieving blood pressure goals. In addition, in a 1-year follow-up study of a subset of DASH-Sodium participants, those that were on the DASH diet continued to eat more fruits and vegetables; and they retained reductions in blood pressure, compared to the control group, despite increased sodium intake [24]. Now, rather than focusing solely on the intake of sodium for the reduction or prevention of high blood pressure, the National Heart, Lung, and Blood Institute promotes the DASH eating plan which includes whole grains, poultry, fish, and nuts and has low amounts of fats, red meats, sweets, and sugared beverages. It is also high in potassium, calcium, and magnesium, as well as protein and fiber [25].

In cases where dietary supplements are the preferred routes for administering nutrients, then dosing is another important factor to consider. At dietary doses, gut proteins are believed responsible for mediating the absorption of vitamin E, but, at pharmacological doses, absorption is likely passive [19]. The daily value for vitamin E is 30 IU (approximately 20 mg of α-tocopherol). Yet, doses of vitamin E in intervention studies tend to be >100 IU per day [26]. Supplementation of nutrients (e.g., vitamin E) is considered most effective when administered at dietary doses, early in the life stage, and

over a multiyear period. The authors of the SU.VI.MAX (Supplementation en Vitamines et Mineraux Antioxydants) study acknowledged these criteria in the study design to test the efficacy of a combination of antioxidants to reduce the incidence of cancer and ischemic cardiovascular disease [27]. A total of 12,741 participants were randomized to receive a daily multivitamin and mineral capsule containing dietary doses of vitamin C (120 mg), vitamin E (30 mg), beta-carotene (6 mg), selenium (100 μg), and zinc (20 mg) or a placebo. The study was double-blinded, and the participants represented a general population of *healthy* French adults aged 35–60 years. The group was stratified according to sex, and the median follow-up time was 7.5 years. No differences were observed in the incidence of ischemic CVD between the participants receiving the combination antioxidant capsule and the placebo. However, in men, a significant protective effect of the antioxidant capsule was shown in overall cancer incidence and all-cause mortality. The lack of effect observed in women was attributed to the higher baseline levels of nutrients in the women participants vs. men. Unlike prior prevention trials that found no effect or deleterious effects of antioxidant supplementation on cancer incidence, this study differed in the choice of antioxidant combination, doses, recruitment methods, selection criteria, and characteristics of the study population. In most of the prior trials, pharmacological doses of the nutrients were administered, the supplements contained individual or (at most) paired antioxidants, the study populations included high-risk individuals, and/or the participants were characterized with very low baseline micronutrient status [27].

Assessments of Nutrient Status and Dietary Intake

Biomarkers are complementary and essential components of assessments of nutrient status and dietary intake. A biomarker is defined as a distinct biological or biologically derived molecule that can be detected in blood, other bodily fluids, or tissues; and it serves as an indicator of exposure, process (i.e., aging), condition, or disease. The validity of a biomarker is determined, in part, by its application. For example, an exposure marker may or may not be valid for assessing dietary intakes of a specific nutrient, and it may or may not be valid for assessing status, risk, or susceptibility. Still, regardless of application, the emergence of omics technology has provided evidence that validation of any candidate biomarker requires careful measures of suitability in various subpopulations. For example, some efforts had been made to assess the intakes of vitamin E from supplements only, and not from foods, via measurements of plasma concentrations of α-tocopherol [28]. For every tenfold increase in α-tocopherol intake (from supplements), plasma concentrations of α-tocopherol were expected to double, whereas concentrations of γ-tocopherol were expected to decrease. As such, the potential for plasma levels of α-tocopherol as a biomarker for exposure to vitamin E supplements was considered. However, a follow-up study showed that healthy individuals supplemented with α-tocopherol did not have equal increases in plasma α-tocopherol concentrations. The increase in plasma α-tocopherol 12 h after an intake of 75 mg of d_6-RRR-α-tocopherol ranged from 0.3 to 12.4 μmol/L [28]. These data clearly show that the optimal supply of vitamin E can differ greatly from one individual to the next; and the data underscores the need to validate potential biomarkers in various subpopulations as a criteria to determine applicability.

Government Funding of Nutrition Research

In 1983, an Interagency Committee on Human Nutrition Research (ICHNR) was created. Representatives of the ICHNR include the departments of Agriculture (USDA), Health and Human Services (HHS), and Defense (DoD), the Federal Trade Commission (FTC), the National Aeronautics

and Space Administration (NASA), the National Science Foundation (NSF), the Agency for International Development (USAID), the Environmental Protection Agency (EPA), the Veterans Health Administration (VHA), and the White House Office of Science and Technology Policy (OSTP). Once convened, the group established the following definition of human nutrition research:

> Human nutrition research is the pursuit of new knowledge to improve the understanding of nutrition as it relates to human health and disease and, as here defined, encompasses studies in five major areas: biomedical and behavioral sciences, food sciences, nutrition monitoring and surveillance, nutrition education, and impact on nutrition and intervention programs and socioeconomic factors. [29]

As of 2013, a primary aim of the ICHNR was to increase the overall effectiveness and productivity of federally supported or conducted human nutrition research. The Committee has recognized a need for a written strategic plan to identify critical human nutrition research gaps that could be addressed within 5–10 years; and in 2016, the ICHNR released its National Nutrition Research Roadmap [30]. Based on the premise that improved nutrition could be among the most cost-effective strategies to address mortality, morbidity, and economic burden associated with noncommunicable chronic diseases in the USA and globally, the Roadmap focused on 3 primary questions and 11 key topic areas for coordinating nutrition research, training, and career development opportunities (Table 31.2) [30]. It is worth mentioning that challenges associated with nutrient intake and bioavailability, phenotype-based nutrient requirements, and assessments of nutrient status and dietary intake (as previously described) are common themes throughout the key topic areas.

Of the US federal government agencies, the National Institutes of Health (NIH) has historically been the largest funder of nutrition research, primarily through grant mechanisms offered by virtually all of its components (institutes, centers, and offices). The most recent report of NIH funding for 2013–2014 noted that annual NIH funding for nutrition research is in the $1.5–1.6 billion range and that its investment is very broad – reflected in the fact that 24 of the NIH ICOs reported funding grants

Table 31.2 National Nutrition Research Roadmap: key research priorities for 2016–2021

Question 1: How can we better understand and define eating patterns to improve and sustain health?
Topic 1: How do we enhance our understanding of the role of nutrition in health promotion and disease prevention and treatment?
Topic 2: How do we enhance our understanding of individual differences in nutritional status and variability in response to diet?
Topic 3: How do we enhance population-level food- and nutrition-related health monitoring systems and their integration with other data systems to increase our ability to evaluate change in nutritional and health status, as well as in the food supply, composition, and consumption?
Question 2: What can be done to help people choose healthy eating patterns?
Topic 4: How can we more effectively characterize the interactions among the demographic, behavioral, lifestyle, social, cultural, economic, occupational, and environmental factors that influence eating choices?
Topic 5: How do we develop, enhance, and evaluate interventions at multiple levels to improve and sustain healthy eating patterns?
Topic 6: How can simulation modeling that applies systems science in nutrition research be used to advance exploration of the impact of multiple interventions?
Topic 7: How can interdisciplinary research identify effective approaches to enhance the environmental sustainability of healthy eating patterns?
Question 3: How can we develop and engage innovative methods and systems to accelerate discoveries in human nutrition?
Topic 8: How can we enhance innovations in measuring dietary exposure, including use of biomarkers?
Topic 9: How can basic biobehavioral science be applied to better understand eating behaviors?
Topic 10: How can we use behavioral economics theories and other social science innovations to improve eating patterns?
Topic 11: How can we advance nutritional sciences through the use of research innovations involving big data?

within that timeframe (www.niddk.nih.gov/about-niddk/strategic-plans-reports/Documents/NIH_Nutrition_Research_Report_2013_2014.pdf). Some notable examples of NIH-funded studies investigating nutrient supplementation include:

- SELECT (NB negative outcome re selenium and vitamin E and prostate cancer)
- VITAL (NB ongoing trial of vitamin D and omega-3 to prevent CVD and Ca)
- D2d (NB ongoing trial of vitamin D to prevent type 2 diabetes)

The SELECT, VITAL, and D2d trials are each important and relevant as they were designed to address specific questions and/or challenges that are pertinent to the nutrition sciences. While no certainty of outcome is claimed, clearly some study designs are stronger than others in regard to the potential scope of the impact. Of course, even the more focused (smaller scaled) studies contribute significantly to the totality of evidence for advancing the science. Three examples of NIH-funded nutrient studies (SELECT trial, VITAL trial, D2d trial) were characterized (Table 31.3) according to approaches in the study designs for addressing phenotype and baseline characteristics, rationalization of dose with respect to diet (or baseline exposure) and nutrient bioavailability, and assessments of adherence (dietary intake) and nutrient status. In addition, given that adverse event monitoring is a crucial component in all clinical trials (but often overlooked in nutrition trials), each study's approach to adverse event monitoring was also tabulated. Major differences in study design were noted when comparing studies targeting nutrient therapeutic strategies for risk reduction in high-risk populations versus studies investigating the benefits of nutrient intake for health maintenance. The former tended to experiment with nutrient doses that were magnitudes higher than the average daily intake in a general population, and oversampling of minority populations or higher-risk populations was more often implemented in recruitment strategies.

The SELECT trial is an example of a study designed to investigate the benefits of nutrient intake for health maintenance. The objective of the SELECT trial was to determine the long-term effect of vitamin E and selenium on risk of prostate cancer in relatively healthy men [31]. More than 35,000 healthy men aged 50 years or older representing the general population, with average risks of developing prostate cancer, participated in the study. The 2 × 2 factorial design allowed for nutrient-nutrient interactions between vitamin E and selenium to be assessed and deemed significant. In addition, long-term follow-up and safety monitoring at 7 years post-randomization revealed a significant increased risk of prostate cancer in individuals receiving 400 IU/d vitamin E supplements [31]. Given that 50% of individuals in the general population aged 60 years or older frequently take supplements containing vitamin E and 23% of those take at least 400 IU/d, the scope and impact of this study are considered to be quite broad.

In contrast, the scope of the D2d trial is expected to be somewhat limited. The study appears to be designed to target nutrient therapeutic strategies for risk reduction in high-risk populations. The objective of the D2d trial is to test whether vitamin D supplementation is safe and effective at lowering the risk of progression to diabetes in people at high risk for type 2 diabetes [32]. An oversampling of nonwhite, prediabetic individuals aged 30 years or older is proposed, and very large doses of vitamin D, 4000 IU, are to be administered daily for 48 months. Study participants are also expected to take 600–1000 IU/d vitamin D outside the D2d protocol. These levels exceed the established upper limit for vitamin D intake, and the study's eligibility criteria do not require participants to be vitamin D deficient prior to enrolling. Baseline assessment of vitamin D status was not an inclusion criterion in the study design. Instead, blood levels of 25(OH)D will be collected at yearly intervals to assess nutrient status at completion of the study, and adherence will be assessed via pill counting [32]. The study design does allow for nonspecific monitoring of adverse events, and tolerability will be assessed via the number of participants who discontinue the study pills. The outcomes of the study may be useful in providing evidence on the therapeutic value of high-dose vitamin D exposure in reducing the risk of diabetes in a subpopulation of individuals that are often underrepresented in clinical trials. On the other hand, limitations in the study design may preclude its relevance to healthy populations with average vitamin D exposure.

Table 31.3 Objectives and challenges of some NIH-funded nutrient studies

		Challenges addressed			
NIH-funded nutrient studies	Objective	Phenotype and baseline characteristics	Rationalization of dose with respect to diet (or baseline exposure) and nutrient bioavailability	Assessments of adherence (dietary intake) and nutrient status	Adverse event monitoring
SELECT trial	To determine the long-term effect of vitamin E and selenium on risk of prostate cancer in relatively healthy men	35,533 healthy men aged 50 years or older with average risk of prostate cancer Baseline prostate-specific antigen (PSA) and digital rectal examination (DRE) obtained	Relevant to 23% of adult male population that take vitamin E supplements >400 IU/d Nutrient-nutrient interactions between vitamin E and selenium assessed	Baseline blood and toenail specimens as well as a 5-year blood sample were collected for future studies of overall nutrient status	Long-term follow-up and safety monitoring
VITAL trial	To investigate whether taking dietary supplements of vitamin D3 (2000 IU) or omega-3 fatty acids reduces the risk of developing cancer, heart disease, and stroke in people who do not have a prior history of these illnesses	16,956 individuals aged 50 years or older from diverse regions and ethnic population groups were enrolled with oversampling in African American population Baseline blood samples were optional and collected from 65.5% of total study population	Experimental doses (2000 IU/d) investigated and are not translatable to current RDA's or average supplement intake	Follow-up blood samples for assessment of adherence and/or nutrient status provided by 35% of study participants	6% of study participants (representing a less diverse subcohort) received full health exams at baseline and at 2 years follow-up
D2d trial	To test whether vitamin D supplementation is safe and effective at lowering the risk of progression to diabetes in people at high risk for type 2 diabetes	2382 targeted enrollment from multicenter, multi-region recruitment of prediabetic adults aged 30 years or older with oversampling of nonwhite individuals Baseline data on FPG, 2hPG, and hemoglobin A1c assessed via 75-g oral glucose tolerance test (OGTT)	Experimental doses investigated (4000 IU/d) and are not translatable to current RDAs or average supplement intake	Blood levels of 25(OH)D collected at yearly intervals and measured at study completion to assess nutrient status Adherence assessed via pill counting	Generalized adverse events monitored every 3 months for 48 months The number of participants who discontinue study pills assessed as measure of tolerability of high-dose vitamin D supplementation

The question of safety, and perhaps efficacy, of high-dose vitamin D supplements in *healthy* populations might be better addressed by the VITAL trial. The objective of the VITAL trial is to investigate whether taking dietary supplements of vitamin D (2000 IU/d) or omega-3 fatty acids reduces the risk of developing cancer, heart disease, and stroke in people who do not have a prior history of these illnesses [33]. Nearly 17,000 individuals were enrolled in the study. The participants were aged 50 years or older, from diverse regions, and oversampled with African Americans [33, 34]. The authors of the VITAL trial acknowledge that very low but also very high levels of vitamin D may contribute to health risks in the general population (e.g., cardiovascular disease and certain cancers). As such, a strength of the VITAL trial design is its approach to adverse event monitoring and assessment of nutrient status. The study design allows for monitoring of blood levels of calcium, 25(OH)D, parathyroid hormone, and kidney function. In addition, a subcohort of participants received a full clinical exam that included measurement of height, weight, blood pressure, oral glucose tolerance testing, spirometry, 2D echocardiography, bone mineral density testing, and structured cognitive and mood assessments [33]. Outcomes from this study may provide important evidence to address the significance of the pleiotropic tendencies of vitamin D with respect to risk reduction in healthy populations.

Clearly, no one study satisfied all criteria for adequately addressing each of the challenge areas.

Members of the ICHNR acknowledge that not only are more rigorous intervention study designs needed to better examine the causal relationships of diet, nutrition, and health status but improved methodologies are also needed for evaluating and integrating observational evidence to help fill the gaps where controlled research designs are limited [29, 30]. Observational cohorts provide long-term follow-up and allow for extensive and repeated measures of dietary and nutritional assessments. Such observational studies can be designed to examine the influence of diet and other health behaviors on various disease outcomes over the life cycle. Several short-term and long-term initiatives for encouraging new approaches for objectively evaluating the quality and integrating the totality of available evidence to address the complexities of nutrition research were presented in the National Nutrition Research Roadmap [30]. Some examples of important initiatives in this area are provided below.

- National Heart, Lung, and Blood Institute observational studies – data collection from observational studies on factors associated with the development of overweight and obesity, as well as their relation of overweight and obesity to heart disease and its risk factors, pulmonary diseases, and sleep disorders.
- National Cancer Institute Cohort Consortium supports meta-analysis across multiple national and international cohorts. Involving more than 7 million people, the cohorts are international in scope and cover large, rich, and diverse populations. Extensive risk factor data are available on each cohort, and biospecimens, including germline DNA collected at baseline, are available on approximately 2 million individuals. Investigators team up to use common protocols and methods and to conduct coordinated parallel and pooled analyses.

The potential of omics-based approaches was recognized in the National Nutrition Research Roadmap for addressing questions related to phenotype variability in response to diet and nutritional status [30]. Omics-based approaches cover a broad range of technologies ranging from proteomics, metabolomics, genomics, to epigenomics. Incorporating the use of omics technologies into current nutrition research methods may further advance the study of interactions between eating patterns and human metabolic processes that may contribute to individual or phenotypic variations. Standardization of the data captured from omics technologies may be essential for the development of novel biomarkers of baseline status, nutrient intake, eating patterns, microbiome function, and environmental exposures. Some examples of important initiatives in this area are provided below.

- PhenX Toolkit provides standardized measures to assess complex diseases, phenotypic traits, and environmental exposures, including those related to individual dietary intake and the food environment. For research on exposures that may be important in identifying disease-risk phenotypes, the

use of PhenX measurements facilitates combining data from a variety of studies and can help investigators expand a study design beyond the primary research focus.

- Big Data to Knowledge (BD2K) is established to capitalize on the exponential growth of medical datasets by promoting innovative advances in big data resources, analytics, and training. BD2K engages with partners in academia, nonprofits, and other government organizations to coordinate the access to, linkages between, and analysis of diverse and multimodal biomedical datasets.
- NIH Health Care Systems (HCS) Research Collaboratory Program works to strengthen the national capacity to implement cost-effective, large-scale research studies that engage healthcare delivery organizations as research partners. This effort aims to rethink clinical trial design and provide a framework of implementation methods and best practice that will enable the participation of many healthcare systems in clinical research.

The National Nutrition Research Roadmap highlights the notion that human nutrition takes place in a complex ecosystem influenced by many factors [30]. Too often, nutrition research has focused on a single component of the system which tended to result in misleading or incorrect outcomes. When deciding which interventions to implement to achieve a desired outcome, the interactions of many components of the system(s) must be considered. Applying systems science methodologies to nutrition research has the potential to stimulate testable hypotheses of how, when, and where an intervention in a system would be expected to have the greatest benefit or the greatest impact toward the desired outcome. The National Institutes of Health has highlighted the utility of systems science methods in several funding opportunity announcements, an example of which is provided below.

- "Systems Science and Health in the Behavioral and Social Sciences" – This FOA called for research projects that were applied and/or basic in nature (including methodological and measurement development), had a human behavioral and/or social science focus and featured systems science methodologies.
  Systems science methodologies are specific methodological approaches that have been developed to understand connections between a systems structure and its behavior over time. "Systems science methodologies" is an umbrella term to refer to a variety of such methodologies including (but not limited to), agent-based modeling, microsimulation, system dynamics modeling, network analysis, discrete event analysis, Markov modeling, many operation research and engineering methods, and a variety of other modeling and simulation approaches.

The National Institutes of Health and federal agencies of the Interagency Committee on Human Nutrition Research have joined to address strategies that might help to improve public confidence in nutrition research outcomes. Initiatives to help better define the role of nutrition, as a whole, in a healthy life span have been fully elucidated in the National Nutrition Research Roadmap. Some common themes among the new initiatives included (1) assessments of nutritional status and dietary intake, (2) phenotypic variations in nutrition requirements (e.g., life stage, sex, genetics, and/or epigenetic differences), and (3) interrelationships between intake (via supplement or food) and true nutrient bioavailability. Increased collaborative efforts with healthcare delivery organizations, nonprofits, academia, and government agencies as well as an increased emphasis on systems science approaches appear to be central to the overall advancement of nutrition research.

Public-Private Partnerships and International Collaborations

Trends in nutritional sciences funding suggest that future initiatives will likely expand beyond collaborations between federal agencies and include more public-private partnerships as well as international collaborations, to better capitalize on access and use of biomedical "big data." Big data refers

to a complex array of very large multimodal data from a large number of data sources. To date, some examples of public-private partnerships and international collaborations, such as those below, show promise for improved organization, shared use, and analyses of big data that may enable new directions for biomedical and/or nutrition research.

- Accelerating Medicines Partnership (AMP) is a cross sector partnership between 2 US federal government agencies, 12 biopharmaceutical life science companies, and 13 nonprofit organizations. The partnership focuses efforts on gaining a better understanding of biological targets and identification of valid biomarkers for three disease areas: Alzheimer's disease, type 2 diabetes, and autoimmune disorders, such as rheumatoid arthritis and lupus. A critical component of the partnership is that all partners have agreed to make the AMP data and analyses publicly accessible to the broad biomedical community such that the number of new therapies is increased for patients and the time and cost for development are reduced [35].
- The Biomarkers Consortium (BC) focuses on rapid identification, development, and validation of high-impact biomarkers to enable improvements in drug development, clinical care, and regulatory decision-making. The BC offers a unique environment in which the resources, scientific expertise, and data of its partners (2 federal agencies, 24 for-profit organizations, 12 not-for-profit organizations) are freely shared [36].
- European Union Joint Programming Initiative (EUJPI): A Healthy Diet for a Healthy Life (HDHL) strives to understand the most effective ways of improving public health through specific interventions targeting dietary and physical activity behaviors. The variety of foods consumed and the diverse ethnic groups across Europe provides an important opportunity to study the relationship between food intake and health among groups that are, to date, relatively poorly examined. The initiative will strengthen cross border collaborations and facilitate standardized data collections and harmonized data pooling [37].

As opportunities improve for researchers in the nutrition sciences to obtain access to and participate in the sharing and analyses of biomedical big data via national or international partnerships, prospects for a more integrated population-based and individualized approach to nutrition gain appeal.

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P. Weber et al. (eds.), *Vitamin E in Human Health*, Nutrition and Health,
https://doi.org/10.1007/978-3-030-05315-4